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The Cell and Environmental Temperature

Proceedings of the
International Symposium on Cytoecology

*The Role of Cellular Reactions
in Adaptation of Multicellular Organisms
to Environmental Temperature*

EDITOR-IN-CHIEF

A. S. TROSHIN

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PREFACE

AN International Symposium on "The Role of Cellular Reactions in the Adaptation of Multicellular Organisms to Environmental Temperatures" was held in Leningrad, U.S.S.R., from May 31 to June 5, 1963. The Symposium was sponsored by the Academy of Sciences of the U.S.S.R. and of UNESCO. Responsibility for its organization was entrusted to the Institute of Cytology of the Academy of Sciences of the U.S.S.R. Fifty-two Soviet and fifteen foreign specialists on cytoecology participated. The foreign participants were from Bulgaria, Czechoslovakia, France, the German Federal Republic, Japan, India, Poland and the United States of America. A considerable number of the members of the Symposium were younger research workers. About 400 guests attended the meetings.

The organizational work necessary to determine those institutions, laboratories and scientists most directly concerned with the various aspects of the subject and the establishment of the program was conducted by an organizing committee consisting of Prof. V. Y. Alexandrov, Prof. L. K. Losina-Losinsky, Prof. G. I. Poljansky (Vice-chairman), Dr. K. M. Sukhanova, Correspondent member of the Academy of Sciences of U.S.S.R., A. S. Troshin (Chairman), Correspondent member of the Academy of Sciences U.S.S.R., I. I. Tumanov, Dr. B. P. Ushakov and Dr. A. V. Zhirmunsky (Secretary).

During recent decades biologists have shown an increasing interest in the analysis of adaptation mechanisms. This interest has been stimulated not only by the theoretical significance of the problem but also because of its practical importance in agriculture and medicine.

A thorough analysis of the mechanisms of adaptation to environmental factors requires a broad approach at varying levels of biological organization. Many adaptive reactions are achieved by the organism as an integrated whole. Other adaptive reactions function at cellular levels and have been demonstrated to play an essential role in many plants and animals.

The study of the role played by cells in the adaptation reaction of an organism constitutes a specific branch of investigation called cytoecology, the interface between the two biological disciplines of cytology and ecology. These cellular adaptive changes presumably involve molecular transformations which occur both in the cytoplasm and the nucleus, and they may be apparent only at the cellular level of organization.

For some time past a large body of experimental material on temperature adaptation has been accumulated in the U.S.S.R. and elsewhere and deserves detailed discussion. Since several cytoecological investigations were being

carried out in some of the laboratories of the Institute of Cytology of the Academy of Sciences of the U.S.S.R., that Institute accepted the invitation from UNESCO and the Academy of Sciences of the U.S.S.R. to conduct an International Symposium devoted to temperature adaptation at the cellular level of organization. In the following Proceedings both the presentations as well as abbreviated transcripts of the subsequent discussions have been recorded. The Proceedings also provide abstracts of papers which are not reproduced in full but which were discussed in the course of the Symposium.

The editors of the Proceedings feel that this publication will be of special interest to biologists concerned with the cytology, physiology and ecology of plants and animals. Since the cold stability of cultivated plants is, to a certain degree, a cytoecological problem, these Proceedings should also be of interest to specialists dealing with practical aspects of agriculture. In addition, questions of temperature adaptations may also be of importance to the medical and veterinary sciences as well as for the new biological speciality of cosmic biology.

OPENING ADDRESS

Professor A. S. TROSHIN

Institute of Cytology of the Academy of Sciences, U.S.S.R.

THE development of modern biology has been characterized by substantial achievements derived from investigations of cell regulatory mechanisms. One current trend of cytological investigations is toward the elucidation of the mechanisms by which living organisms adapt to their environment. Both cellular and molecular adaptive mechanisms are being studied under a variety of environmental conditions and increased attention is being paid to homeostasis phenomena at the cellular level.

Of great importance is the observation that a correlation often exists between the degree of cell resistance to an environmental factor and the importance of that factor in the environment. It is becoming apparent that the degree of resistance of both cells and intracellular components can have adaptive significance.

Examination of this correlation can be of great theoretical and practical significance. A study of cell thermoresistance, for example, has been recently applied to the study of species and species development. The study of adaptive resistance may also indicate the means by which this resistance may be increased artificially, contributing to our ultimate understanding of acclimatization.

For some time past, both in the U.S.S.R. and other countries such as Austria, the German Federal Republic, the U.S.A. and Japan, there has been developing a new direction in biological investigations which we in the U.S.S.R. have termed cytoecology. Cytoecology is a disciplinary interface between cytology and ecology. The purposes of this new field are, first, to gain understanding of the cellular and molecular mechanisms of organismic adaptation and, second, to study cellular adaptations themselves. Particular attention has been paid by cytologists to environmental temperature factors and this is not surprising. A temperature factor is an unavoidable and obligatory condition of existence for any living organism. It is one of the universal factors, for all processes taking place in living systems are under its influence. Furthermore, it is a directly acting factor since in many living organisms it has particular influence on the rate and characteristic of life processes. It is the particular importance of the influence of environmental temperature that has led to its choice as the principal subject of this Symposium.

Originally the following six questions were proposed for discussion:

1. Heat- and cold-resistance of animal and plant cells of taxonomically close species existing under differing temperature conditions.
2. The effect of environmental temperature on the heat- and cold-resistance of animal and plant cells of the same species.
3. External factors influencing cell resistance to high and low temperatures.
4. Cellular responses to high and low temperature.
5. Biochemical mechanisms underlying both cell resistance and adaptations to high and low temperatures.
6. Homeostatic processes occurring in cells during temperature fluctuations.

The titles of proposed contributions initially submitted to the Organizing Committee revealed that not all these subjects were under investigation to an equal degree. Several contributions, in fact, although clearly applicable, did not deal directly with any of these specific subjects. This led ultimately to a division of the Symposium into subjects concerned with animals or plants and with high or low temperature effects.

We appreciate that the agenda of this Symposium is crowded and that any one of the four sections of the program could occupy an entire symposium. However, as can be seen merely from the titles of contributions, the responses of animals and plants to the influence of both heat and cold have much in common and their parallel discussion should prove both desirable and fruitful.

The financial support of this Symposium has been provided by the Academy of Sciences of the U.S.S.R. and by UNESCO. On behalf of all members of the Symposium, allow me to express to these respective organizations our sincere gratitude.

In closing, I should like to welcome our foreign colleagues from Bulgaria, Czechoslovakia, France, the German Federal Republic, Japan, India, Poland and the U.S.A. who have come to participate in this Symposium. I am also pleased to welcome here the many Soviet cytologists.

I have full confidence in the success and fruitfulness of this Symposium and I take great pleasure in opening this International Symposium on Cytoecology devoted to "The Role of Cellular Reactions in the Adaptation of Multicellular Organisms to Environmental Temperature".

ADDRESS OF WELCOME

Dr. A. KEPES

UNESCO, France

I AM very glad to have this opportunity to welcome you, the scientists who are gathered together in the Hall of the Academy of Sciences of the U.S.S.R. for this Symposium sponsored by UNESCO.

The recent development of cell biology has been rapid with many more people working in the field each year.

New techniques are created; new branches, and new disciplines are differentiated from the main body of biology. The problem of maintaining the basic unity of scientific knowledge in biology arises. To maintain this unity, UNESCO has made an effort to maintain a steady flow of scientists from one country to another, from one discipline to another, in order that science may remain one.

This Symposium is one of the projects undertaken to achieve this aim. If I can judge by the number of distinguished scientists who are attending this meeting, by the interest which they bring to the subjects to be discussed, I am full of hope that the efforts of UNESCO will not be in vain.

I wish you very fruitful scientific work during this session.

THE FROST-HARDENING PROCESS OF PLANTS

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THE winter-hardiness laboratory of the K. A. Timiryazev Institute of Plant Physiology has studied the hardening of spring and winter grain crops, fruit and forest species; plants which possess different frost-resistance. High frost-resistance is the result of a long and complicated preparation of plants for winter. In addition to specific genetic factors the following conditions are necessary:

1. the presence of a dormant stage or of a comparatively long vernalization stage;
2. a first hardening stage at low positive temperatures;
3. a second hardening stage under slow cooling at sub-freezing temperatures (Tumanov, 1940, 1960).

The plants should pass through all these stages in the above-mentioned succession. Each stage proceeds under certain environmental conditions and raises resistance to a higher level, being a preparatory step to the next stage. Plants can endure winter even at an extremely low temperature after successfully passing through all hardening stages.

An initial requirement is that the plants must possess a good hardening ability. Even the most winter-hardy species are not readily hardened in a growing state. For example, intensively growing (under continuous lighting) birch seedlings, even put under favourable environmental conditions, raise their resistance only from -5 to -15°C (Tumanov, 1960). Vigorous growth cannot be combined with high frost-resistance; the former and the latter should not coincide in time. Plants grow during warm months, developing their frost-resistance in the cold season of the year (Tumanov, 1955).

High hardening ability in tree species develops after they have entered the stage of dormancy. The organism's properties sharply change during this stage. It is unable to grow and instead, accumulates great reserves of starch and other nutrients through photosynthesis.

In the process of hardening, these nutrients are mobilized by the organism and converted into protective compounds. The stage of dormancy creates favourable conditions for the next stage, hardening at low non-freezing temperatures. For example, in autumn, when pine, birch and lime branches are dormant, their frost-resistance rises after the first hardening stage to -35°C ,

but under the same environmental conditions after the period of dormancy, only to -15°C (Tumanov and Krasavtsev, 1955).

Many tree species enter the period of dormancy under the influence of the shorter days of autumn. In the laboratory, we can produce plants of great size in short periods of time. We can grow trees under continuous lighting. Then, when it is considered desirable, we can establish the ten-hour day, transferring them from growth to dormancy. The duration of the period of dormancy is important for hardening. Siberian and Far East trees, for example Siberian apple trees or Ussurian pear trees, possess a short period of dormancy but enough for the areas where they are growing. In their native land they remain frozen during the whole winter and spend much time in forced dormancy. If these species are grown in areas with lasting autumns and frequent winter thaws these potentially frost-hardy species are easily frozen to death. Under such conditions they are unable to remain dormant and consequently are not susceptible to hardening. In some years, climatic conditions may prevent plants from entering the period of dormancy. It is also observed that when southern plants are grown in northern areas they cannot accomplish their growing during a short warm season and they freeze and die. The same plant often can have lignified and unlignified parts. The latter are poorly hardened for their growing processes are not completed. Thus, winter frost-resistance may be predetermined by the conditions of the previous warm season.

Not all wintering plants have a stage of dormancy. Winter crops do not possess it and are less able to harden. In the laboratory, swollen winter rye seeds with a high water content (70 per cent of dry matter) were slowly and regularly cooled from -3 to -60°C , then stored in liquid nitrogen. After thawing they showed 71 per cent germination and, after exposure to a still lower temperature (liquid hydrogen) – 43 per cent (Samygin, Varlamov and Matveyeva, 1960). So, dormant cells of winter grain crops, before they have renewed growth, are able to pass safely the second hardening stage. Like northern forest species they can withstand even the lowest temperatures. This permits the prediction that winter crops may be hardened to such a degree that they will endure any winter, the only condition being the inhibition of growth processes. Even swollen seeds of spring wheat can be hardened to survive at -60°C . Winter and spring grain crops have an adequate mechanism for the second hardening stage but it cannot work efficiently only because growth processes cannot be fully stopped.

Other plants have no such physiological mechanism for hardening. For example, swollen corn seeds frost-kill even under optimal conditions of the second hardening stage. These seeds cannot be made highly frost-resistant even after inducing a period of dormancy. Warmth-loving plants make poor use of favourable environmental conditions for hardening. A period of dormancy alone is not enough for development of high frost-resistance since they lack a physiological mechanism for efficient hardening.

Inhibition of autumn growth in winter plants is usually associated with an adequate duration of the vernalization stage. In warm months spring and winter plants are growing at the same rate. When the temperature is slightly above 0°C the growth of spring plants is more vigorous than that of winter ones (Vasiliev, 1939). When the stage of vernalization is accomplished, winter plants are growing in cold months as vigorously as spring ones. That is why winter plants, grown from vernalized seeds become hardened less well than similar plants from unvernalized seed (Tumanov, 1940). In regard to the frost-resistance of plants, the stage of vernalization is analogous to the stage of dormancy, the difference being that the former inhibits growth only at lower temperatures while the stage of dormancy stops growth even more efficiently despite the absence of low temperatures.

The first hardening stage requires low positive temperatures. This period is characterized by the accumulation of protective substances and the inactivation of growth processes. The accumulation of protective substances can proceed in different ways. The first stage in trees is characterized by hydrolysis of polysaccharides. Winter plants have no stored starch, they accumulate sugars by the end of autumn through photosynthesis (Tumanov, 1931). During the first stage of hardening therefore these plants require light.

The essential role of sugars in developing frost-resistance in winter plants is proved by experimental application of these substances as an additional plant nutrient (Tumanov and Trunova, 1963). By this means it is possible to make plants go through the first hardening stage even in darkness. Feeding plants with sugars from the external solution in darkness resulted in the increase of sugar percentage in winter up to 75 per cent of dry matter or 22 per cent of water. Such sugar content is usually found in the best sugar beet roots before harvesting. Frost-resistance is more seriously affected by the amount of sugars in the cell than by their chemical specificity. Sugar protects winter plants only at high but not toxic concentrations.

It is not enough for cells to accumulate sugars in order to pass the first hardening stage. By the end of the growing period ripe grapes and sugar beet roots may accumulate high sugar concentrations but they do not harden easily. Feeding winter plants with sugars from the external solution can result in accumulation of sugar in cells in great amounts at temperatures which are higher than the hardening temperatures, for example, at +15°C. After such enrichment of cells with protective substances, the frost-resistance of winter plants increases due to the rise of the osmotic concentration, but resistance does not rise as intensively as when the sugar accumulation in cells proceeds at about 0°C. Enrichment with sugars at temperatures higher than the temperatures of hardening, raised the frost-resistance of winter wheat after the second hardening stage to -13°C, while the accumulation of the same amount of sugars at the temperature of about 0°C increased its resistance to -25°C (Tumanov and Trunova, 1963). Under natural conditions, wintering plants accumulate protective substances at low temperatures in autumn. Enrichment

of cells with protective substances can proceed more quickly than the first hardening stage. All these facts prove that there is a specific physiological mechanism in the cells of wintering plants. It is with its help, in the presence of high sugar concentrations and at the temperatures of about 0°C, that the protoplast undergoes the changes which result in the inactivation of growth processes.

Experiments show that swollen winter wheat seeds do not require the first hardening stage. They acquire high frost-resistance without the exposure near 0°C. It is enough to cool such specimens slowly and steadily at negative temperatures in order to enable them to survive, even extremely low temperatures.

In Samygin and Varlamov's experiments, 18 per cent of seeds which had begun to germinate survived at -20°C after the second hardening stage alone, and 85 per cent survived after the first plus the second stages. The role of the first stage is more important for seeds more advanced in germination. For example, of seedlings with 2-3 mm coleoptiles, all died at -20°C after the second stage alone, while 78 per cent survived after two stages. Thus the first stage is necessary to inactivate growth processes. If it is already achieved, the first hardening stage may prove unnecessary. The favourable effect of the first stage diminishes for winter wheat seedlings of greater size. This stage efficiently inactivates growth processes only when they are not very intensive. It is known that cells of winter plants are poor in auxins after the accumulation of sugars at about 0°C (Tumanov and Trunova, 1958). Low temperatures alone, without enriching plants with sugars, are not sufficient.

Swollen seeds of winter plants which have not yet begun to germinate can be trained to endure -250°C by making them pass only the second hardening stage without the first. For tree species which have entered the period of dormancy, the above treatment cannot make them winter-hardy to the same extent since they need the first hardening stage. There is no visible growth during the period of dormancy; nevertheless, growth processes still take place. They are very slow and can be inactivated more easily and more fully at the first stage. Tree species pass the first stage more successfully than winter plants. The period of dormancy alone without hardening raises frost-resistance in birch seedlings from -7 to -15 and -20°C. The first stage that follows raises their resistance by 15-20 degrees more. After this treatment the objects are able to survive at -35°C. Winter plants, which have no period of dormancy, after the first stage endure only -10 and -12°C. Growth processes in trees are inactivated by two stages: at the time of entering the period of dormancy and at the first hardening stage. Growth processes in winter plants are inactivated only by the latter method. They are unable fully to inactivate their growth.

The first hardening stage allows the organism to pass safely the subsequent hardening stage in a frozen condition. It raises its frost-resistance to such a level that plants can survive severe frosts. It changes the organism's physiology so that frosts which have killed plants before the first stage are harmless or

even useful after it. The organism is responding to the same environmental factors in different ways.

The rise in the frost-resistance of plants is especially high after the second hardening stage during the slow lowering of below-freezing temperatures (Tumanov and Krasavtsev, 1959). As frosts become more and more severe the resistance of cells becomes higher and higher. Eventually, very low temperatures will injure them unless they become sufficiently dehydrated to be unfreezable. The second stage may proceed within a wide temperature range depending on the success of the preceding conditioning. Winter plants are able to make use only of mild frosts, to -20 and -30°C , in developing their frost-resistance. Northern tree species are able to develop their frost-resistance at any negative temperature which can be met on earth, enabling them to withstand abrupt changes of temperatures. Rapid hardening helps too. During the second stage birch trees become resistant to -195°C for 36 hr, pine trees—for 12 days. The Antonovka apple tree raises its resistance to -60°C after 24 days (Krasavtsev, 1961).

During mild frosts the second hardening stage is partly lost; when frosts become more severe again it is restored. Thus, a certain correlation is established between the frost-resistance of plants and the severity of winter. After thawing, the second stage is lost but it can return if thawing has saved the first stage.

How can frosts raise frost-resistance in plants? Observations of freezing plant cells on the cold stage microscope showed that the formation of ice in the protoplast always killed the plant (Samygin and Matveyeva, 1960). An organism resistant to frosts is not achieved if the cells have high water-holding capacity. Such plants can survive only in comparatively mild frosts. Bound water is always a useful factor. In this case the water does not freeze all at once, rather freezing occurs at a wide range of negative temperatures. This provides cells with an opportunity to release water to intercellular spaces and to change the basic properties of the protoplast.

Though hardening improves the capacity of plants to vitrify, resistance is not based on this ability (Tumanov and Krasavtsev, 1962). Hardened plants can be vitrified under laboratory conditions and can be spared freezing injury by this method, but under natural conditions this phenomenon is of no importance. Efficient vitrification requires quick cooling and similarly quick thawing. The essential requirement of the second hardening stage is quite the contrary: the exposure of plants to temperatures which produce crystallization rather than vitrification. Besides, vitrified objects can be kept only at extremely low temperatures while hardened plants can remain frozen for a long time at any temperature.

The protection of plants against the formation of ice in the protoplasm is based on depriving cells of all water that can freeze at a given temperature. Water must fill intercellular space in due time and turn into ice only there. Successful cell dehydration requires that the temperature fall be slow and

gradual. During the second hardening stage as well as during the first (Levitt and Scarth, 1936) the water permeability of cells increases. Calorimetric determinations of ice content show that water from well-hardened cells is filling intercellular space at the same rate as from cells killed before freezing (Tumanov and Krasavtsev, 1959). Water translocation can result in almost complete dehydration after which ice does not form even at lowest temperatures.

Experiments show that the development of high frost-resistance in plants requires more time than water translocation from cells into the intercellular space. Even when some degree of dehydration is achieved the protoplast needs a considerable period of time to accomplish its inner changes. After this, cells acquire new properties: high permeability for



FIG. 1. Black currant. Left: two 3-year-old plants from cuttings frozen to -253°C ; right: control plant (unfrozen).

water and resistance to slow dehydration and mechanical deformations. In this way plants are protected both from intracellular ice formation and the harmful consequences of intercellular ice formation. Dehydration, during the second hardening stage, usually develops at temperatures sufficiently low to spare plants from injurious biochemical changes. It is presumed that all the above-mentioned features result from changes in the submicroscopic structure of protoplast during the second hardening stage. The factors acting are:

1. Slow dehydration which brings the molecules in the protoplasm so near to each other that they begin to affect one another and acquire certain orientation; very quick dehydration may be fatal.

2. Low temperature which, by lowering the thermal motion of molecules, helps to retain the structure which has formed in the protoplasm.

Reorientation of big protein particles is possible only on the condition that the protoplasmic viscosity is not too great. That is why moderately low temperatures, from approximately -5 to -30°C , are most efficient for the second hardening stage. Below -60°C this process cannot be seen. This presumed



FIG. 2. Ulyanovska winter wheat. Plants hardening under laboratory conditions survived at -20 , -23 , -25 and -32°C .

change of the protoplast structure also explains the effects of the second hardening stage after thawing. After thawing and the reabsorption of water by the cells, their frost-resistance abruptly falls. Nevertheless, some portion of the resistance is lost more slowly. It is possible that the reversal of the structural change of the protoplasm requires more time than the absorption of thawed water. Not all plants are able to change the structure of their protoplasm, therefore not all of them can be made intracellularly unfreezable. Such ability develops in plants only after the cessation of growth. Hardened plants, which have reached the unfreezable state, cease their physiological processes at extremely low temperatures and the organism turns into an inert body. In order to restore the normal functions of the organism it is necessary that the structure of the protoplasm be viable in a frozen and highly dehydrated state.

This hardening schedule is confirmed by the ability to develop in plants under laboratory conditions such high frost-resistance as has not been met in nature. In our experiments, northern forest species, for example of black currant and birch, induced to dormancy and hardened, raised their frost-resistance from -5 to -253°C (Fig. 1). Other plants do not acquire such high

resistance: winter wheat -32°C (Fig. 2), apricot -60°C (Fig. 3). Even such resistance would be enough to enable cultivated plants to survive the winter in fields and orchards in the areas of their cultivation. A laboratory hardening



FIG. 3. Apricot seedlings hardened under laboratory conditions survived at -60°C (separate branches have frozen and died).

cannot be applied to field conditions, it is an urgent challenge for the full utilization of available knowledge to develop practical methods to prevent freezing injury of plants.

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A PRINCIPLE OF THE FROST-RESISTANCE MECHANISM IN PLANT AND ANIMAL CELLS

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IT IS well known that various animals and plants can survive freezing. A number of theories have been expounded on the mechanism of such frost-resistance. However, it has also been well known that there are clear discrepancies between the hypotheses on frost-resistance advanced by botanists and by zoologists (Levitt, 1958). The purpose of this brief comment is to outline a common principle of frost-resistance found under natural conditions in both animals and plants. In regard to the protoplasmic factor in frost-resistance, a more detailed description will be made of some experimental evidences in sea urchin egg cells since the nature of frost-hardening in this egg cell is very unique. Since the death of an animal or plant results from frost injury to a single tissue, in the present paper the frost-resistance of individual cells will be mainly discussed.

A. Mechanisms Preventing Intracellular Freezing

In an extensive variety of living cells, intracellular freezing has been found to be fatal, although a few reports in disagreement with this view have recently been published. The prevention of intracellular freezing is presumed to be necessary in order to preserve life in the frozen state. When an organism is subjected to freezing, ice generally forms first outside of the cells. At moderate sub-freezing temperatures the initiation of intracellular freezing, if any, takes place as a result of inoculation with ice crystals from outside the cells (Asahina, 1961). In such a case, the protoplasmic surface membrane is the best barrier for preventing ice inoculation into the cell. However, after the cell has come in contact with ice, if the cell surface is quickly cooled far below the freezing point of the cell fluid, the surface protoplasm is very liable to be seeded with ice crystals. Consequently, in a cell freezing extracellularly, any factor which can reduce the rate of cooling at the cell surface should be effective in preventing intracellular freezing (Asahina, 1962).

In various cells of frost-hardy organisms the following beneficial factors can be found for this purpose.

- A1. Initiation of freezing of the outside medium by natural ice seeding at a high near-freezing temperature.

- A2. Initiation of freezing of a large amount of body fluid prior to cell freezing. This factor is probably involved in the fact that almost all insects which have been found to be frost-resistant are of pupal or larval stages.
- A3. High permeability of the cell to water.
- A4. High ductility of the cell surface. When an extracellularly freezing cell is cooled at a moderate rate, the last two factors, by increasing the velocity of dehydration of the cell, reduce the cooling rate at the cell surface where ice is forming through the liberation of latent heat.
- A5. A solution of highly hydrophilic substances in the surrounding medium also protects a cell against ice seeding since, by forming a layer of concentrated solution over the ice front, it interferes with the intimate contact of the ice front with the protoplasmic surface.

In addition, in very drought-resistant organisms severe desiccation is reasonably effective in preventing intracellular freezing.

B. Mechanisms Preventing Excessive Dehydration of an Extracellularly Freezing Cell

Although an extracellularly frozen cell may have reached a steady state at a given low temperature without intracellular ice formation, if the freezing is too severe or prolonged the cell protoplasm is still susceptible to injury. The frost-injury following such extracellular freezing occurs as a result of dehydration of the fluid both inside and outside the freezing cell. To protect cells against such a type of frost-injury the following factors are considered to be beneficial.

- B1. The occurrence of some structural changes in the protoplasm enabling it to resist a severe dehydration at a low temperature. This has an intimate correlation with the above factors A3 and A4.
- B2. An increase in protective substance such as glycols or sugars in the cell.

The last factor has been amply demonstrated in natural animal and plant cells (Heber, 1958, 1959; Parker, 1962; Sakai, 1962; Salt, 1961), while a relatively small number of theories have been expounded relative to the former. In hardy plant cells, both herbaceous and woody, morphological changes in certain cell components have been known to occur (Heber, 1959; Parker and Philpott, 1961). In such cells it is reasonable to presume that structural changes in the protoplasmic protein tending to increase its resistance to dehydration may take place. In this regard a new hypothesis concerning the behavior of sulfhydryl groups in the protoplasmic protein of hardened cells is notable. Levitt and his collaborators report in plant tissue a gradual increase in sulfhydryl groups during the first several days of hardening (Levitt *et al.*, 1961).

In the egg cell of the sea urchin, on the other hand, the increase in frost-resistance characteristically occurs instantly just after fertilization without the production of a known protective agent (Asahina and Tanno, 1963). The remarkable fluctuation in the degree of frost-resistance in the egg cell is clearly shown in Fig. 1. Upon fertilization, the frost-resistance rapidly increases, reaching a maximum without about 5 min after insemination, then decreasing as development proceeds. It is of interest to note that, only one minute after insemination, the cellular frost-resistance has increased remarkably. Within

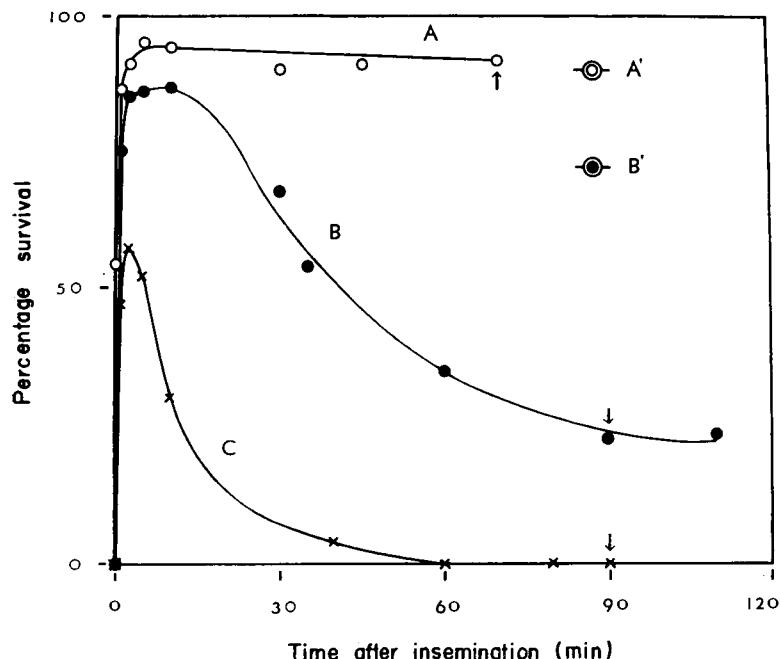


FIG. 1. Changes in frost-resistance in eggs of *Strongylocentrotus nudus* during the first cleavage. Arrows show the time of cleavage. A (○), eggs were inseminated at 20°C and frozen at -20°C for 4 hr; A' (◎), eggs treated with urea solution previous to the freezing at -20°C for 4 hr; B (●), eggs inseminated at 16°C and frozen at -22°C for 19 hr; B' (◎), eggs treated with urea solution previous to the freezing at -22°C for 19 hr; C (×), eggs inseminated at 16°C and frozen at -25°C for 18 hr (Asahina and Tanno, 1963).

this short period cortical change occurs. It was expected, therefore, that in an egg cell in which cortical change alone had taken place without insemination, frost-resistance should be enhanced to as high a degree as in a fertilized egg. This was tested by applying Motomura's urea method (Motomura, 1941). As anticipated, urea treatment applied to an unfertilized egg resulted in a remarkable increase in cellular frost-resistance (Fig. 1). Cytochemical investigation by Kawamura and Dan showed that in fertilized egg cells of the sea

urchin, the distribution of protein-bound SH groups, shown by colour intensity, changed remarkably during the first cleavage (1958, 1960). Our cytochemical study, using the same staining method as that employed by Kawamura and Dan, confirmed that finding. The stainability in egg cytoplasm promptly increased upon fertilization. Before the mitotic stage the colour intensity was nearly uniform in the entire cell, while during mitosis the astral centres and spindles were stained deeply. It should be noted that the colour intensity in cytoplasm is highest about 10 min after insemination and then apparently decreases as development proceeds (Fig. 2). The egg cell in which cortical change was artificially induced by urea treatment, also exhibited a distinctly high stainability in cytoplasm (Fig. 2).

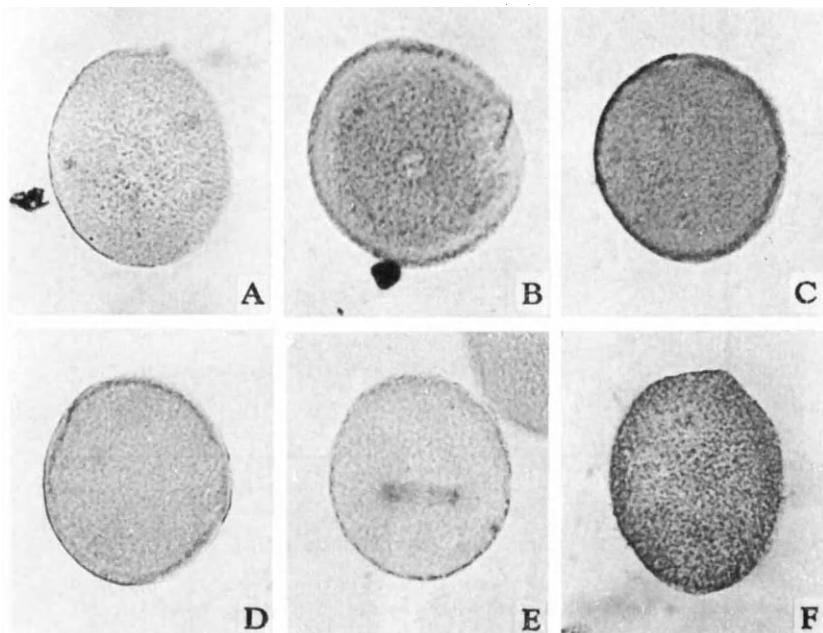


FIG. 2. Eggs of *Strongylocentrotus nudus* fixed with 5 per cent TCA and stained with Bennett's reagent ($\times 260$). A, Unfertilized egg; B, 1 min after insemination; C, 2·5 min after insemination; D, 10 min after insemination; E, anaphase (80 min); F, unfertilized egg treated with urea solution.

These results suggest that in the sea urchin egg cells, the increase in frost-resistance is expected under conditions in which at least some component of cytoplasm is rich in protein-bound SH groups. However, whether the rapid increase in cellular frost-resistance is associated with an increase in the amount of total protein SH in the entire cell is as yet uncertain. Quantitative studies of SH groups have failed to show any appreciable increase in total protein

SH in a fertilized egg cell during the first several minutes after insemination (Kawamura, 1960; Sakai, 1960).

Frost-killing after a comparatively long period of extracellular freezing may be attributable to some imbalance in cell metabolism. To minimize this factor, metabolic activity in the freezing cell should be decreased. As an artificial method this can be achieved by cooling the cell to the lowest tolerable temperature. In overwintering insects the best means to this end is deep diapause.

Almost all the above-mentioned factors leading to increased frost-resistance in living cells have been found to be well controlled by environmental factors, especially by temperature.

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RELATION OF INTERNAL AND EXTERNAL FACTORS ON THE INCREASE OF FROST-HARDINESS IN WOODY PLANTS

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THERE are two fundamentally different types of cell freezing, extracellular and intracellular freezing. In non-hardy cells, intracellular freezing readily takes place, even under slow cooling, and is always fatal to such cells. In hardy cells, the penetration of ice crystals into the cell interior is prevented at the cell surface. Ice formed on the external surface of the cell wall continuously grows, withdrawing water from the cell interior as the temperature descends. Cells frozen in this way undergo a remarkable dehydration and concentration. Extracellular freezing is not fatal to cells within certain limits of temperature and time of freezing. The limit depends upon the degree of their frost-hardiness.

I have found that the twigs of willow, birch, and poplar were not injured in winter even when cooled from -5 to -96°C at a slow cooling rate. It is clear, therefore, that with a slow cooling rate after freezing, hardy twigs can be continuously cooled down to very low temperatures without damage. On the other hand, when immersed directly from room temperature into alcohol below -15°C , even the hardest twig cannot withstand such a rapid freezing. From this it can be considered that the twig from which all the easily freezable water has been previously removed by slow extracellular freezing might survive even after immersion in liquid nitrogen (-196°C). I have succeeded in recovering twigs of willow and poplar alive after immersion in liquid nitrogen, provided they are prefrozen at temperatures below -30°C (Sakai, 1956, 1960).

In addition, using this prefreezeing method, we have also succeeded in recovering twigs of willow and poplar alive after immersion in liquid helium (-269°C) (Sakai, 1962a). These facts indicate that almost all the easily freezable water can be drawn from the cell interior by extracellular freezing at about -30°C and that the cells and the tissues in that state are not injured even when immersed directly in liquid helium provided that they have survived the prefreezeing at -30 to -40°C . Below this temperature the intensity of cold seems not to exert any important effect upon living cells.

It has long been known that frost-hardiness in woody plants, as a rule, shows remarkable periodicity through the cycle of a year. As was mentioned above, in winter the twigs of willow and poplar can survive freezing at very low temperatures, while in summer they cannot withstand freezing at -5°C even for a short period. In general, the hardier the plant the greater the seasonal variation of the frost-hardiness. It is also known that many plants increase their frost-hardiness by chilling. However, the growing twigs of woody plants are neither frost-hardy nor able to increase their frost-hardiness, even when subjected to low temperature. Soon after the growth of the twig ceases, these twigs become frost-hardy without being subjected to a low temperature. As long as they are kept at a higher temperature (15°C), their frost-hardiness cannot increase further beyond a definite level (about -7°C), but when subjected for several days to about 0°C the twigs considerably increase their frost-hardiness (Sakai, 1955).

Soon after the growth of a twig ceases, especially in deciduous trees, considerable changes take place in the cells of the twigs from late August to early September. There is a decrease of both water content and activity in the cambium cells and an increase in both starch granules and osmotic concentration. Without such changes, particularly the remarkable decrease of water content, twigs cannot be hardened either naturally or artificially. Before late August, all the cells in twigs frozen at -5°C were completely killed by intracellular freezing. After early September the capacity to prevent intracellular freezing in cells gradually increased; in late October, even comparatively rapidly frozen cells were not frozen intracellularly.

Simultaneously with this change, the resistance to denaturation from extracellular freezing also increased from early September to winter.

In hardy cells, the effective temperature for hardening is below $+10^{\circ}\text{C}$, but the effectiveness of hardening increases with decreasing temperature, within the limit of the temperature which the twig can withstand. At a given temperature, the longer the exposure the more effective within a certain limit. However, the effective temperature for frost-hardening of a twig is considerably different from the degree of its frost-hardiness. In a twig, which can withstand a continuous frozen state for several days, exposure to subzero temperatures is more effective in improving frost-hardiness than exposure to 0°C . For the purpose of maintaining the highest value of frost-hardiness in winter, in Sapporo, storage at about -5°C is the most effective.

In natural and artificial frost-hardening, the increase of frost-hardiness has been associated with fluctuations in the content of various substances. In particular, a parallelism between sugar content and frost-hardiness has generally been accepted by most investigators. However, there is no direct evidence as yet that the increase of the sugar content alone, in cells, results in the rise of frost-hardiness. I have investigated the causal relationship between the sugar content and the frost-hardiness in the cell in the same stage of given species and conclude that the increase of sugar in hardy cells directly results

in the increase of their frost-hardiness whether through frost-hardening or the artificial introduction of sugar. In addition, some polyhydric alcohols as well as acetamide showed a protective action against frost injury when artificially introduced (Sakai, 1962b). These results suggest that protection against frost injury is not unique to sugars, but other substances which can penetrate in great amounts without damage also exhibit a protective action against frost injury. However, potassium chloride and calcium chloride showed no preventive effect because of their toxic action upon the cells. In nature, the substances which are most effective in preventing frost injury in trees are sugar and polyhydric alcohols. Unlike insects, the glycerol content in plants is very small.

Unlike hardy cells, non-hardy cells such as those in the bulbs and tubers of gladiolus, dahlia and potato do not increase their frost-hardiness as a result of exposure to low temperatures, although the sugar content in the cells is increased remarkably by chilling. In the growing twigs of woody plants, the same relationship was also found. These facts show that if the cells and the tissues are not in a hardy state, the increase of sugars in the cells results in little or no increase in their frost-hardiness. From these facts, it seems very probable that the establishment of a certain protoplasmic condition is necessary for the tolerance of freezing and only in such cells can protective substances be effective in increasing their frost-hardiness.

It is reasonable to emphasize, therefore, that in discussing the process of frost-hardening and the relationship of sugar content to frost-hardiness, that the stage and conditions of the plant tissues as well as their sugar content must be taken into consideration.

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DISCUSSION

Discussion of É. Asahina's Paper

G. A. SAMYGIN: Which is the more serious obstacle to ice penetration into the cell: the protoplasmic membrane or the cellulose wall of the cell?

É. ASAHLNA: The protoplasmic membrane, as can be seen from the micro-photographs.

G. A. SAMYGIN: What is the main reason for cell death during extracellular freezing?

É. ASAHLNA: Dehydration of the protoplasm is a more significant cause, than the mechanical injury caused by ice crystals.

L. K. LOSINA-LOSINSKY: Do you not see any contradiction in the fact that both high permeability and a decrease in the dehydration rate of cell protoplasm increase frost-resistance?

É. ASAHLNA: Both of these processes are significant, and the very predominance of either process depends on certain conditions, the freezing rate, in particular.

L. K. LOSINA-LOSINSKY: May I make some suggestions in respect to the problems touched upon in the interesting paper of Prof. É. Asahina. According to his opinion, the freezing of a great amount of insect body fluids precedes cell freezing and therefore almost all frost-resistant insects have the greatest frost-resistance at the larval and pupal stage.

On the other hand, some insect species among Diptera were found to have the highest frost-resistance at the imago stage while others show highest resistance at the egg stage. In general the winter stage possesses the highest degree of frost-resistance, but different species are not similar at this stage. Also, I do not think, that it has been proven that freezing of insect body fluids occurs without intracellular freezing. There are reasons to feel that a few insects which survive freezing, can endure intracellular formation of ice crystals.

Nevertheless, I agree with a number of assumptions made by Prof. É. Asahina. To my mind his most important statement concerns the fact that in the presence of hydrophilic substances in the environmental medium adjacent to the ice front, a layer of concentrated solution develops to protect the cell from freezing. The protective action of glycerol may be the result of this phenomenon.

I agree that the general principles and mechanisms of low temperature resistance both in animal and plant cells need to be looked for. However, we shall fail to do this if we disregard those specific peculiarities by which the frost-resistance of cells of animals differs from that of plants.

BIOCHEMICAL AND PHYSIOLOGICAL ASPECTS OF PLANT FROST-RESISTANCE

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IT HAS become a custom in the field of frost-resistance of plants to follow the biochemical changes, which take place in the cell during hardening and de-hardening, in order to learn something about the mechanism of the frost-resistance of cells.

We followed this well-established custom and compared the fluctuations of sugars, proteins and amino acids with changes in frost-resistance. However, after a few years we became somewhat confused: we found, as had other authors before us, a general agreement between sugar content and frost-hardiness. We did not find much correlation between proteins and frost-hardiness. We found still less or no correlation between amino acids and hardness. Obviously the living cell is much too complicated to reveal by such simple experiments the way in which it achieves protection against frost.

Since the living cell seems to be too complicated, we tried to obtain simpler systems: we broke the cell down and investigated the response of a few cell fractions to freezing. In this way we arrived at a number of results which shall be dealt with in this report.

If one freezes solutions of cell proteins, precipitation of a considerable part of the proteins occurs. This phenomenon has been observed by a few investigators, but the actual meaning of it has, to my knowledge, not been made clear. It may or may not reflect the coagulation of proteins in the protoplasm of frost-killed cells *in vivo*.

We have been able to isolate those proteins, which are precipitated by freezing, from other proteins by sedimentation in the ultracentrifuge. The frost-sensitive protein fraction comprises about 60 per cent of the total protein of the leaf cell and contains a high amount of lipoidal material. It can be termed, therefore, a lipoprotein fraction. In the field of animal research Lovelock (1957) has shown that lipoproteins are frost-sensitive.

Addition of sugars to the frost-sensitive protein fraction leads to protection of the proteins against the action of freezing, i.e. against precipitation. There is an inverse correlation between the amounts of sugars added and the amount of protein precipitated by frost. Protection is not afforded by high molecular weight carbohydrates such as starch, or by salts. Based on our findings, we published in 1958 a protein denaturation theory of frost-resistance very

similar to that proposed recently by Levitt (1962). However, we did not specify the role of SH, since lipoproteins contain, besides SH groups, other reactive centres, which might be affected by freezing and which can be protected by sugars and other compounds. We are not the first to consider protein denaturation as a possible cause of injury. This had already been done 60 years ago by Gorke (1906) and much support has come in recent years from Alexandrov and co-workers. Our theory is founded on the behaviour towards frost of isolated cell proteins.

TABLE 1. THE EFFECT OF FREEZING ON CYCLIC AND NON-CYCLIC PHOTOPHOSPHORYLATION BY BROKEN CHLOROPLASTS OF SPINACH

Results are expressed in μ moles phosphate uptake or ferricyanide reduction per mg chlorophyll/hr. Experimental procedure: Chloroplast isolation in 0.05 M tris(hydroxymethyl)aminomethane containing 0.35 M NaCl, 0.01 M phosphate, 0.01 M ascorbate, 0.005 M cysteine, pH 7.8. Subsequent washing in the same medium, but without ascorbate and cysteine. Osmotic rupture of the chloroplasts in water yielding "broken chloroplasts". Freezing for 3 hr at -25°C , if not indicated otherwise. After thawing addition of chloroplasts containing about 60 μg chlorophyll to the following reaction mixture (μ moles/ml): tris(hydroxymethyl)aminomethane 14, NaCl 30, phosphate 2.2; adenosine diphosphate 2.2, MgCl_2 3.5; pH 7.8; total volume 1.2 to 2.4 ml. Addition of 2 to 4 μ moles ferricyanide or 0.3 μ moles phenazine methosulphate respectively. Illumination for 5 to 10 min. Determination of ferricyanide reduction spectrophotometrically at 400 m μ and of phosphate uptake according to Berenblum and Chain (non-cyclic photophosphorylation) or Fiske and Subbarow (cyclic photophosphorylation).

Experiment	Cyclic photophosph. phosphate uptake	Non-cyclic photoph. phosphate uptake	Ferricyanide reduction (total)
1. Unfrozen	304	143	344
Frozen	5	0	606
2. Unfrozen	242	138	506
Frozen	20	12	464
3. Unfrozen	186	112	479
Frozen	0	1	475

But even after a theory is published the question may be raised, whether it is sufficiently supported by experimental facts. In our special case, has the precipitation of proteins observed after freezing *in vitro* anything to do with the frost-killing of the cells *in vivo*? We have tried to answer this question by a number of biochemical experiments both with intact cells and with cell fractions.

As a test system we often use isolated chloroplasts. During photosynthesis electron transport takes place in the lamellae of the chloroplasts. In the course of this electron transport, light energy is converted into chemical energy, which is stored in part in the pyrophosphate bonds of adenosine triphosphate.

From the important work of Arnon two reactions are known, in which ATP is synthesized in the light from ADP and phosphate, namely by non-cyclic

photophosphorylation and cyclic photophosphorylation. In the former oxygen is evolved and an oxidant is reduced as is the case during photosynthesis in the intact cell. In the latter ATP synthesis is not accompanied by gas production or consumption. Both reactions provide the main part of the energy necessary to drive the living machinery of autotrophic cells.

During freezing, the synthesis of ATP in cyclic and non-cyclic photophosphorylation becomes impaired. After freezing of isolated chloroplasts from spinach, synthesis of ATP can no longer be observed, although, at least in non-cyclic photophosphorylation, electrons are still transferred in the light to the respective acceptors (Table 1). Electron transport is not only not inhibited by freezing, but actually stimulated (Table 2). Therefore the suppression of photophosphorylation reflects an uncoupling of ATP synthesis from electron transport.

TABLE 2. EFFECT OF FREEZING ON RATES OF FERRICYANIDE REDUCTION BY BROKEN CHLOROPLASTS OF SPINACH IN THE LIGHT

Experimental procedure as outlined in legend to Table 1, but short illumination times to permit estimation of initial rates of reduction.

Experiment number	Maximal reduction rates in μ moles/mg chlorophyll per hr		
	Unfrozen	Frozen	Unfrozen Frozen
1	1520	1580	0.96
2	950	1175	0.81
3	1520	1675	0.91
4	935	1078	0.86
5	560	880	0.64
6	736	970	0.75
7	344	606	0.57

TABLE 3. UNCOUPLING OF NON-CYCLIC PHOTOPHOSPHORYLATION AFTER DIFFERENT TIMES AT -25°C

Experimental conditions as in Table 1.

Freezing times in min	0	30	60	90	120	180
Coupling rate (= % of the stoichiometric rate of phosphorylation)	54	46	20	18	5	0

The uncoupling of photophosphorylation by freezing shows the following characteristics: Uncoupling takes place at various temperatures below the freezing point of water. At -25°C uncoupling is completed after about 2 hr (Table 3). Uncoupling is prevented by the addition of sugars prior to freezing. Two per cent of sucrose in the solution is sufficient to afford complete protection (Fig. 2). Besides sucrose, other sugars are also effective. The protective

action of sugars on photophosphorylation is very similar to the protection afforded against frost precipitation of susceptible proteins (compare Fig. 1 and Fig. 2). It is interesting to note, that a sugar concentration of 1 to 2 per cent, which is required for maximal protection in photophosphorylation, is available, on a fresh weight basis, in resistant cells.

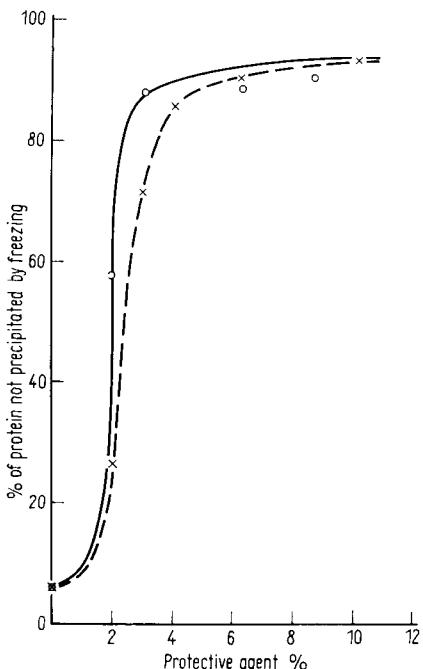


FIG. 1. Protective influence of different concentrations of glucose and bovine albumin on the precipitation of frost-sensitive proteins of winter wheat by freezing. Frost-sensitive proteins were obtained from leaf homogenates by centrifugation at 30,000 g and were re-suspended in dilute buffer. Frost-precipitated proteins were determined gravimetrically after brief centrifugation at 100 g. Freezing temperature -20°C . \times glucose; \circ bovine albumin.

In the leaf cell, ATP is formed not only by photophosphorylation in the chloroplasts, but also during the oxidation of respiratory material in the mitochondria, while in cells lacking chlorophyll, respiration is the main source of ATP production. Oxidative phosphorylation of respiration takes place in the mitochondrial membrane.

We have investigated the response to freezing of mitochondria isolated from cauliflower and spinach. Freezing of washed mitochondria always results in the loss of oxidative phosphorylation. Frozen mitochondria may or may not take up oxygen. This indicates that, besides a possible uncoupling of phosphorylation due to freezing, the electron transport of mitochondria is affected.

Loss of the ability of rat liver mitochondria to form ATP after freezing has, in connexion with other investigations, already been observed by Porter and co-workers.

As is the case in photophosphorylation, addition of sugars to isolated mitochondria protects oxidative phosphorylation against freezing.

It had still to be shown that destruction of oxidative phosphorylation, which has been observed *in vitro* in chloroplasts and mitochondria, also occurred during the freezing of intact cells. To show this, we performed the following experiments: we killed leaves of wheat and spinach by freezing them to -20°C

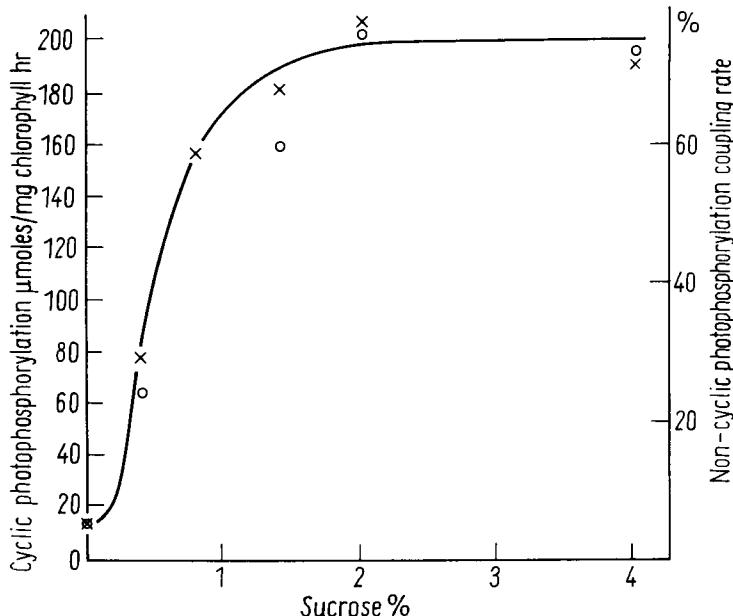


FIG. 2. Protective effect of sucrose on uncoupling of photophosphorylation by freezing. \times cyclic photophosphorylation; \circ non-cyclic photophosphorylation. Experimental conditions as outlined in Table 1 except that broken chloroplasts were frozen in solutions containing various amounts of sucrose.

and isolated immediately afterwards chloroplasts from the frozen material. These chloroplasts proved to possess only a very limited capability of synthesizing ATP in the light as compared with chloroplasts isolated from unfrozen leaves (Table 4).

Furthermore, in frost-killed leaves there is only very little or no photosynthetic $^{14}\text{CO}_2$ incorporation (which requires ATP) into organic compounds.

In another experiment frozen and unfrozen leaves were supplied with inorganic phosphate containing ^{32}P . Only the unfrozen leaves were able to incorporate ^{32}P into ATP and other compounds derived therefrom.

That mainly phosphorylation reactions are affected by frost-killing of leaves is indicated by the following experiment: living and frost-killed leaves were supplied with radioactive sucrose. In both cases the sucrose is converted into a number of organic compounds including organic acids. The soluble enzymes responsible for these transformations are obviously not affected by freezing, while the insoluble enzyme systems of photophosphorylation and of oxidative phosphorylation are affected.

TABLE 4. CYCLIC PHOTOPHOSPHORYLATION OF INTACT AND BROKEN CHLOROPLASTS, WHICH WERE ISOLATED FROM FROZEN (FROST-KILLED) AND UNFROZEN LEAVES OF WINTER WHEAT AND SPINACH

Chloroplast isolation and determination of phosphorylation as indicated in Table 1.
Freezing of leaves at -20°C .

Experiment number and material	Photophosphorylation in $\mu\text{moles}/\text{mg chlorophyll per hr}$, and chloroplasts isolated:	
	From frozen leaves	From unfrozen leaves
1. Wheat	Intact chloroplasts 17.7	63.3
2. Wheat	Intact chloroplasts 10.4	42.2
3. Wheat	Broken chloroplasts 17.6	47.0
4. Wheat	Broken chloroplasts 7.1	16.2
5. Spinach	Intact chloroplasts 0.0	326.0
6. Spinach	Intact chloroplasts 4.0	304.0

Thus freezing of intact cells yields results very similar to those observed with cell fractions and with isolated cell organelles. It can be concluded, therefore, that destruction due to freezing, of the phosphorylation reactions which provide the energy necessary to maintain life, takes place *in vitro* and *in vivo*.

Uncoupling or destruction of the ATP synthesis in photosynthesis or respiration can be prevented by sugars. From the *in vitro* experiments it is clear that contact between the protective agent and the sensitive protein is required for protection. No protection is afforded if the sensitive proteins and the protective agents are separated by a semipermeable membrane. In order to be protective, the sugars must therefore be located in the protoplasm of the intact cell, while the sugars in the vacuole are ineffective or, at best, only of indirect influence. With the aid of a method of chloroplast isolation specially developed for this purpose we succeeded in demonstrating that the protoplasm of resistant plants is capable of accumulating sugars and soluble proteins (Heber, 1957 and 1959). These findings may explain, at least in part, the infrequent correspondence between sugar content and frost-resistance in intact cells. Direct correlations are to be expected only between the amounts of protective agents in the protoplasm and its resistance. The amount of protective agents in the vacuole of the plant cells may overshadow such a positive correlation.

The destruction of oxidative phosphorylation and of photophosphorylation during freezing leads to the incapability of the cell to produce amounts of ATP sufficient to maintain metabolism. All energy-producing or energy-requiring processes in the cell are connected, directly or indirectly, with the turnover of ATP. Lack of ATP and the incapability of the cell to synthesize ATP results in the breakdown of metabolism and is, therefore, sufficient to cause the death of the cell.

Enzymes are responsible for the formation of ATP from ADP and phosphate. Freezing, or dehydration due to freezing, apparently alters the state of the proteins involved in phosphorylation in such a way that activity is lost. This effect thus deals clearly with the molecular level of life.

Ilijin (1933) concluded, on the other hand, that frost causes mechanical damage to the cells. Levitt (1956) followed the same line in his earlier mechanical theory of frost-resistance in that mechanical stress is supposed to cause the death of the cell. However, our results do not fit in any way the mechanical theory of frost-resistance, if this theory refers to mechanical damage in its usual gross sense, i.e. to damage caused by mechanical disruption of biological structures. Without denying the existence of tensions, to which the protoplasm is exposed during freezing, we propose another interpretation of the action of frost. In addition, we would like to put our protein denaturation theory of 1958 on a more solid experimental base.

Chloroplasts are capable of forming ATP in the light even after they have been disintegrated by osmotic rupture and by ultrasonic treatment beyond microscopical visibility. Removal of water due to freezing, however, results in the complete abolition of the ability to synthesize ATP. Clearly a mechanical interpretation of this phenomenon is not possible. Rather the primary action of frost consists in a destruction of sensitive lipoprotein structures and, at the same time, of biochemical processes, which are of the utmost importance in maintaining the life of a cell. This destructive effect is caused by dehydration. Sugars and, probably to a lesser degree, other substances provide protection to sensitive proteins by direct interaction or indirect interaction via bound water. Possibly hydrogen bonding plays a major role in the destructive effects of freezing and in the protection afforded by sugars and other compounds. A prerequisite of protection consists in the direct contact of the protective agent and the sensitive sites.

In our view hardening requires: (1) the production of amounts of soluble sugars and/or other protective substances sufficient to afford protection, and (2) a suitable distribution of those substances between the protoplasm or the sensitive parts of the protoplasm and the vacuole. Both processes may be interrelated in plants capable of hardening. A simple production of sugars would not be enough to result in the rise of resistance, since the sensitive structures within the protoplasm may not be readily accessible to sugars and other protective agents except in the case of hardening.

The survival of the cell is thus made possible by the protection of sensitive protein structures against the destructive effects of freezing.

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THE AUTOFLUORESCENCE OF CELLS OF SOME NORTHERN FOREST PLANTS WITH REGARD TO THEIR FROST-RESISTANCE

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CELLS of many forest plants have a bluish autofluorescence. Using fluorescence as a test we can observe the accumulation and transformation of fluorochromes in a cell.

Cells of the bark parenchyma of red-berried elder (*Sambucus racemosa*), cherry (*Prunus cerasus*) and goat willow (*Salix caprea*) have been studied and tested. Bark parenchyma of elder which consists of rather large layered cells

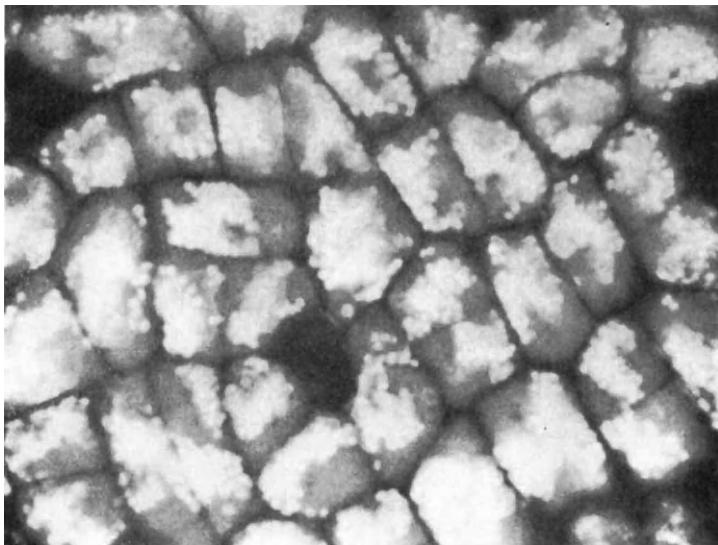


FIG. 1. Fluorescence of bark parenchyma cells of elder in the state of dormancy
in winter.

is a most convenient subject for microscopic study. The layers of cells are readily separated from each other almost without damage.

Protoplasmic streaming is observed in such preparations.

Some substances with a blue fluorescence were separated from extracts of cherry, elder and willow bark parenchyma by means of chromatography. These substances have a maximum radiant absorption within 275–285 m μ , and in some features they are similar to flavonoids and glucosides. The polyphenol nature of these substances cannot be doubted (Krasavtsev, 1962).

According to available published data, the content of polyphenol substances in forest plants changes with the season (Levitt, 1956; Paech, 1950; Parker, 1957). The same is true of fluorescent substances. A bluish-grey fluorescence in cells of bark parenchyma is clearly observed in late autumn and in winter.

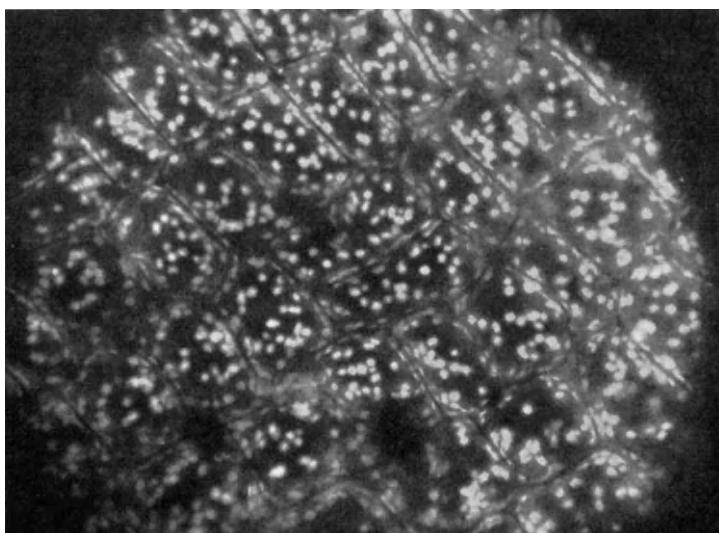


FIG. 2. Fluorescence of bark parenchyma cells of elder in the growing state in summer.

In growing shoots the chloroplasts fluoresce in summer (Figs. 1 and 2). Accumulation of fluorochromes in living cells coincides with the inhibition of their growth. This fact suggests the idea that these substances bear the character of growth inhibitors.

The inhibiting effect of fluorochromes was determined by biotests on wheat coleoptiles (Boyarkin, 1947, 1948). Fluorochromes were separated into bands on a chromatogram. Fluorescing bands were eluted by water and their concentration was determined spectrophotometrically.

The data in Table 1 shows that fluorescent substances of bark parenchyma have the properties of growth inhibitors.

It is known that, having stopped their growth and having entered the period of dormancy, plants increase their frost-resistance, especially during hardening (Tumanov, 1960). Some data, cited in this paper, show the correlation between fluorochrome content in cells and their frost-resistance.

TABLE 1. EFFECT OF FLUOROCHROMES OF BARK PARENCHYMA ON THE GROWTH OF WINTER HEAT COLEOPTILES

Number of fluorochromes	R_F on descending chromatogram. Butanol-acetic acid-water	Colour of fluorescence	Fluorochrome concentration in mg/ml	Increase of coleoptiles in % of control
<i>Cherry fluorochromes</i>				
4	0.85	Light-blue	5×10^{-3}	78
<i>Elder fluorochromes</i>				
1	0.53	Bluish-white	3×10^{-2} $8-10^{-2}$	65 36
2	0.65	Blue	3×10^{-3} 10^{-2}	35 43

1. Correlation between Fluorochrome Content in Cells and Their Frost-resistance

The microscopic study made in August showed that in this month cells of different elder shoots and tissues had different intensities of fluorescence. Freezing of pieces of elder tissues in water and in 10 per cent sucrose solutions revealed a change in frost-resistance parallel to the change in fluorochrome content (Table 2).

TABLE 2. FROST-RESISTANCE OF VARIOUS ELDER TISSUES IN AUGUST

Object	Intensity of fluorescence	Results of freezing (°C)			
		In water		In 10% sucrose	
		Living	Dead	Living	Dead
<i>Bark-parenchyma</i>					
2-yr shoots	Intense	-10	-15	-60	
1-yr shoots with growth complete	Weak	-4	-7	-20	-25
1-yr growing shoots	Almost invisible	-4		-4	-4
<i>Epidermis</i>					
1-yr growing shoots	Weak			-4	-7
					-10

In further tests the content of fluorochrome in the cells was increased artificially. Pieces of parenchyma were placed into an eluate of the fluorescing bands cut out of the chromatogram. As a result of such treatment the cells displayed an observable fluorescence. After artificial fluorochroming, the cells were frozen in a 10 per cent sucrose solution. In some cases these cells sur-

vived a temperature of -30°C in a sucrose solution while unfluorochromed cells died at -4°C . A preliminary sucrose treatment gave a weaker effect (Table 3). The treatment with a weak tannin solution resulted in some increase in frost-resistance.

TABLE 3. FROST-RESISTANCE OF PARENCHYMA CELLS OF ELDER AND CHERRY GROWING SHOOTS AFTER THEIR TREATMENT WITH FLUOROCHROMES

Concentration: elder fluorochromes—no. 1 2×10^{-2} , no. 2 8×10^{-3} ; cherry fluorochromes—no. 4 3×10^{-2} mg/ml.

Method of treatment	Results of freezing in 10% sucrose solution ($^{\circ}\text{C}$)	
	Living	Dead
<i>Elder</i>		
No treatment	—	-4
Fluorochrome no. 1—3 hr at 2°C	-20	-25
Fluorochrome no. 2—3 hr at 2°C	-30	—
<i>Cherry</i>		
No treatment		-5
Fluorochrome no. 4—6 hr at 3°C	-20	-25

TABLE 4. FROST-RESISTANCE OF WINTERING CELLS OF BARK PARENCHYMA AFTER THEIR TREATMENT WITH FLUOROCHROME

Concentration: cherry fluorochrome no. 4 9×10^{-2} ; elder fluorochrome no. 2 8×10^{-2} mg/ml.

Method of treatment	Results of freezing (in water) ($^{\circ}\text{C}$)			
	Rapidly (1°C per 2-3 min)		Slowly (5°C per 24 hr)	
	Living	Dead	Living	Dead
<i>Elder</i>				
No treatment	-25	-30	-60	
Elder fluorochrome no. 2, 48 hr	-40	-45	-30	-40
The same plus 10% sucrose, 24 hr	-20	-25	-60	
Cherry fluorochrome no. 4, 24 hr	-5	-10	-20	-30
The same plus 10% sucrose, 24 hr	-10	-15	-60	
<i>Cherry</i>				
No treatment	-15	-20	-40	
Cherry fluorochrome, 24 hr	-10	-15	-15	-20
The same plus 10% sucrose, 24 hr	-15	-20	-30	-40

After the treatment with fluorochromes the shape of plasmolysis was different from normal with the same plasmolytic agents: 5 per cent sodium chloride or 30 per cent sucrose. In untreated pieces of parenchyma from grow-

ing shoots, lasting concave plasmolysis was observed. After fluorochroming we observed rapid convex plasmolysis. Many scientists found similar differences in the forms of plasmolysis during vegetation or in the state of dormancy depending on the season (Levitt, 1956; Parker, 1960; Genkel and Oknina, 1948; Sitnikova, 1950).

It should be noted that the cells in the fluorochrome solution died if they remained there more than 48 hr. In the case of higher fluorochrome concentrations the cells died more rapidly, and a yellow-green fluorescence of protoplasm was observed.

After keeping the wintering cells in the fluorochrome solutions, their fluorescence intensity increased. In this case they retained their ability to plasmolyse, but their frost-resistance decreased. The activity of various fluorochromes is probably specific for different species of plants (Table 4). The negative effect of the greater amount of fluorochromes is more evident during rapid freezing.

In the case of slow freezing during the second hardening stage (Tumanov, 1960), the toxicity of an artificially induced excess fluorochrome content in cells decreased.

Tissue treatment with a sucrose solution, especially during further hardening, decreased the harmful activity of fluorochromes.

Low negative temperatures probably resulted in the change of state of the fluorochromes in the cells.

2. Change of State of the Fluorochromes in the Cells during Freezing

At positive temperatures, the fluorochromes of living cells are accumulated mainly in the vacuoles (Klein and Linsér, 1930; Larcher, 1953; Oppenheimer and Jacoby, 1961; Krasavtsev, 1962). Almost the whole cell has a bluish auto-fluorescence which serves as a background for the red spots of the chloroplasts.

During freezing, the fluorescence becomes greenish. Living cells, when frozen have a greenish-white fluorescence. The temperature drop results in an increased brightness of the fluorescence. The bright green-yellow fluorescence may serve as a criterion of the cells' death.

Formation of intracellular ice results in a green-yellow fluorescence of the whole cell. If ice is formed in the intercellular space the cells will shrink and change their shape. In this case some parts of the cell do not fluoresce, but form dark spots. These spots are located in the vacuole areas. Thus, the fluorescence of the vacuoles is partially lost. The protoplasmic portions of the cell, on the contrary, fluoresce more brightly. From these observations it was concluded that during freezing fluorescing substances left the vacuoles for the protoplasm (Krasavtsev, 1962). However, in frozen tissues the redistribution of the fluorochromes in the cell does not clearly manifest itself.

This phenomenon was studied by cell fixation, with the use of freeze-substitution (Pearse, 1962).

The fluorescence of the frozen tissues does not disappear after fixation with acetone as a solvent. Fixation with acetone at room temperature results in fluorochrome diffusion inside the cell. Due to this fact, a control procedure was based on fixation after rapid freezing (vitrification) in liquid nitrogen.

Control objects were quickly plunged in acetone cooled to -60°C after they had been plunged in liquid nitrogen.

Experimental pieces of bark parenchyma were slowly or rapidly frozen to -60°C , and afterwards they were plunged into acetone at the same temperature. In an additional procedure objects were frozen first only to -10°C , and then they were consecutively plunged into liquid nitrogen and cooled acetone.

After fixation (for 5 days) the temperature of the acetone was raised to room temperature, and the preparations were consecutively transferred to benzene, benzene-paraffin oil mixture, and paraffin oil. The survival of cells after various freezings prior to fixation were estimated in parallel tests.

The comparison of fixed tissues with the tissues in a frozen state showed that fixation did not change the shape of the cells. In the test of rapid freezing down to -60°C , intracellular ice was partially formed. The cells filled with ice after fixation had a complete yellow-green fluorescence (Fig. 3).

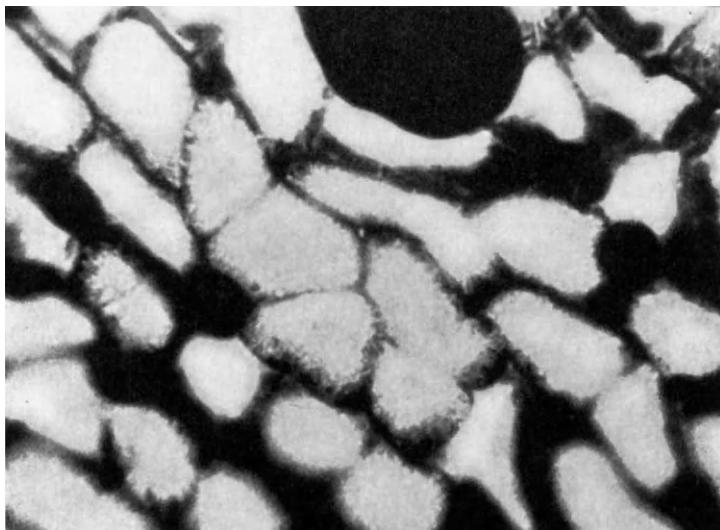


FIG. 3. Fluorescence of bark parenchyma cells fixed after rapid freezing to -60°C . Partly intracellular and partly extracellular ice.

During slow freezing, the tissues did not die and the ice was accumulated outside the cells. After fixation, the greenish fluorescence was brighter as a result of slow freezing to -60°C than as a result of freezing to -10°C . The

greenish fluorescence was not complete. There were unfluorescing parts in every cell, and the fluorescing part of the cell retained the same shape as in the frozen state (Fig. 4).

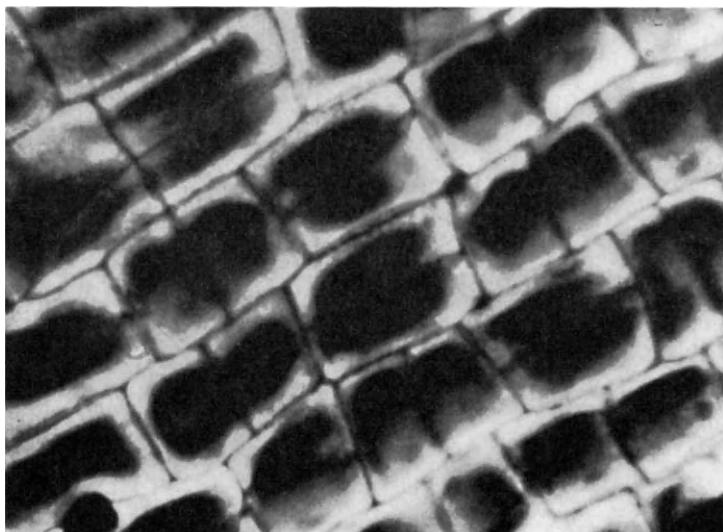


FIG. 4. Fluorescence of bark parenchyma cells fixed after slow freezing down to -60°C . No fluorescence in the central part of cells (vacuoles). Protoplasm is fluorescing.

Fixed preparations were studied with the help of immersion lenses and phase-contrast devices. A nucleus, chloroplasts (colourless as a result of chlorophyll extraction) and smaller elements of protoplasm were found in the fluorescing part of the cell. These tests corroborated the conclusion that the protoplasm possessed fluorescence.

In pieces of bark parenchyma vitrified in liquid nitrogen, after thawing in warm water (30°C), most cells preserved the ability to be plasmolysed. Cells fixed after vitrification did not differ in their form and distribution of elements from the picture observed in living cells at a positive temperature.

The main mass of cells in preparations, fixed after vitrification, had almost no fluorescence. Cell walls had a very weak fluorescence, and protoplasm a still weaker one. Fluorochromes, present in the vacuoles during fixation, were more or less fully extracted by acetone. Fluorochromes soluble in acetone were not absorbed by the protoplasm. During fixation acetone might penetrate into the protoplasm and the vacuoles till the very moment when water began to leave the cell.

The results of the tests with fixation in a frozen state confirm the earlier conclusion (Krasavtsev, 1962) that there is a translocation of fluorochromes

inside the cell during freezing. Fluorochromes leave the vacuoles and are more or less closely bound in the protoplasm. Fluorochromes are easily extracted from the vacuoles by acetone. The process of extraction from protoplasm is more difficult. The fluorescence of protoplasm disappears when preparations are kept for a long time in a 10 per cent solution of ice-cold acetic acid in acetone, in methyl alcohol, and in water. The fluorescence of protoplasm disappears very quickly in aqueous solutions of sodium or potassium hydroxide (0.1 per cent).

What is the mechanism of the interrelation of fluorochromes and protoplasm? The data (Krasavtsev, 1962) indicate that yellow-green fluorescence of the protoplasm appears during the interaction of blue fluorochromes with substances containing amino-groups. Thus, according to this interpretation, the presence of fluorochromes in protoplasm must result in the blocking of amino groups.

To determine the presence of free amino groups the preparations were fixed after freezing and were immersed in a 0.5 per cent ninhydrin solution for 3 hr, then they were transferred into paraffin oil. In fixed cells, after vitrification, its protoplasm showed a violet-blue stain. After slow freezing down to -10 and -60°C, a weaker coloration was observed but not in all cells. In the test of rapid freezing down to -60°C the above-mentioned did not produce any staining with ninhydrin.

After treating preparations with a 10 per cent acetic acid solution in acetone, the protoplasmic fluorescence grew very weak. In all tests, including that of rapid freezing down to -60°C, blue staining by ninhydrin was observed.

Thus, the idea that the fluorochromes available in the protoplasm can block amino groups is confirmed.

In 1 per cent chloride solutions in all kinds of fixation after preliminary freezing (rapid and slow down to -10 and -60°C) a grey-green staining of the protoplasm appeared indicating a considerable amount of substances of a polyphenol nature. When fixed, after vitrification, with ferrous chloride only a light orange coloration was observed.

The mechanism of interaction of the fluorochromes and the protoplasm should be different for the cells killed by freezing and for hardened ones. Migration of the fluorochromes from the vacuoles into the protoplasm takes place both on injury due to freezing and at low temperatures which do not cause any damage.

In the latter case the displacement of fluorochromes may be reversible.

The reasons for the fluorochrome displacement inside the cell follow:

1. diffusion of the cell sap as a result of water release;
2. a rise in fluorochrome concentration in the cell sap as a result of cell dehydration during ice formation in the intercellular spaces;
3. a rise in tonoplast permeability at low temperatures.

In the case of frost-killing the fluorochrome displacement may be stimulated by the damage to the protoplasm.

On the basis of tests with artificial fluorochroming, we can conclude that quick penetration of fluorochromes into protoplasm in excess quantities may be harmful to it.

Conclusions

During the inhibition of the growth of shoots and their entrance into dormancy, a bluish fluorescence appears in the cells of the bark parenchyma of some woody plants. This phenomenon should be attributed to the accumulation of fluorescing substances of a polyphenol nature.

Artificial introduction of these substances into parenchyma cells of growing shoots results in a bluish fluorescence. A small amount of fluorochromes may increase the frost-resistance of the cells of growing shoots. Excess fluorochroming, however, is harmful.

Both the treatment of tissues with sucrose and cold-hardening decrease the toxicity of excess fluorochromes.

In living cells, at positive temperature, the fluorochromes are accumulated mostly in the vacuoles. During the microscopic study of frozen and fixed tissues it was found that at negative temperatures the fluorochromes left the vacuoles for the protoplasm, where the fluorochromes could block amino groups. The state of other active groups may be changed as well.

The study of this phenomenon may bring forth new ideas of the mechanism of frost-killing and hardening against frosts.

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PROTOPLASMIC DEHYDRATION AS ONE OF THE CAUSES OF CELL DEATH FROM THE FORMATION OF EXTRACELLULAR ICE

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PROTOPLASMIC dehydration is considered to be one of the causes of cell death by extracellular ice (Müller-Thurgau, 1886; Molisch, 1897; Kessler, 1935; Scarth, 1944; Asahina, 1956; Heber, 1958). But this theory is in contradiction to the results of freezing experiments on sections in protective solutions. The cells survive in these solutions at much lower temperatures and, consequently, at a higher degree of plasma dehydration. Our investigations were undertaken to clear up the problem of the significance of protoplasmic dehydration by freezing.

The experiment was as follows:

Sections of kale stems (*Brassica oleracea* L. var. *acephala*) were divided into three groups: (1) infiltrated with water; (2) infiltrated with a glycerin solution about 0.5 M and then gradually transferred to a glycerin solution, $\Delta t^\circ = 5.6^\circ$; (3) without infiltration.

Sections of the first group were placed in test tubes on wet filter paper and frozen to different temperatures at the rate of $1^\circ\text{C}/10-20$ min, ice being added at -1°C . These sections were kept at the final temperatures for 18 hr, then slowly thawed and placed into beakers with water in order to determine, by decoloration, the degree of their injury.

Sections of the second group were divided into two subgroups. One subgroup was frozen at different temperatures, by being placed into tubes on the paper which was wetted with the glycerin solution, $\Delta t^\circ = 5.6^\circ$; the rate and the duration of cooling was the same and ice was added at -7°C . After slow thawing the sections were placed into beakers with the same solution and then gradually transferred to water. After each 20-40 min the sections of the second subgroup were placed into more concentrated glycerin solutions, which differed from the previous one in Δt° by $2-5^\circ$, until the sections reached one of the concentrations, the Δt° of which was equal to one of the final freezing temperatures of the sections of the first subgroup of this group.

As the sections were placed into more concentrated solutions the beakers with these sections were exposed to lower temperatures from -4 to -30°C ; the solutions, however, were prevented from freezing. The sections were left in the final solutions for 18 hr, then they were transferred from the more concentrated solutions into weaker ones, accompanied by a slow rise in their temperature, and finally transferred to water.

Sections of the third group were placed on watch glasses in sealed beakers, which held a layer of sodium chloride or sulphuric acid solutions of fixed concentrations in order to maintain the relative humidity of the beaker's atmosphere stable. At 0°C the sections were air-dried to the humidity of the beaker's atmosphere for 3 days. Then they were placed for one day into a humid chamber and then on wet paper and into water.

After all these treatments and one day's retention in water, the area of the sections which retained its coloration was visually determined (to the nearest 10 per cent) and the mean of the experimental values was derived (each series consists of 5–6 sections); the coloured area was considered alive. The experiment described made use of the sections taken from well-hardened plants (growing at $+2^{\circ}\text{C}$) and slightly hardened ones (growing at $+10$ to $+15^{\circ}\text{C}$). To save space we will not describe the results of this experiment in detail, but have summarized them in Table 1. This lists the values which cause 20 and 70 per cent killing of cells. These values give the Δt° of the solutions used during drying (Δt°_1) or for plasmolysis (Δt°_2), or the actual freezing temperatures (t°). When $t^{\circ} = \Delta t^{\circ}_1 = \Delta t^{\circ}_2$ the sections were affected by equal forces of dehydration and consequently cells were losing the same amounts of water. The final temperatures and Δt° of the solutions for different treatments were checked at $3\text{--}5^{\circ}$ intervals.

TABLE 1. CHARACTERISTICS OF CONDITIONS WHICH CAUSE THE INJURY OF KALE STEM SECTIONS DURING FREEZING, DRYING AND PLASMOlySIS

Plant hardening	t° during freezing		Δt° of solutions during:	
	In water	In glycerin with $\Delta t^{\circ} = -5.6^{\circ}$	Drying	Plasmolysis
Less than 20% of dead cells				
Weak	-8	-45	-7	-45
Good	-16	-50	-12	-60
More than 70% of dead cells				
Weak	-10	-55	-9	-55
Good	-20	-60	-20	-60

The data in Table 1 show that there is a correlation between the levels of cell dehydration which cause cell death during freezing and drying, and there is a higher resistance to dehydration in hardened cells. However, cells in

protective glycerin solutions withstood much lower temperatures, i.e. greater protoplasmic dehydration. This means that during freezing without protective solutions and during drying, protoplasmic dehydration cannot be the main cause of the death of cells for the protoplasm is able to withstand a more intensive dehydration than this. The phenomenon may be attributed to mechanical factors which affect protoplasm more and more injuriously as the cells are losing water (Iljin, 1933; Tumanov, 1940; Scarth, 1944; Levitt, 1956; Samygin and Matveyeva, 1961, 1963).

The correlation of the degree of the dehydration of cells which leads to their death during freezing in the protective solutions and during osmotic dehydration shows that in both cases there is a common cause of death, i.e. dehydration of the protoplasm up to the critical level; the hardened cells being more resistant in this case too. In other experiments they survived in glycerin and saccharose solutions at -190°C and even at -250°C ; but the unhardened cells were always killed. This fact proves that the protoplasm is able to withstand very great dehydration at such low temperatures. It was shown by Maximov's experiments (1913) that the protective action of solutions parallels their concentrations; we observed the survival of kale cells, at such low temperatures as in the preceding experiment, only in solutions with Δt° of about -4° or lower.

We found in our other work (Samygin and Matveyeva, 1963) that the higher protective action of the solutions associated with their higher concentration can be explained by a weakening of the mechanical effects on the protoplasm. However, the concentration of the protective solution should also influence the reverse absorption of water by the protoplasts during thawing. The higher the concentration of the solution, the less is the amount of water reabsorbed by the protoplasts during thawing, also the extension by the swelling vacuole (i.e. the mechanical effect is less), and the less is the degree of swelling of the protoplasmic colloids. In addition, the swelling itself as well as the absorption of water by the whole protoplast proceeds more uniformly during the whole period of thawing when the solution concentrations are higher, and not so uniformly when concentrations are low (Table 2).

The influence of the concentration of the solutions on the water absorption by protoplasts during thawing should alter the degree of injury after freezing. This is proved by the following experiments (Table 3). On paper wetted with protective solutions in beakers we froze kale stem sections infiltrated with the same solutions. Before thawing, these sections were placed into another beaker with ice which was at the same temperature as the sections (Table 3). Because of this the sections were in water as soon as the temperature had reached 0°C ; this resulted in the rapid absorption of water by protoplasts, increasing their size and swelling the protoplasm. Control sections were thawed without being transferred to ice.

Table 3 shows the condition of all the sections after their gradual transfer to water. It is clearly seen that the injury of the sections thawed on ice was

greater than that of the control ones except when these had died, as happened with two weaker solutions at -30°C . After exposure to -25 and -20°C , thawing on ice did not injure the sections infiltrated with the solution of the higher concentration. After -20 and -25°C it was observed that the weaker the concentrations of the infiltrated solutions, the more evident was the harmful effect of thawing on ice.

TABLE 2. AMOUNT OF ICE CONVERTED TO WATER AT DIFFERENT TEMPERATURE INTERVALS DURING THAWING OF SACCHAROSE SOLUTIONS OF DIFFERENT CONCENTRATIONS (CALCULATED DATA). (PER CENT OF THE WHOLE AMOUNT OF WATER IN THE SOLUTION)

Solution concentrations	Amount of ice converted to water during the temperature rise indicated in each column							
	-20 to -14°	-14 to -10°	-10 to -6°	-6 to -4°	-4 to -2°	-2 to 0°	in all	
10	-0.5	1	2	4	4	10	70	91
18	-1.0	2	3	6	7	25	40	83
34	-2.0	3	5	12	14	36	0	70
48	-3.0	5	7	15	18	15	0	60
61	-4.0	7	7	18	20	0	0	52

TABLE 3. EFFECT OF PRE-THAWING TRANSFER TO ICE, OF FROZEN SECTIONS INFILTRATED WITH PROTECTIVE SOLUTIONS

Freezing conditions of sections			Percentage injury to sections after thawing on:				
Freezing temperature	Saccharose concentration (g/cm ³ of water)	Δt°	Ice	Saccharose solutions (g/100 ml of water)			<100
				17/100	31/100	57/100	
-15	0 (water)	-	100	<100	<100	100	-
-20	17/100	-0.9	70	0	-	-	-
-20	31/100	-1.8	40	-	10	-	-
-20	57/100	-3.7	20	-	-	<10	-
-25	17/100	-0.9	<100	50	-	-	-
-25	31/100	-1.8	50	-	<10	-	-
-25	57/100	-3.7	0	-	-	0	-
-30	17/100	-0.9	100	100	-	-	-
-30	31/100	-1.8	<100	-	90	-	-
-30	57/100	-3.7	<100	-	-	20	-

This may be explained by the fact that the weaker solutions have a lower protective effect and so the injury to the protoplasm during freezing is greater. Rapid water absorption by the protoplasts results in even greater injuries so that finally the cells die (during thawing on ice). With slow water absorption

(during thawing in solutions) the injury does not become greater (at -20°C) so that the result is the same as with stronger solutions, if these injuries have not already reached a serious level during freezing (at -30°C).

TABLE 4. INFLUENCE OF THAWING RATE ON INJURY OF KALE SECTIONS FROZEN IN SACCHAROSE SOLUTIONS

Solution concentration (g./cm ³ of water)	Δt°	Percentage injury to sections after:					
		Thawing			Deplasmolysis		
		-20°	-25°	-30°	-20°	-25°	-30°
Thawing at $+2^{\circ}\text{C}$							
17/100	-0.9	0	100	100	80	100	100
31/100	-1.8	0	0	0	0	80	100
57/100	-3.7	0	0	0	0	0	20
Thawing at $+30^{\circ}\text{C}$							
17/100	-0.9	40	100	100	90	100	100
31/100	-1.8	0	90	100	40	100	100
57/100	-3.7	0	0	0	--	100	100

The influence of reabsorption of water by the cells during thawing was also observed during experimental changes of the thawing rate (Table 4). In this experiment stem sections of the hardened kale plants, infiltrated with saccharose solutions of three different concentrations, were slowly frozen in tubes to the temperatures shown in the table and then thawed at different rates: either slowly to $+2^{\circ}\text{C}$, or rapidly, by placing the tubes in water heated to $+30^{\circ}\text{C}$ with a simultaneous addition to the tubes of the same solutions also heated to $+30^{\circ}\text{C}$.

The data show that rapid thawing is much more harmful than slow thawing but to different degrees depending on the freezing temperature and the solution concentration. Rapid thawing also had a negative effect on the sections which were placed in the high-concentration solutions, though it became evident only after transferring these sections to water. Ice thawing in this solution did not lead to a considerable increase in the size of the protoplasts, for during freezing their size remained almost the same thanks to strong plasmolysis even before freezing. In this case the injurious effects of rapid thawing can be explained only by the influence on the rate of swelling of the protoplasm (dehydrated by freezing).

Microscopic observations of the thawing of sections of onion scales frozen in protective solutions showed that the loss of the semi-permeability of the protoplasts (judging by the release of anthocyanin) and consequently their death took place not during freezing, but during thawing, especially at its final stages when the temperature was -6°C or higher, and even more often—after the ice had thawed (Figs. 1, 2, 3 and 4). Thus, Fig. 2 which represents the

situation at -3°C after freezing to -30°C (Fig. 1) shows that the protoplasts still retain their form and size as well as their semi-permeability though all the ice in the section has already thawed (Δt° of the saccharose solution = -3.7°) and the intercellular water has been absorbed by cells. It is interesting

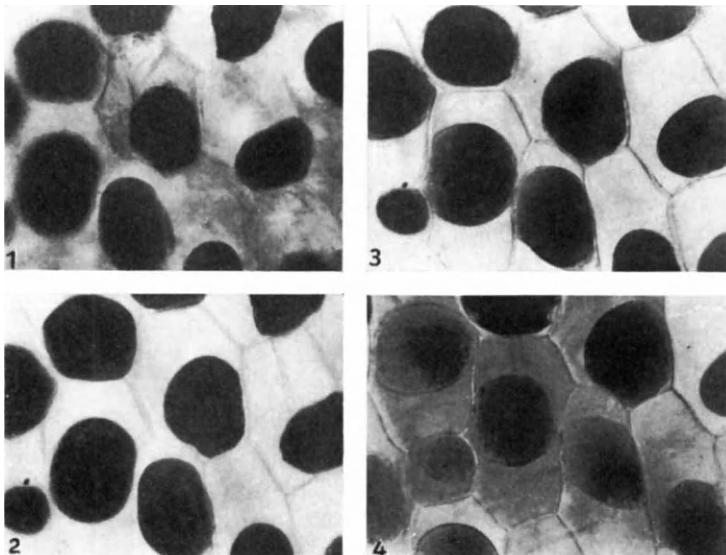


FIG. 1. Epidermal cells of onion frozen down to -30°C in saccharose solution (57 g per 100 cm^3 of water; $\Delta t^{\circ} = -3.7^{\circ}$); ice in one cell, the rest without ice.

FIG. 2. The same cells at -3°C after -30°C . Ice has thawed.

FIG. 3. The same cells at $+1^{\circ}\text{C}$ after -30°C . Protoplasts begin to release anthocyanin.

FIG. 4. The same cells at $+10^{\circ}\text{C}$ after -30°C . Anthocyanin is released by protoplasts in most cells.

to note that in the cell with ice between its walls and the protoplasts at -30°C (the protoplast itself had no ice) the release of anthocyanin from the protoplast was not observed even at -3°C . Its release became visible at $+1^{\circ}\text{C}$ (Fig. 3) in two cells (one of them contained ice) and only at $+10^{\circ}\text{C}$ (Fig. 4) was it clearly observed in many cells. Two cells which did not release anthocyanin had injured protoplasts, these injuries were absent at -3°C . Such protoplasts can retain their semi-permeability while they are in the solution, but die during deplasmolysis (as is true of protoplasts with slightly visible changes). All the cells of this section became decolorized in water.

It is from this fact that the increased injury of sections after deplasmolysis, shown in Table 4, can be explained. It compelled us always to take into consideration the degree of injury of the sections frozen in protective solutions not only after thawing, but after deplasmolysis as well. This operation, if

carried out slowly, is not dangerous for cells of unfrozen sections: for we have seen deplasmolysis without injury after the solutions of much higher concentrations than those which have been used for protection during freezing. The death of cells in thawed sections only after deplasmolysis shows that there are slightly visible protoplast injuries which begin during freezing and become greater even during slow saturation of protoplasts with water which lead to death.

The absence of significant changes in the form and size of protoplasts during thawing in this experiment (Figs. 1-4) proves the absence of serious mechanical deformations both during their thawing and freezing; due to this fact their injury and death cannot be caused by injurious mechanical effects; plasma dehydration during freezing to -30°C being the real cause of their injuries. It is interesting to note that as the release of anthocyanin began in the cell with ice simultaneously with the other cells, the probable cause of the death of this cell may also be attributed to protoplasmic dehydration; the ice around the protoplast not being responsible for any additional mechanical damage to the latter.

It seems to us that the experiments described in this paper permit the following conclusions. The comparison of the degrees of dehydration, which lead to cell death during freezing in protective solutions and during osmotic dehydration, as well as microscopic studies, which show that cells die without any deformation of protoplasts, suggest that cells may die only from protoplasmic dehydration during their freezing in protective solutions. Death occurs at low temperatures (-25°C and below) at which the cells and the protoplasm retain only small amounts of water. Therefore, cell injuries occur when the protoplasmic colloids are deprived of the small amounts of water which remain at such low temperatures; while the loss of greater amounts which occurs at higher temperatures is not dangerous. It can be explained by the fact that water which is left in the protoplasmic colloids at low temperatures belongs to the "structural" water, the loss of which leads to irreversible changes in the properties of the colloids.

However, the injurious effect of the intensive protoplasmic dehydration displays itself only in the reverse process of its swelling during thawing as has been proved by microscopic observations during thawing as well as by the experiments on the effect of the thawing rate and the concentration of the cell's surrounding medium during thawing. Unfavourable conditions of thawing increase the injurious effect of the protoplasmic changes caused by its dehydration which can result in the death of cells; under favourable thawing conditions cells can retain their viability in spite of the protoplasmic changes caused by its dehydration unless these changes have been lethal. Rapid swelling of the protoplasm, already altered by dehydration, is probably accompanied by a change in the normal progress of the process which finally results in a disturbance of the protoplasmic semipermeability and death of the cells.

We shall ask now if cells can die from protoplasmic dehydration during their freezing without protective solutions. We have already mentioned that the main cause of the death of cells from extracellular ice may be attributed to mechanical deformations which affect protoplasts due to the loss of water by cells, usually at comparatively high temperatures (-10 to -20°C) when protoplasmic dehydration has not yet reached the injurious level. But there are frost-resistant plants which die only at -30°C or below. Is not protoplasmic dehydration the main cause of their death since their cells can withstand serious mechanical deformations?

However, these deformations can increase even at such low temperatures if cell dehydration continues; so the death of cells from mechanical factors cannot be excluded even for highly frost-resistant plants. In addition, it is quite possible that cells of frost-resistant plants are highly resistant to protoplasmic dehydration and can withstand any level of it. We have shown at the beginning of the article that even the cells of the hardened kale plants, which are much less resistant than our tree species, can endure practically any protoplasmic dehydration, surviving in protective solutions even at -250°C . Resistance to protoplasmic dehydration increases with an increase in frost-resistance as has been shown above by the comparison of hardened and unhardened kale plants. Epidermal cells of onion died from protoplasmic dehydration even at -25 to -30°C in protective solutions, which correlated with their lower frost-resistance than that possessed by kale. So it seems at first sight hardly possible that protoplasmic dehydration is the cause of the death of cells in frost-resistant species.

However, it must be taken into consideration that high resistance of cells to protoplasmic dehydration in protective solutions should be attributed to the fact that dehydration of the protoplasm proceeds slowly and, as we have shown above, that rapid swelling is accompanied by critical injuries. Under natural conditions cells are surrounded not with solution but with ice alone. In this case the conditions for swelling of the highly dehydrated protoplasm are less favourable than during experimental freezing in solutions, permitting protoplasmic dehydration to display its injurious effect even at not very low temperatures. Therefore, it is impossible to exclude completely the effect of this factor during freezing without protective solutions. Similar conclusions were also drawn by Tumanov and Krasavtsev (1962). Furthermore, protoplasmic dehydration can affect the resistance of cells to mechanical factors, as has been suggested by Tumanov (1940) and Levitt (1956), but our knowledge in this respect is scanty. Death of cells of frost-resistant species also can be caused by the development of small amounts of intracellular ice because of the slower release of the remaining water from cells at very low temperatures (Tumanov and Krasavtsev, 1959).

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ON SOME METABOLIC CHANGES IN SHOOT APICES OF WHEAT PLANTS AT LOW TEMPERATURES

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AS PART of a larger study concerning the development of shoot apices of several wheat varieties cultivated under different external conditions, some metabolic changes taking place in these organs during wintering of the plants were examined. In our experiments wheat varieties of the Czechoslovak selections, differing as to their developmental properties, were used, winter, half winter and spring varieties, as well as alternating wheat. The half winter and alternating wheat varieties are the most hardy Czechoslovakian wheat varieties.

Free sugars and amino acids were analysed by means of paper chromatography. Proteins soluble in phosphate buffer at pH 7 were tested by a spot method with bromophenol blue (Kutáček and Kratochvíl, 1958). The following histochemical reactions were also performed: the test for ascorbic acid with silver nitrates, the nitroprusside reaction for free sulphydryl groups, the Nadi reaction for cytochrome oxidase, the test for peroxidase with the Bojarkin benzidine reagent (Bojarkin, 1951), the tetrazolium reaction for dehydrogenases using succinate, malate and citrate as substrates. The ability of the shoot apices to be stained with neutral red was considered a measure of their acidity. The histochemical enzyme reactions were checked by means of specific inhibitors.

A relationship was observed between the dynamics of growth and development of the shoot apices, the fluctuations of their sugar content and the cold-resistance of the plants.

In the autumn, cold-resistant varieties stopped growth and development of the shoot apices sooner than less resistant ones. As was shown by further experiments, this is caused by the greater sensitivity of hardy varieties to the short autumnal days as compared with less hardy ones.

At a time when no further morphological changes were taking place in the shoot apices, free sugars began to accumulate in these organs. This accumulation began, therefore, sooner in the shoot apices of the half winter and alternating wheat varieties than in those of the winter varieties which stopped

developing later. The spring variety went on to develop for the longest time, as its sensitivity towards short days is lowest.

As long as the temperature was relatively high in autumn, the sugar level was subjected to certain fluctuations, the level of monosaccharides changing more markedly than that of sucrose. While the concentration of this last sugar rose more or less steadily, that of the monosaccharides increased considerably even with slight lowerings of temperature and decreased again with its rise. In winter, the effect of even greater temperature changes on the content of monosaccharides was much weaker (Fig. 1).

Besides monosaccharides and sucrose, polyfructosans and raffinose appeared in the shoot apices in autumn. These obviously act as reserve substances (Schlubach, 1953). They accumulated in larger amounts in varieties stopping their development sooner in autumn than in varieties continuing to develop for a longer time. In the beginning of winter the sugar content reached its maximum.

In most years the winter conditions are not steady in Czechoslovakia. Frost periods alternate with periods of thawing weather. Under these conditions the sugar level of the shoot apices fluctuated according to the temperature. During frost periods it increased, during a rise in temperature it decreased. These fluctuations proved to be stronger, the less resistant a given variety was. Therefore, in the shoot apices of half winter and alternating wheat varieties the smallest fluctuations in sugar content took place. In the shoot apices of spring varieties the fluctuations were very pronounced.

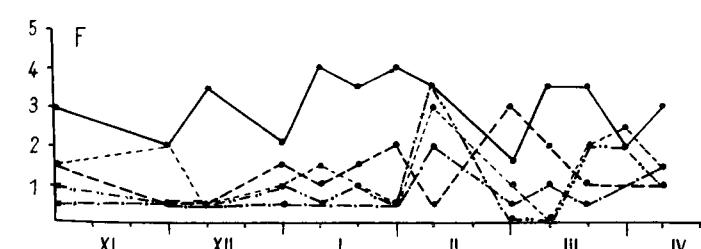
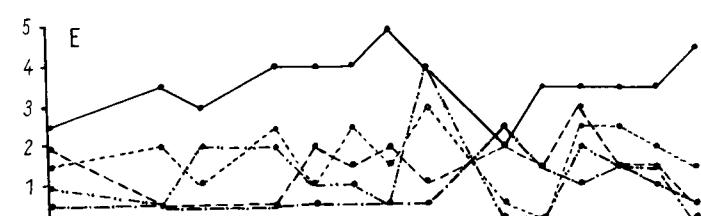
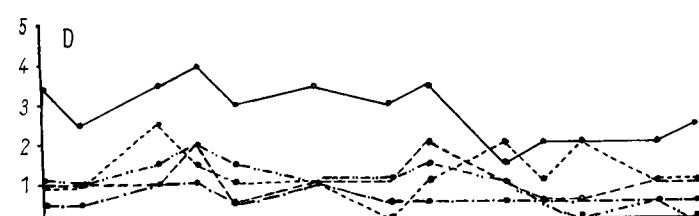
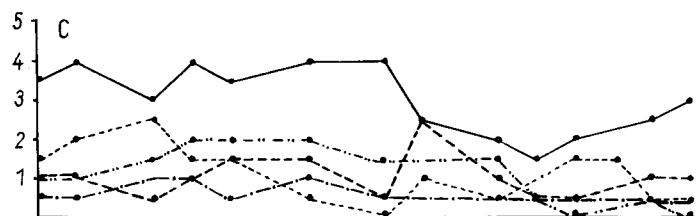
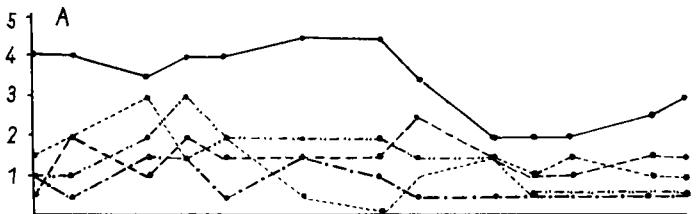
The decrease in sugar level during periods of thawing is probably due to a rise in metabolic activity of the plants, e.g. an increase in the rate of respiration at higher temperatures. This increase was higher in shoot apices of less resistant varieties, which are less sensitive towards short days; whereas sensitive varieties which are cold-resistant under our conditions are not able to resume their development in winter and, therefore, they expend less sugars. As a consequence, the concentration of sugars in their shoot apices did not fluctuate to such a pronounced degree.

No relationship was established between the total amount of sugars and the cold-resistance of the plants, there only exists a connexion between the dynamics of these substances and the latter property.

In shoot apices of varieties relatively adapted to the conditions of wintering in Czechoslovakia the fluctuations of the sugar level during winter were mainly due to changes in the concentration of monosaccharides. In foreign

FIG. 1. (*opposite*) Dynamics of free sugars in the shoot apices of different wheat varieties (1959/60). Abscissa shows the month. Ordinate shows sugar content:

— sucrose, -·-·- fructose, -·-·-·- raffinose, -·- glucose, ······ polyfructosans. Varieties: A. Chlumecká 12—Half winter variety, B. Česká přesivka—Alternating wheat, C. Pyšelka—Czechoslovak winter wheat variety, D. Kaštická osinatá—Czechoslovak winter wheat variety, E. Hadmerslebener IV—German wheat variety, F. Ratoborská—Spring variety.



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poorly adapted varieties, the sucrose level was also subjected to strong fluctuations.

In winter the amount of polyfructosans and raffinose decreased with the rise in monosaccharide and sucrose level and vice versa. Only during the strongest frosts did the concentration of all the sugars rise. Sugars were probably translocated to the shoot apices from other parts of the plant during these periods.

In early spring the metabolic activity of the shoot apices rose with the renewal of their development, and their sugar level decreased at the same time. This decrease was more rapid, the earlier in spring a given variety resumed its development. Such varieties quickly lose their cold-resistance and are, therefore, often damaged by late frosts. In shoot apices of varieties which resume their development later the sugar level decreased more slowly.

In spring, polyfructosans and raffinose disappeared from the shoot apices. This occurred later in more resistant varieties than in less resistant ones.

In the shoot apices 17 free amino acids were detected which were essentially the same as those quoted by Steward *et al.* (1951) for the apical meristem of *Lupinus albus*. During wintering quantitative changes in the concentrations of most amino acids were observed. Thus, for instance, the amount of glutamine decreased, and that of α -alanine and serine increased.

At the onset of frost periods proline was detected in the shoot apices. With a rise in temperature this amino acid disappeared. This phenomenon has not yet been explained. According to Heber (1958) proline can also be found in wheat leaves in winter. Our results agree with those of Dr. Heber as we also did not find any relationship between the dynamics of free amino acids and the cold-resistance of the plants.

As to the proteins soluble in phosphate buffer at pH 7, it was observed that their concentration decreased somewhat after the onset of low temperatures and increased after the rise in temperature. These fluctuations were less marked in hardy varieties than in non-hardy ones.

The intensity of the histochemical reactions for peroxidase and sulphhydryl groups (Fig. 2) decreased during periods when morphological changes were no longer taking place in the shoot apices. This happened sooner with resistant varieties than with non-resistant ones. With a rise of temperature during winter the intensity of the reaction increased, with a lowering of temperature it decreased.

The above-mentioned substances seem to be related to the rise in metabolic activity of the shoot apices. Therefore, during winter the intensity of the reactions fluctuated more markedly in shoot apices of non-resistant varieties than in those of resistant ones. The reactions were very intense at a time when development was resumed in the spring. The acidity of the shoot apices behaved in a similar way. It increased during thawing weather and decreased during frost periods. In winter the shoot apices of most varieties could not be stained with neutral red for a certain time, this period being the longer, the

more resistant a given variety was. The shoot apices of spring varieties could be stained during the whole winter. During periods when the shoot apices could not be stained by neutral red, they reacted in a different way with some oxidation-reduction indicators than shoot apices which could be stained with

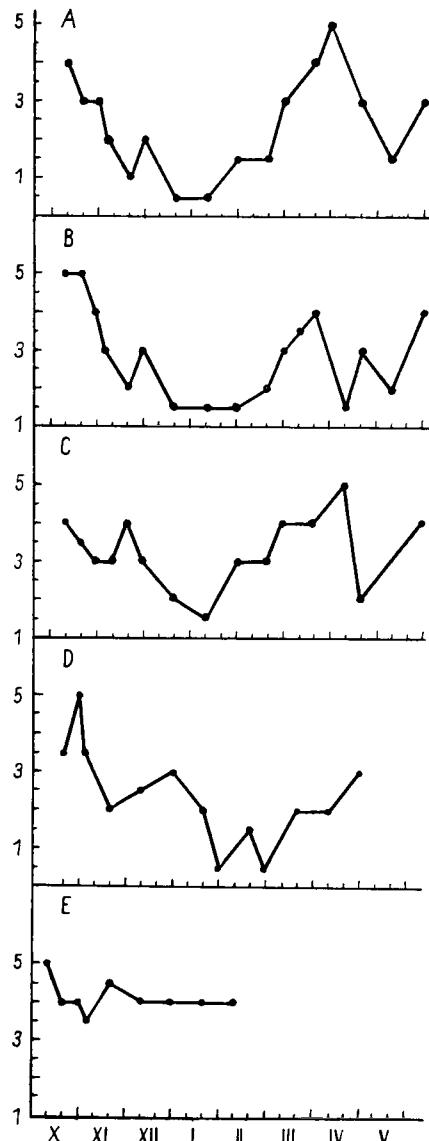


FIG. 2. Dynamics of the reaction of SH in shoot apices of different wheat varieties (1957/58). Abscissa shows the month. Ordinate shows SH content.

neutral red during the same time. It might be concluded, that the failure of the reaction with neutral red is related to a change in rH in the shoot apices.

The Nadi reaction was more intense in most cases during periods, when the reaction for peroxidase was weak, and vice versa. It is therefore, more intense in quiescent shoot apices than in actively developing ones.

No relationship could be observed between the dehydrogenases examined and the cold-resistance of different varieties. In non-resistant varieties there existed, however, a certain relationship between the intensity of the reaction for dehydrogenases and the dynamics of the sugars. In varieties starting to accumulate sugars late in autumn the intensity of the reaction for dehydrogenase fluctuated in winter in an opposite direction to the changes of the sugar content. In spring the intensity of the reaction for dehydrogenases strongly increased.

All the above-mentioned methods were also applied to the shoot apices of plants belonging to the resistant varieties, which were cultivated under conditions of permanent illumination in the field from the time of their emergence. The shoot apices of such plants behaved like shoot apices of non-resistant varieties.

Shoot apices of plants grown from vernalized and non-vernalized seed were also tested by means of the same methods. Only vernalized and non-vernalized shoot apices not differing as to their morphological state were compared. In the shoot apices of vernalized plants belonging to varieties which were promoted in their further development by vernalization, the reactions for ascorbic acid, sulphydrol groups and peroxidase were more intense and the acidity was higher than in the shoot apices of non-vernalized plants. The reaction for cytochrome oxidase was stronger in non-vernalized plants than in vernalized ones. In the shoot apices of varieties whose development is not promoted by vernalization (spring varieties, alternating wheat) no such differences were observed. The reaction for dehydrogenases was more intense in vernalized plants of all the varieties examined. It may be assumed, therefore, that the first group of reactions is specific for vernalization, while the more intense reaction for dehydrogenase is probably due to a non-specific effect of low temperatures as such.

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MORPHOLOGICAL AND FUNCTIONAL CHANGES OF CHLOROPLASTS AFTER COOLING OF LEAVES OF *Cucumis sativus* L.

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IT WOULD seem important to follow the sequence and the character of the injuries to separate cell structures and functions under cooling conditions, in order to find out the reasons for the injury and destruction of plants sensitive to cold, under the influence of low positive temperatures. The after-effect of low positive temperatures on cucumber leaves (var. *Klinskye*) has been investigated. The excised leaves were kept in a moist chamber at a constant temperature of +1.8 to +2.1°C for different periods of time. After the cessation of cooling the photosynthetic intensity of the cooled leaves was measured by the radiometric method (Zalensky *et al.*, 1955) at a temperature of 20°C and a light intensity of 25,000 lux. Simultaneously the leaves were examined under light and luminescent microscopes and preparations were made for electron microscope investigations (OsO_4 brought to pH 7.4 with a veronal-acetate buffer). It was established that functional and morphological injuries in chloroplasts (repression of photosynthetic capacity, decrease in fluorescence of chlorophyll and changes in chloroplast structure) are the earliest signs of cold injury. These signs develop simultaneously and become more evident as the period of cooling is prolonged.

The leaves kept at +2°C and a light intensity of 500 lux for 3–5 hr are already characterized by considerably decreased photosynthetic capacity. Their photosynthesis at +20°C constitutes only 30–60 per cent of the controls kept at room temperature. At the same time the reduction in fluorescence becomes evident and the chloroplast structure changes. The most characteristic changes observed with the light microscope are the following (Fig. 1a): the chloroplasts swell, become round and the part containing chlorophyll acquires the form of a cup and shifts to the periphery of the chloroplasts; the rest of the volume is filled with transparent colourless substances which faintly differ from the surrounding cytoplasm. The envelop of the chloroplasts becomes obvious. At this time they are very similar to the vacuolized chloroplasts which have been observed under various other conditions (Küster, 1956; Lindner, 1960). After a more prolonged period of cooling the chloroplasts stick together.

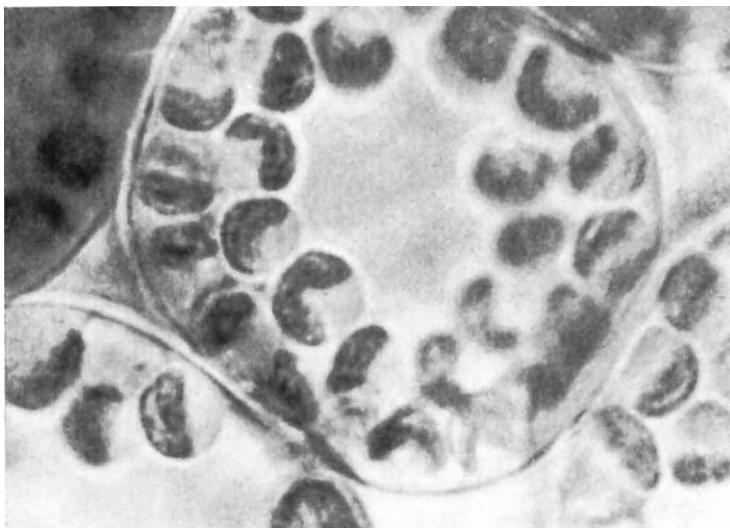


FIG. 1a. Chloroplasts of leaf of *C. sativus* kept for 25 hr in the light at +2°C. $\times 90$, Hamal 3. Photographed through the epidermis of a leaf disk infiltrated with water.

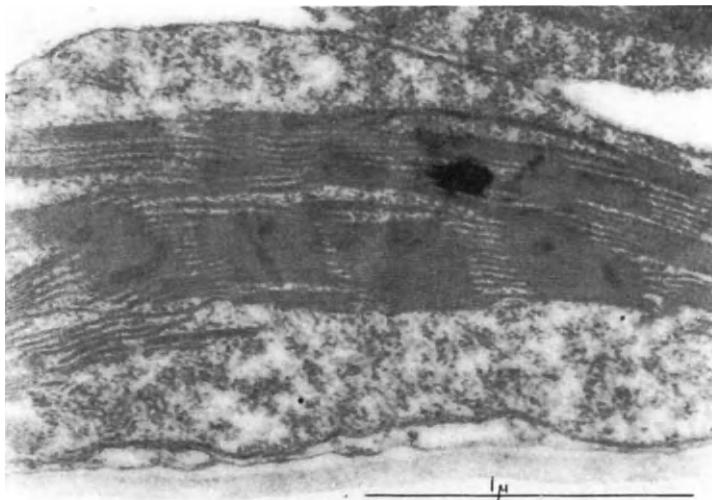


FIG. 1b. Chloroplast of the leaf of *C. sativus* kept for 7 hr in the light at +2°C. Electron optical magnification $\times 16,000$.

Electron microscope investigations have shown that changes in the chloroplast structure begin with the swelling of the stroma (Fig. 1b). Probably it is caused by the flow of water into the chloroplasts. In the chloroplasts of leaves subjected to cooling at +2°C for 25 hr in the light the stroma increases con-

siderably in volume and all the lamellar system shifts to the periphery of the chloroplast and becomes deformed as a result of the bending of the stroma lamellae (Fig. 1c). Thus the transparent part of the swollen chloroplasts seen with the light microscope is not a vacuole but a swollen stroma. Weier and Stocking (1962) observed the formation of such "cup plastids" with the curved lamellae in the leaves of *Nicotiana rustica* after they were subjected to darkness and due to the changes in the tonicity of the isolation medium.

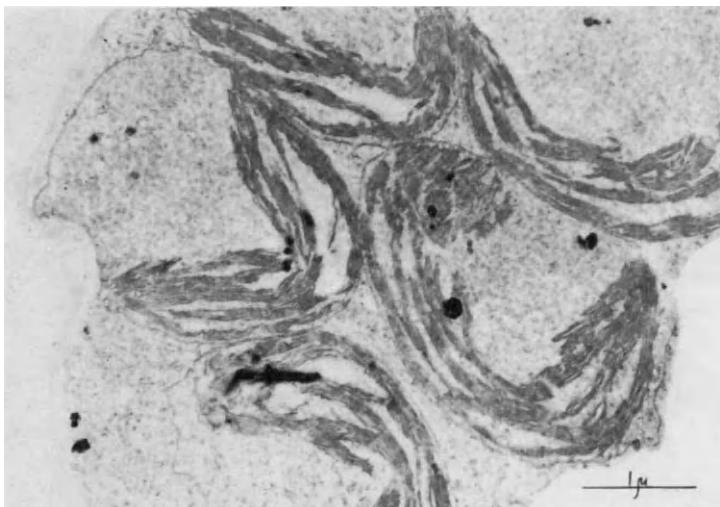


FIG. 1 c. Chloroplasts of leaf of *C. sativus* kept for 25 hr in the light at +2°C.
Electron optical magnification $\times 11,200$.

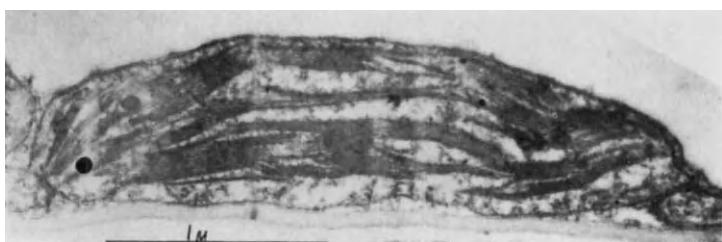


FIG. 1 d. Chloroplast of the leaf of *C. sativus* kept for 25 hr in the dark at +2°C. Electron optical magnification $\times 13,200$.

In this state the chloroplasts almost entirely lose their photosynthetic ability and are characterized by a considerable repression of chlorophyll fluorescence. The fact that it is not a destruction but a shift of its elements, can explain the good reversibility of these cold injuries. In our experiments the photosynthesis of leaves cooled for 25 hr in the light after the termination of cooling

was only 6 per cent, but after they were kept for 40 hr at room temperature it amounted to 83 per cent of the photosynthesis of the controls. Almost all of the chloroplasts which immediately after cooling have a structure represented in Fig. 1c regain their normal structure in 40 hr as was seen under the light microscope. During the same period the normal intensity of the chlorophyll fluorescence is restored. Thus the pronounced morphological and functional changes in the chloroplasts appeared to be entirely reversible.

Our experiments have shown that changes in the photosynthetic apparatus can be prevented to a large extent by darkening of the leaves during the cooling. Figure 2 shows the curves for the intensity of photosynthesis of leaves cooled

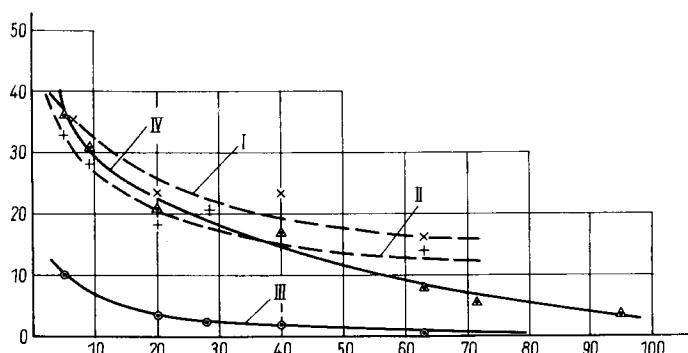


FIG. 2. Photosynthetic intensity of leaves of flowering plants of *C. sativus* at 20°C and 25,000 lux depending on the previous conditions. Abscissa: duration of exposure of leaves at the given temperature (hr). Ordinate: intensity of photosynthesis in mg CO₂ for 1 g of dry weight in 1 hr. I—+16°C in the light; II—+16°C in the darkness; III—+1·8°C in the light; IV—+1·8°C in the darkness.

in the light (500 lux) and in the darkness and that of the controls exposed to light and to darkness at room temperature. The darkened control leaves show decreased photosynthetic ability. But at the same time, leaves subjected to darkness during the period of cooling, after transfer to normal light and temperature show a higher intensity of photosynthesis than leaves cooled in the light. A more prolonged period is required for the same repression of photosynthesis in the darkened leaves than in those subjected to light. A 5–20-hr cooling, which is always followed by a considerable decrease in the photosynthetic ability of the leaves subjected to light during the cooling, nevertheless caused a stimulation of photosynthesis in the darkened leaves in a number of cases. Such factors as the swelling of the chloroplasts and the reduction in fluorescence of chlorophyll which develop simultaneously with the decrease in photosynthetic ability also appear later in the leaves subjected to cooling in the darkness. Figure 1d shows that 25 hr cooling of cucumber leaves in the darkness is not followed by any pronounced changes in the

chloroplast structure. Only a few swollen chloroplasts appear in the cells, the majority of them maintaining their normal structure. In the darkness, the injury to the chloroplasts develops more slowly and for the irreversible changes a more prolonged period is required than in the light.

It has been established that the injury to the cells caused by a short-term heating results simultaneously in a repression of photosynthetic ability and a cessation of protoplasmic streaming (Alexandrov, 1955; Lyutova, 1958, 1962). Both of these characteristics in contrast to such signs of injury as the repression of respiration and the destruction of selective semipermeability appear at the first stages of heat injury. In the case of the cold injury even the complete and irreversible repression of photosynthesis is not followed by the cessation of protoplasmic streaming. Ability to continue protoplasmic streaming at room temperature disappears in the epidermal and mesophyll cells only after 100–120 hr of cooling. Soon afterward, complete protoplasmic coagulation takes place. The cessation of protoplasmic streaming and the coagulation occur approximately simultaneously in the course of the effect of low temperatures in the light and in the darkness. Thus darkness protects only the photosynthetic apparatus from injury and keeps it intact for a longer period of time than cooling in the light. If we stop the cooling of leaves before the disappearance of the capacity for the protoplasmic streaming but after the irreversible injury to the chloroplasts, the leaves will be destroyed later at room temperature. This death can be prevented by cooling the leaves during the same period of time not in the light but in the darkness. For instance, after 50 hr of cooling in darkness, chloroplasts revive and the leaves remain alive, while the same period of cooling in the light results in destruction of the leaves within 3–4 days after their transfer to room conditions. This is caused by the irreversible injury to the chloroplasts which occurred during the cooling.

The injurious effect of light on the chloroplasts is apparently connected with the photo-oxidation of some sensitive constituents of the chloroplasts.

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EFFECT OF LOW TEMPERATURE ON MITOSIS IN PLANTS

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TEMPERATURE is one of the principal external factors affecting plant growth and cell division. Frost-resistance of some plants is connected with cessation of growth and cell division at a certain time in autumn. The influence of low temperatures on mitosis and meristematic cell structures were studied.

1. Methods for determination of the "threshold temperature" (i.e. the lowest temperature at which cell division was still possible) were developed. It was demonstrated in a number of plants (*Allium cepa* L., *Crepis capillaris* Wall., *Haplopappus gracilis* (Nutt.) Gray, *Hordeum sativum* var. *nutans* R. Reg., *Triticum vulgare* L., *Trillium ovatum* Pursh., *T. kamtschaticum* Ledeb., etc.) that, although there was some intraspecific variation, all the species examined showed a very similar behaviour as regards their threshold which was found to be at or above 0°C.

2. Differences in the behaviour of dividing cells attributable to the varied rates of temperature decrease have been discovered. When the temperature was slowly decreased to the threshold and beyond, the completion of the mitotic cycle took place in meristematic cells, but no new mitosis was initiated. In case of a rapid drop in temperature, cell division was completely suppressed so that mitosis appeared to stop at any phase.

3. When the mitotic cycle is completed under conditions of slowly decreasing temperature, meristematic cells enter a state of dormancy which probably results in an increased cold-resistance.

4. With a rapid drop in temperature below the threshold value the processes of cell division and cell elongation were arrested in every part of the plant. The threshold temperature was found to be the same for all the meristems of a given plant.

5. A sudden drop in temperature from the optimal to a temperature slightly above the threshold value sometimes temporarily arrests mitosis. But in such cases cell division can be resumed later on, continuing from the very mitotic phase at which it was stopped.

6. The completion of mitosis at a temperature below the threshold value is not connected with the cessation of some other life processes. It was shown

that in the winter-crop cereals, with the arrested cell divisions, a slow process of vernalization can take place. Therefore, the growth and division of cells is not necessary for vernalization.

7. Some authors disregard the possibility of mitosis being arrested at any phase if the temperature drops abruptly. They come accordingly to a false conclusion assuming that mitosis proceeds at temperatures far below 0°C.

8. In cases when cell division stops without completing the mitotic cycle, the following alterations in meristematic cells were observed: chromosomes were markedly shortened, thickened, and their volume increased at the metaphase, they coalesced at the anaphase, etc. also, heterochromatic segments appeared in metaphasic chromosomes.

Apart from morphological alterations in the chromosomes, changes in the cell cytoplasm were also observed. The degree of all the changes was found to depend on the temperature, on the duration of the treatment and on the individual peculiarities of the organism.

9. It was found that changes in the chromosome morphology when mitosis stops short are caused by rapid cooling. When, however, cell dividing is resumed after a temperature rise, all the changes caused by the arrest of mitosis disappear.

10. It may be suggested that similar changes in the chromosomes induced by such factors as high temperature, chemical substances etc. may also be regarded as evidence of interrupted mitosis.

CELL RESISTANCE TO LOW TEMPERATURES AS A FUNCTION OF THE VITAMIN FACTORS

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THE importance of the vitamin factor in the ability of a cell, a tissue or a body as a whole to endure low temperatures has not yet been thoroughly studied. The role of vitamin C has been relatively better studied. The analysis of materials pertaining to this question shows the following.

There is a tendency to an increased accumulation of ascorbic acid in plants growing in the North or at high altitudes.

The content of ascorbic acid in the needles of coniferous trees is considerably higher during the colder seasons of the year.

The more cold-resistant varieties of plants contain more ascorbic acid. There is a synthesis of ascorbic acid at low temperatures.

When some isolated tissues of warm-blooded animals are incubated at a temperature of about 0°C, the amount of ascorbic acid falls steadily. But at the same time the amount of dehydroascorbic and diketogluconic acids increases in quantities indicating synthesis and not simple transformation of ascorbic acid. This phenomenon is observed while the tissues still remain alive (and this was confirmed in tissue cultures).

Later on with more prolonged storage of isolated tissues some decrease in the amounts of the substances was noted and this decrease coincided with the destruction of the cells.

The preceding facts allow us to suppose that vitamin C (probably in the form of dehydroascorbic acid) may be one of the factors protecting the cells from the unfavourable action of low temperatures.

CYTOCHEMICAL PECULIARITIES OF WOODY PLANTS AND THEIR WINTER-HARDINESS

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U. HEBER in this symposium presents interesting data about the damage to the apparatus of photophosphorylation and oxidative phosphorylation in the cells of plants during freezing. Such damage was also observed during the investigations of other influences on the plants as well.

However, the dissociation of phosphorylation from oxidation cannot be considered in a negative connexion only and it must be co-ordinated with the adaptation of plants to unfavourable conditions. It was ascertained in our laboratory that by using 1 per cent adenosine triphosphate solution, it is possible to break the condition of "deep dormancy" in some woody plants (Sergeev, 1962). Comparing this data with results of three years' estimations of activity of respiratory ferments and bioelectric potentials of action in a year's cycle of woody plants, the following conclusion can be drawn: the uncoupling of oxidation and phosphorylation underlies the "deep dormancy" phenomena. Such a dissociation is reached by a depression of the activity of cytochrome oxidase and increase in activity of polyphenol oxidase (Sergeev and Sergeeva, 1962).

Growth stimulators have an important role in the biochemical mechanism of "deep dormancy" as well. The disruption of "deep dormancy" with the help of solutions of gibberellic acid testifies to that. In our laboratory, it has been possible to break the "deep dormancy" of different woody plants, except apple trees.

Winter-hardy and non-hardy woody plants have typical cytochemical and biochemical differences, which can be used for the purposes of diagnosis (Fig. 1). European birdcherry is a winter-hardy species and large-fruited apple trees in Baskiria are often damaged in winter time.

Cytochemical investigation showed that, in the second part of the vegetative period and in the period of "deep dormancy", winter-hardy species differ from non-hardy ones by a higher activity of polyphenol oxidase (tissues of annual shoots and generative buds). After completion of the period of "deep dormancy" winter-hardy plants achieve a sharp rise in the activity of cytochrome oxidase in comparison with non-hardy ones. These peculiarities of the respiratory apparatus cause a timely cessation of growth and an increase

in resistance of the more winter-hardy species, and an early vegetation and intensive growth of the shoots in the spring of the next year as well.

It was definitely shown that winter-hardy species are characterized by a higher content of ribonucleic acid. One can suppose, that this is connected with a more intense synthesis in the tissues of winter-hardy species, which

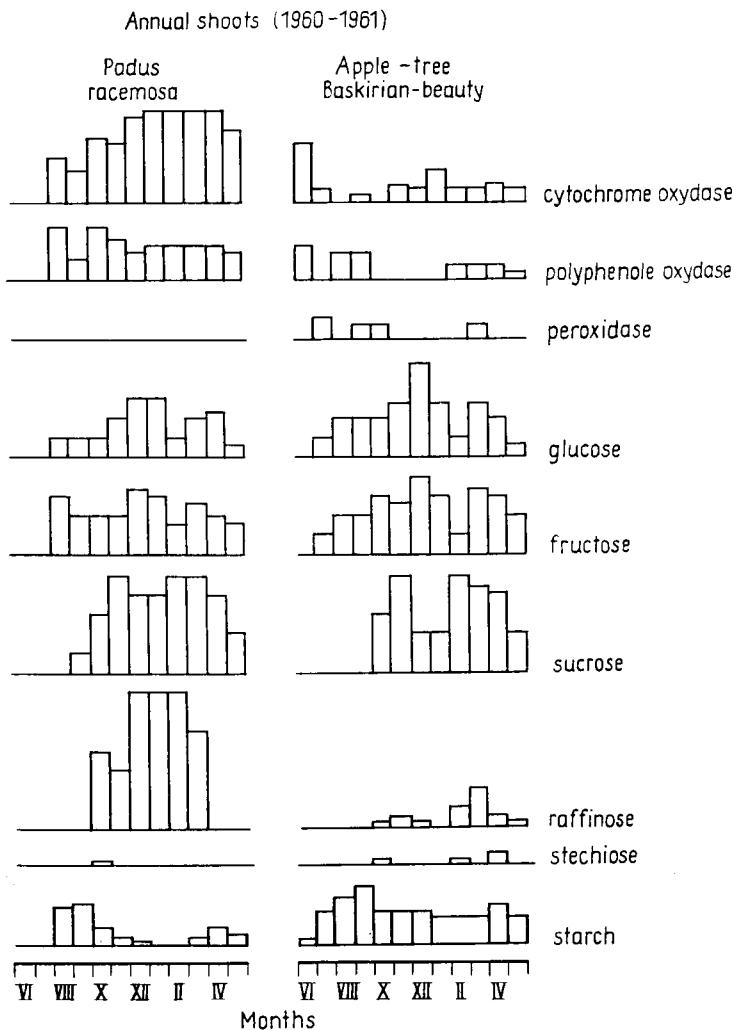


FIG. 1.

underlies the biochemical and biophysical processes, thus increasing their resistance to low temperatures and to other unfavorable factors of winter-spring time. Data of the investigations of carbohydrate and nitrogen metabolism of woody plants testify to this fact.

A study of the dynamics of sugars in a year's cycle of winter-hardy and non-hardy species showed that the former during the period of "forced dormancy" are characterized by a high sugar content and by oligosaccharides which are more varied in structure.

The raffinose content correlates with winter-hardiness most distinctly. Comparative estimation of the oligosaccharides can give an idea about the degree of winter-hardiness of woody species. The content of monosaccharides, according to our data and data of other investigators, does not correlate with the degree of winter-hardiness of woody plants.

The protective role of oligosaccharides, obviously, is connected with their growth-inhibiting properties, which can easily be revealed with the help of simple biotests (germination of wheat grains on weak solutions of different sugars). Inhibition of the growth by solutions of raffinose is connected with the galactose content in its molecule which is noted for this peculiarity among monosaccharides.

The second biochemical peculiarity of winter-hardy species in comparison with non-hardy ones consists in a larger content of albuminous substances. The curve for the content of albuminous substances in a year's cycle of the former species is higher than that of the latter.

One more peculiarity of the nitrogen metabolism of winter-hardy woody plants should be pointed out. This is the appearance of considerable amounts of proline in autumn which, according to our data, inhibits the growth of plants as well.

Data about cytochemical peculiarities of winter-hardy and non-hardy woody species throw light on the nature of winter-hardy plants and are of great practical importance.

THE FIRST PHASE OF FROST-HARDENING OF WINTER CROP PLANTS IN DARKNESS ON SUGAR SOLUTIONS

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DURING the first phase of frost-hardening of winter crops the following two prerequisites are essential: (1) accumulation of sugars, and (2) low positive temperatures.

The above statement was fully confirmed in the experiments with frost-hardening of sections of coleoptiles by keeping them in sucrose solutions at a temperature approximating to 0°C. A similar method was successfully used for the frost-hardening of whole winter wheat plants. The roots and tillering nodes, previously freed from soil by washing with water, were immersed in a highly concentrated sucrose solution in which they were kept in darkness for two weeks at a temperature of about 0°C. As a result, the plants acquired a frost resistance no lower than that developed in the light. In a number of cases it was even higher. Consequently, in the course of frost-hardening of winter crops the light is required exclusively for photosynthesis and can be successfully replaced by the plant intake of sugars from an external solution.

Analyses have shown that the rate of accumulation of sugars in plants, either by means of photosynthesis or through the absorption of sugars by the underground parts is approximately the same.

Survival of plants during severe frosts is possible if they contain a high concentration of protective materials in their cells.

Using this method of frost-hardening an unprecedented frost-resistance of winter wheat plants has been achieved: all the plants studied survived temperatures as low as -32°C.

In the course of experiments with coleoptiles it was established that some groups of sugars increase the frost-resistance of plants, whereas others have no effect. Experiments on frost-hardening of whole plants helped to reveal a number of the hitherto unknown regularities. Their frost-resistance increased due to the intake not only of sucrose, glucose and maltose, but also of rhamnose and lactose as well. The two last mentioned sugars failed to contribute to the frost-resistance of coleoptiles. The frost-resistance of whole plants or their organs may be increased by the uptake of any sugars that pene-

trate the cells, because they are metabolized there and transformed into forms which are intensely accumulated in the cells. Temperature is a very important factor in the frost-hardening of crops. A temperature approximating to 0°C may be regarded as the optimal one for the frost-hardening of winter crops. It is of interest to note that at all temperatures in the range from 15 to -3°C the frost-resistance of plants increases. However, at a temperature of 15°C the frost-resistance increases only by 2-3°, whereas at a temperature approximating 0°C it rises by 18°. Although absorption of sugars from the external solution proceeds satisfactorily at high temperatures (in fact, it proceeds even more rapidly than it does at lower temperatures), nevertheless the increase in the frost-resistance of plants at higher positive temperatures is many times lower as compared with the increase observed at temperatures of about 0°C. This may indicate that to obtain a sharp increase in the frost-resistance of winter crops a long-term exposure to temperatures of about 0°C is needed, apart from the enrichment of cells in sugars.

The positive effect of temperature approximating to 0°C is manifest only in plants with adequate amounts of stored sugars. A low positive temperature by itself (in the absence of stored sugars) was found to decrease the frost-resistance of plants. A very slight increase in frost-resistance was observed in the plants in which the accumulation of sugars took place after the low temperature treatment. In this case the result was about the same as in the experiment, with high temperatures, referred to above.

It appears that the fundamental changes in protoplasts occurring under the influence of low positive temperatures are possible only in the presence of considerable amounts of protective materials accumulated in the plant. The process of enrichment of cells in sugars proceeds more rapidly than the changes occurring in the cells as a result of the action of low positive temperatures.

DISCUSSION

M. I. LYUTOVA: Did you study sugar content in the protoplasm and in the vacuolar sap separately?

U. HEBER: We have tried to compare the sugar content of the protoplasm with that of the vacuole. We proceeded in the following manner: leaves were plunged into liquid nitrogen and subsequently freeze-dried. The freeze-dried material was then ground in a mixture of petroleum ether-carbon tetrachloride. From the suspension, chloroplasts were isolated by sedimentation at different densities of the suspending medium. During this procedure little or no transformation of the soluble compounds within the cell takes place, as has recently been shown by the application of ^{14}C -labelled compounds. Now the sugar content of the isolated chloroplasts was compared with that of the remainder of the cell. A simple calculation reveals the approximate distribution of the sugar within the leaf cell: If γ is that part of the total cell proteins residing in the chloroplasts and a , b and c are the amounts of sugar, on a unit protein basis in the chloroplasts, in the remainder of the cell (cytoplasm plus vacuole) and in the total cell respectively, the distribution of sugar within the cell is represented by the equation:

$$\gamma a + (1 - \gamma) b = c$$

γ can be easily calculated from chlorophyll determinations of the isolated chloroplasts and of the total cell, while the sugar content in chloroplasts and in the total cell can be directly estimated. Only b , the sugar content of cytoplasm + vacuole, is unknown, but can easily be calculated from the above equation. If we now arbitrarily assume the sugar content of chloroplasts and cytoplasm to be about equal, then an approximate figure of the sugar content in the vacuole can be obtained as compared with the sugar content of the protoplasm.

M. I. LYUTOVA: What did you observe under freezing-uncoupling (P/O decrease) or a simultaneous depression of phosphorylation and O_2 uptake?

U. HEBER: I will have to differentiate between the two, to answer this question. In the case of chloroplasts a true uncoupling doubtless takes place as is clearly shown by the fact that non-cyclic photophosphorylation in the absence of sugar is completely suppressed by freezing, while the light-dependent reduction of ferricyanide is even stimulated.

In the case of mitochondria we are not sure, what actually happens. Freezing of washed chloroplasts of spinach and cauliflower always results in the loss of

oxidative phosphorylation. Usually, but not always, the oxygen uptake is also suppressed. We tend to interpret this as follows: first, an uncoupling of oxidative phosphorylation from electron transport may occur, which is, however, followed by a secondary destruction of the electron transport chain.

Even in chloroplasts the electron transport chain is not unaffected by freezing, as indicated by an increased sensitivity of ferricyanide reduction to ultrasonic treatment after freezing.

L. K. LOSINA-LOSINSKY: Do you consider the process of thawing significant?

U. HEBER: Our system is very simple. It consists only of the lamellae of chloroplasts which retain their capacity to synthesize ATP. It responds in the same manner to slow and quick thawing. However, we can make our system more complicated. I have already mentioned that chloroplasts can be protected against freezing by the addition of sugars. Now the protective effect can be reversed by the addition of different salts such as NaCl, Na_2SO_4 , MgCl_2 and KCl. About 0.02 M NaCl can abolish the protective effect of 0.08 M sucrose. Thus a balance can be established. This more complicated system may be supposed to be sensitive against freezing and thawing rates.

Question: Did the proteins of hardy and non-hardy cells differ in their denaturation capacity?

U. HEBER: We have never observed any difference in the electrophoretic properties of proteins from hardy and non-hardy cells or in the behaviour towards protective agents. Photophosphorylation of chloroplasts both from hardy and non-hardy cells was suppressed in the same way by freezing. However, you mentioned denaturation in your question. We have never observed so far denaturation of pure proteins by freezing. What we definitely have observed is the loss of enzymic activity in the lipoprotein system. The fact that lipoproteins are always involved, raises the suspicion that the lipid part of the protein structure may have something to do with the sensitivity of that structure. In fact, today we doubt that a "denaturation" of proteins in the usual chemical sense is the primary cause of what is happening. It may very well be a secondary effect.

V. Y. ALEXANDROV: Have you or Dr. Samygin noticed the isolation of protoplasm from cell walls in cells in the state of winter dormancy at low temperatures?

O. A. KRASAVTZEV: No I did not notice distinct isolation of protoplasm from the cell walls though I cannot insist on the fact that there is no such phenomenon.

L. K. LOSINA-LOSINSKY: Dr. Samygin's work proves that rapid thawing is more injurious for plant cells than a slower one. It is established by other experiments too. In animal cells such as insect cells, mammalian spermatozoa, etc., the opposite effect was observed. The quicker the thawing (at 40–50°C)

the higher the percentage of the survival of cells. When plant cells freeze, water is drawn out of them and it freezes outside the cells. The crystallization of water in animal cells appears to occur inside the cells. Therefore the result of thawing must be different in these two types of cells. Unfortunately, it is not clear in what way rapid thawing protects both animal and yeast cells from damage. In this case it is not the process of vitrification which requires rapid thawing. In the course of rapid warming, the animal itself and its cells are better preserved. I noticed that cytolysis is more frequent after slow thawing. It is obvious that the properties of animal and plant cell walls play a certain role in this process. I should like to advise you not to disregard the conclusion drawn by Dr. Samygin which is: the damage of cells occurs in the course of thawing, not of freezing. Lately it has been often stated that thawing is more dangerous than freezing. My observations of infusoria and insects agree with this. However, one can say that the injury occurs in a frozen state but is displayed later on after thawing. Additional experiments should be made to clear up the matter.

There is much similarity between the cold-resistance regularities of plants and insects and in the reactions of a cell and of the whole organism. I agree with Prof. Asahina who thinks that we must look for common principles and mechanisms of cold-hardiness in plant and animal cells. But one should keep in mind morphological and physiological peculiarities of plant and animals cells which do not allow us to give the same explanation for both of them.

Question (to Dr. Heber): If you believe that protective substances play the main role in frost-resistance, then what role does the protein structure play?

U. HEBER: We certainly do believe that protective substances play the main role in frost-resistance, provided that they come into contact with the sensitive structure within the protoplasm.

As concerns the role of proteins we have a simple working hypothesis which sufficiently explains the known facts. Let us suppose that two lamellar structures face each other. These lipid-containing lamellae are stabilized by hydration. Water may be bound to them via hydrogen bonding. Freezing removes part of that water, which results in the close approach of these two lamellae. They may come so close to one another, that chemical linkage can be formed or that van der Waals forces come into play between the lipids of the adjacent lamellae resulting in a "flowing together". The latter possibility would account for the observation that only lipoproteins are affected by freezing. At any rate, coagulation is the result of the close approach. If, however, sugars are available, close approach of the lamellae cannot take place, since sugars cannot be removed by freezing. Owing to their OH groups sugars may form hydrogen bonds, perhaps via bound water, to the protein part of the lamellae and stabilize them in that way. Therefore sugars, and possibly

other compounds, act as protective agents and prevent frost coagulation and loss of enzymic activities.

T. M. BUSHUYEVA: How do you explain the results of Arnon, who observed that chloroplasts phosphorylated at -8°C and even at lower temperature?

U. HEBER: Arnon and Hall added methanol as a protective agent to their chloroplast preparations. Therefore, phosphorylation could take place.

O. L. LANGE (to the report of I. M. Kislyuk): I want to make a remark on the interesting results of Dr. Kislyuk. She finds that photosynthesis is disturbed in cucumber already after exposure to about 2°C . However, a certain group of plants shows a remarkable adaptation of its assimilatory metabolism to very low temperatures.

We investigated the CO_2 exchange of lichens in relation to their temperature. Our material originated from regions of Germany, from high-mountain regions, from tropical rain-forest, etc. Tropical species (e.g. *Parmelia pachyderma*) have the optimum of CO_2 uptake at temperatures of about $+20$ to 25°C (10,000 lux), CO_2 uptake is nearly zero at 0°C . High-mountain species (e.g. *Letharia vulpina*) behave in a quite different manner. The optimal temperature for CO_2 uptake in this species is about 7°C , at 0°C it is still high, and at -5°C it is still half that of the optimum. Such lichens show CO_2 uptake beyond the experimental limits of error even as far down as -20 to -24°C .

We believe that adaptation to such low temperatures accounts for the fact that lichens are pioneers of vegetation under cold climate condition.

BIOPHYSICAL ASPECTS OF THE ACTION OF LOW TEMPERATURES ON LIVING CELLS AND TISSUES

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OUR principal work in low temperature biophysics has been concerned with the preservation of life in mammals. In this particular field very few techniques are available. Dehydration cannot be used, as has been shown by Lipschutz (1929), and it is necessary to apply artificial cooling.

Remarkable results have been reported from the U.S.S.R. by Borodin, Kalabuchov, Sacharov, Negovski and their co-workers and, from other countries by Gajja, Andjus, Bělehrádek, Parkes, Smith, Adolph, Kayser, Laborit, Adams-Ray and others. Indeed, it has been widely proven that, under certain circumstances, it is possible to cool down an animal or even a human being, to near 0°C and to keep him in a state of complete clinical death for some time. The organism can then be rewarmed slowly and, by an adequate method of reanimation, can resume its previous activity. Artificial hypothermia has introduced notable improvements in the field of surgery and resuscitation and has contributed widely in improving our knowledge of the physiological aspects of death (Smith, 1961; Negovski, 1959).

However, this does not answer the problem of the preservation of life since survival at low temperature is a limited process which must be short to be reversible. Even at -6°C, in the supercooled state, most cells continue to metabolize and, deprived of oxygen and nutrients by the complete stasis of both respiration and blood circulation, they are liable to be injured. Indeed, if one wishes to bring the tissues to a state of absolute stability, much lower temperatures are required. Mere refrigeration is not sufficient and freezing is necessary.

It can easily be understood that the progressive hardening of such a complex system as a living organism, by turning its water content into ice, will produce enormous structural and biochemical changes. Higher animals will never survive such treatment, and their components—organs, tissues and cells—are killed by deep freezing if they are cooled down to very low temperatures without any special care.

Nevertheless, for isolated tissues and cells, it has been possible to develop special techniques which permit the retention of viability after freezing,

storage and thawing. Working along these lines, we have been able to freeze a chick-embryo heart to liquid nitrogen temperature in such a way that it resumes its normal physiological activity after thawing.

It is quite impossible, in a short time, to present a complete review of the enormous research work done in this particular field. We shall therefore restrict ourselves to some specific aspects of the problem. We shall first examine briefly the main effects of cold on tissues from the biophysical standpoint—then, we shall summarize our experimental results with the chick-embryo heart. More specific information on the general background of the effects of cold on organisms and the precise details of our experimental procedures can be found in our publications (Rey, 1959a, Simatos, 1959), or will be shown in our film.

The Different Steps of the Freezing Process

1. Preliminary Cooling and Ice Formation

At the beginning of the cooling process, no special phase changes appear even at subzero temperatures when supercooling takes place. This is a state of high instability and at a given temperature—depending upon the cooling velocity and the nature of the specimen—the supercooled state is broken and ice crystals suddenly develop. They are scattered throughout the tissue, mainly in the extracellular spaces, although they can also appear in the cytoplasm itself. As has been shown by Lovelock (1954) and Rey (1959a), these crystals are composed of pure ice so that an interstitial net of canaliculae filled with hypertonic fluids remains between these ice crystals. In other words, the frozen material is an ice-sponge impregnated with a strong saline solution. The essential parts of the cells, or the cells themselves, if we are dealing with a suspension of cells, bacteria or virus particles, are soaking in the interstitial fluids. The primary effect of cold is initially osmotic and salting out the proteins is likely to occur. As the crystallization progresses, the saline concentration increases and more denaturation takes place. This can proceed down to very low temperatures (-50 or -60°C) under which circumstances it is impossible to store the frozen tissues with survival. The presence of interstitial fluids will induce and maintain both a continuous osmotic degradation as well as a slow but effective biochemical activity. In fact, at -50°C , some enzymes are still active and can provoke deleterious transformations of some cellular components. This is why, in all cases, this temperature zone (-5 to -50°C) can be called a “dangerous zone” and has to be traversed as quickly as possible. Another aspect of the preliminary freezing period is associated with the formation of ice crystals *per se*. Depending upon the cooling velocity, ice can develop in large lumps imposing mechanical stress on the delicate cytoplasmic architecture or it can appear in minute crystals spreading out over the tissue in a feather-like manner. In the latter case, the mechanical effects will be entirely different. However, at first glance, from the work

of Deansley (1960) it seems that the shape of ice crystals is not of the utmost importance as far as the preservation of life is concerned. Some cells which appear entirely disrupted will prove to be viable while a perfect maintenance of the intracellular structure is not always an index of potential physiological activity. This is why we must concern ourselves with the biophysical changes inside the cells rather than their actual appearance in the frozen state.

2. *The Crossing of Intermediate Temperatures.*

Crystallization of "Eutectic Mixtures"

As we have just said, -50 or -60°C is not a sufficiently low temperature to produce complete rigidity as can be shown by electrical measurements. If the electrical resistance of a tissue or a biological medium is recorded in the course of freezing, it will be seen to increase slowly as the ice crystals develop. However, as long as interstitial fluids are present, resistivity will remain proportionally low. With sufficient cooling, the concentrated fluids will crystallize, giving an intricate mixture of ice crystals, hydrates and glassy bodies. By analogy with what is known in mineral chemistry they are usually called "eutectic" or "eutectoid-like" mixtures. When they have hardened, the rigidity of the solution is complete and the electrical resistance jumps to a high level. All chemical reactions then cease and long-term preservation can be expected.

The exact determination of this temperature which has been called "maximum temperature of complete solidification" (Rey, 1960b) is of the utmost importance in order to predetermine the freezing and storage programme. It will vary from one tissue to another and depend on their previous treatment. For instance, in the case of glycerol-impregnated tissues, it is in the neighbourhood of -75°C .

Another interesting observation can be made relative to eutectic crystallization. When the system has hardened, it appears quite stable. Then if it is rewarmed slowly, eutectoid-like mixtures will soften and melt at a specific temperature, which is always the same for each material and which has been called "minimum temperature of incipient melting" (Rey, 1960b). This temperature is very important because it represents the upper limit of the zone of optimum storage. Though it should be the same as the maximum temperature of complete solidification, it is generally much higher. This peculiar phenomenon is typical of the freezing of aqueous systems. During cooling, metastable equilibria are reached, as a result of which interstitial fluids will remain liquid well below their normal crystallization temperature. This metastable state can be broken at a temperature which may vary according to the cooling or the freezing process itself. In fact, from one experiment to another, the maximum temperature of complete solidification will never be the same although, once frozen, the eutectic will always melt at precisely the same temperature in the course of rewarming: the minimum temperature of incipient melting. In other words, for a given range of temperatures, the structure of

the frozen mass will be dependent not only on the temperature but also on the way in which this temperature has been reached. Even for simple aqueous sodium chloride solutions the structure at -30°C will not be the same if freezing has been achieved by progressive cooling from 0°C to -30°C or by deep freezing to -80°C and rewarming back to -30°C .

This is of the utmost importance in the preservation of frozen tissues and shows quite clearly that the thermal history of the specimen must be specified very closely. In practice, deep freezing to very low temperatures will always be required in order to achieve complete hardening of the system, even though much higher storage temperatures might be used. For this study, we have found electrical measurements and differential thermal analysis very reliable tools for the exact determination of the minimum temperature of incipient melting (Rey, 1960b, 1961).

3. Low Temperature Thermodynamic Phenomena

From what has been said before, it could be expected that when hardening is completed, further changes cannot take place. Although this is generally true, in some instances, some kinds of "solid reactions" will occur.

(a) *Migratory Recrystallization*. If the tissue is kept frozen at a temperature above -120°C , ice crystals are liable to form and fuse together into larger ice blocks. This has been called migratory recrystallization by Meryman (1955) and is particularly important above -100°C . It does not seem, however, to affect the viability but it will definitely play a major part if histochemical or histological work has to be carried out on the frozen tissues or tissues frozen and dried at drying temperatures above -100°C (Rey, 1960a). It can be expected that the histological structure of the dry material at the end will not be the same as that existing in the tissue when frozen initially in liquid nitrogen or in liquid propane.

(b) *Vitreous Transformation—Devitrification*. Another kind of "solid reaction" is seen in the low temperature evolution of glasses. As we have shown, glassy bodies (Rey, 1960a) are very often found in the course of freezing and, at very low temperatures, they may undergo transformations. Differential thermal analysis shows that vitreous transformation as well as recrystallization phenomena take place in glycerol-impregnated material at temperatures as low as -130°C in the solid state. This, again, does not seem to have too important an effect on viability but will induce structural changes. Although vitreous transformations can be considered reversible, recrystallization, i.e. devitrification, is a one-way process and will produce a modification of the texture of the frozen mass.

In order to avoid those phenomena, storage temperatures as low as -150°C are required.

4. Storage Period and Thawing Process

It is quite clear that the thawing process is not simply the reverse of freezing and that it plays a very important part in the preservation of life. We shall not

develop, here, this particular point save to mention that, according to our own experience and in full agreement with most other investigators in this field, low temperature storage (below -100°C) and very quick thawing are required.

An Experimental Study of the Preservation of Life Survival of the Chick-embryo Heart

As a short example of the above discussion we shall briefly describe our experiments on the chick-embryo heart.

First we shall summarize the different techniques which can be used for the preservation of life in isolated cells or tissues.

1. Ultra-rapid Freezing and Vitrofusion

According to Luyet and Gehenio (1960), ultra-rapid cooling may produce low temperature stability in cells without structural changes, since water cannot crystallize out. Then, if crystallization is again avoided by rapid warming, viability can be preserved. Due to its special requirements, this technique cannot be applied to large tissue masses but is restricted to very small pieces. Rinfret (1962, 1963) has reported good results with blood cells.

2. Freeze-drying : Lyophilization

Freeze-drying is known to be an adequate technique for the preservation of biological and some living organisms such as bacteria, yeasts and viruses. However, it has failed up to now to preserve life in cells from higher organisms. It is likely that these cells are killed in the previous freezing phase. It is impossible to use glycerol as a protective agent since it cannot be extracted under vacuum and concentrates in the course of drying (Rey, 1960a). However, new experiments which have been carried out in this field by Juscenko (1959) are very promising. Juscenko freezes spermatozoa in the presence of glycerol which is then washed out by low temperature substitution of a mixture of freon and heptane. These liquids are then distilled off with the ice in the course of drying.

3. Protective Agents

The addition of protective agents is so far the most common way to protect cells from the deleterious effects of freezing. Sugars, diethylene glycol and dimethylsulphoxide have been used for that purpose and produce good results. The most important work has been done with glycerol, the remarkable protective action of which was discovered by Jean Rostand (1946). Later, Polge *et al.* (1949) confirmed the previous experiments of Rostand.

We have been working primarily with glycerol.

It is not our intention to report our experiments in detail as they have already been described (Rey, 1957-1959) and will be shown on our film.

However, we shall summarize the main results (see Figs. 1-5).

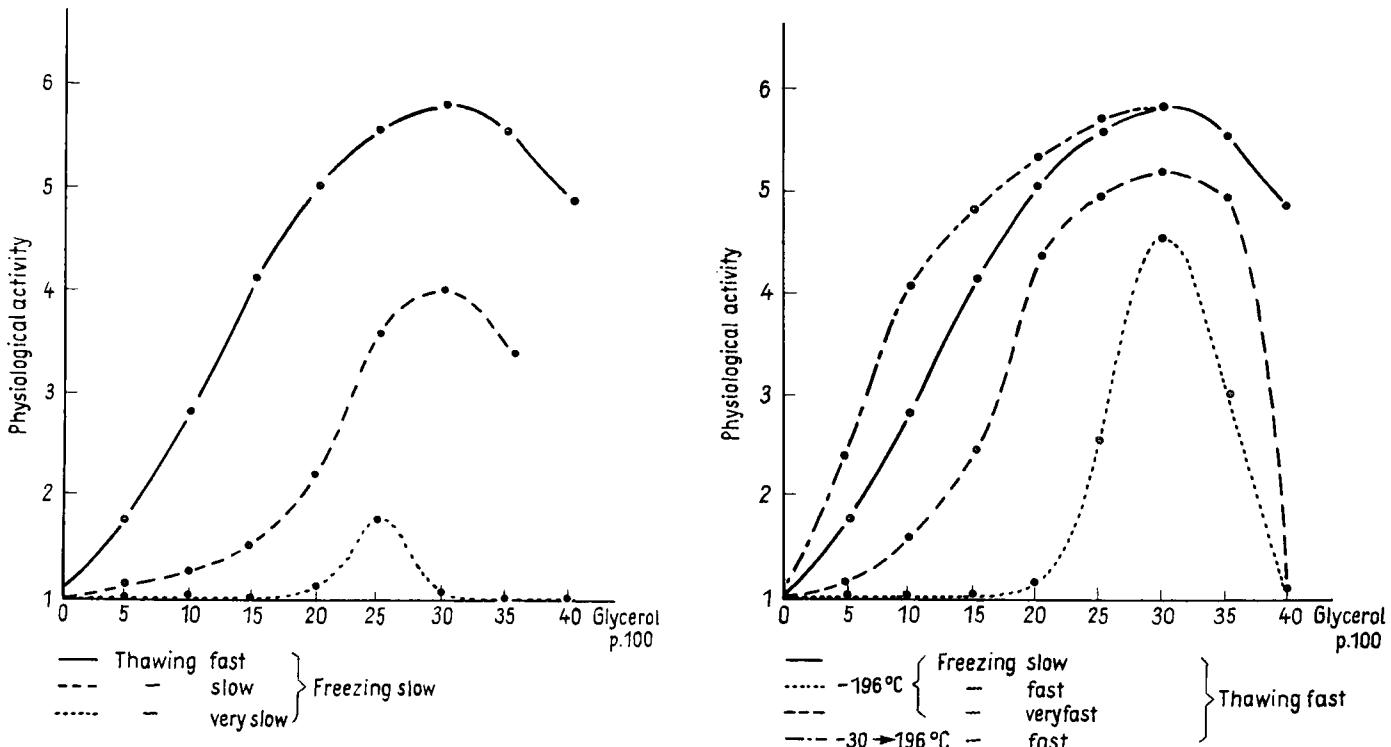


FIG. 1. Variation of the physiological activity of the chick-embryo heart according to the velocities of freezing and thawing. The activity is plotted on an arbitrary scale ranging from no activity —(1)— to co-ordinated auriculo-ventricular contractions—(6).

Hearts taken out of 6- to 8-day-old chick embryos are isolated and soaked in 30 per cent glycerol in a physiological balanced salt solution, they are then frozen in liquid nitrogen down to -196°C over periods varying from 1 min to $\frac{1}{2}$ sec.

They can be then stored at -196°C or at higher temperatures up to -75°C for some months or more. To check the viability, thawing is effected very

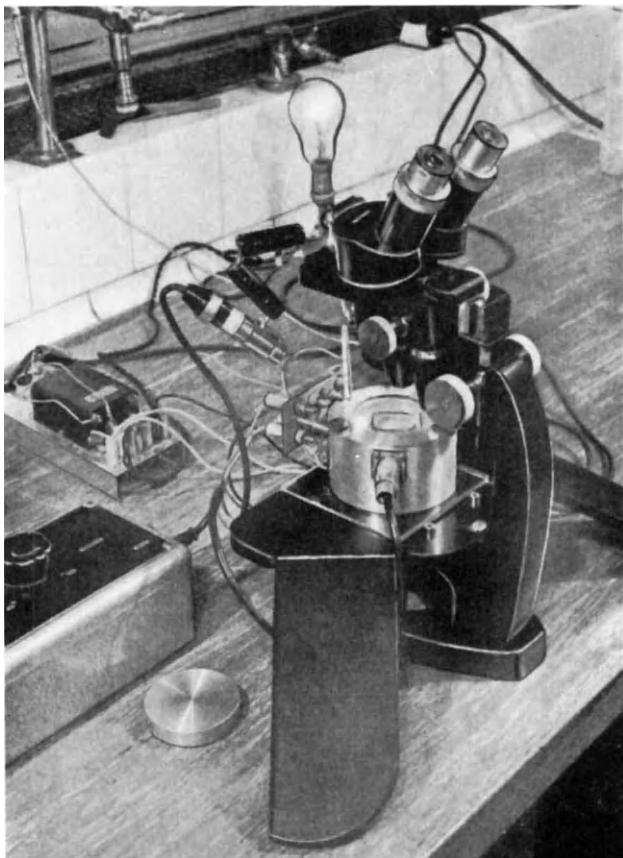


FIG. 2. Experimental set-up to record the electrogram of the chick-embryo heart. The heart is cultured *in vitro* in organ culture in aseptic conditions and can be examined directly during the whole experiment.

quickly by direct immersion of the heart in warm ($+38^{\circ}\text{C}$), balanced salt solution. Hearts are then cultured *in vitro* in organ or tissue culture.

After they have been studied for nearly one week, they show normal physiological activity, including growth of their cells (in tissue culture), regular contractions and normal electrocardiograms in organ culture. The evolution of

the electrocardiogram during cultivation is similar to that of control non-frozen hearts and shows the same developmental patterns as are seen *in vivo*.

From the biophysical standpoint, the protection afforded by glycerol can be outlined as follows:

In the presence of glycerol, the crystallization of ice is more regular. The velocity of ice growth is markedly reduced and a smooth feather-like freezing is effected.

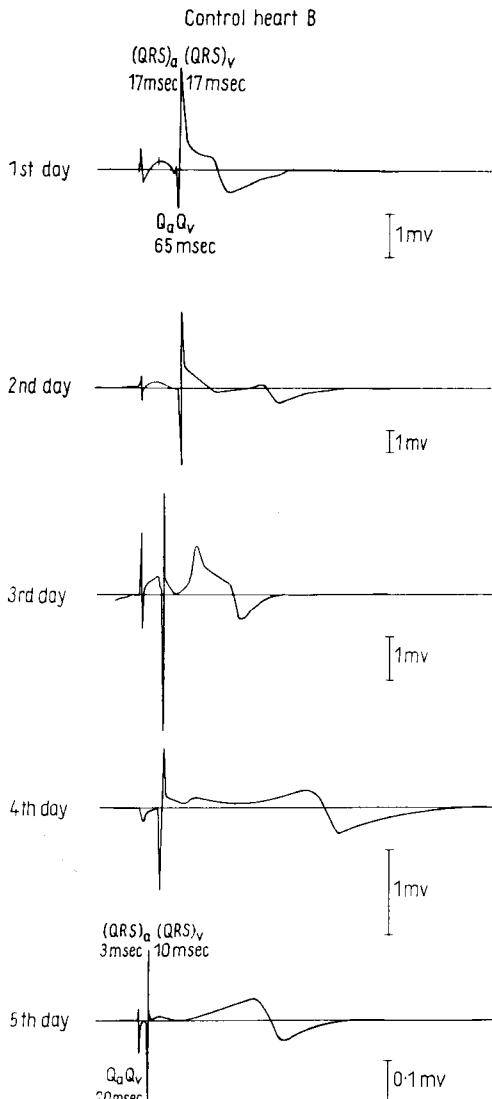


FIG. 3. Evolution of the electrocardiogram of a 5-day chick-embryo heart cultivated *in vitro* without any previous treatment.

As water separates out in the form of ice, glycerol remains in the interstitial fluids. Thus it helps in reducing the concentration and, as it permeates the cells, it prevents osmotic shock.

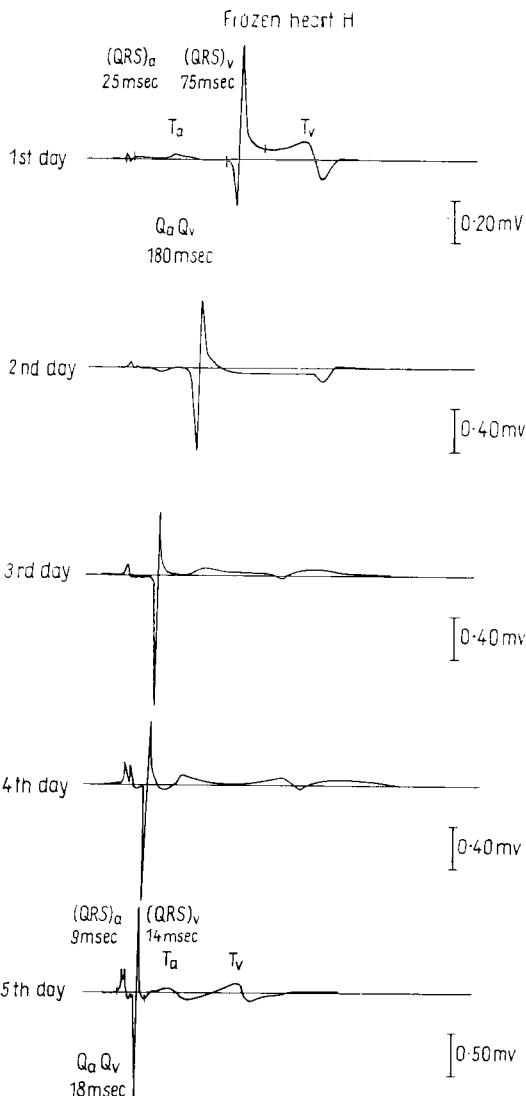


FIG. 4. Evolution of the electrocardiogram of a 5-day chick-embryo heart cultivated *in vitro* after freezing in liquid nitrogen (-196°C), storage and quick thawing. The heart has been pretreated with 30 per cent glycerol in balanced salt solution.

Eutectic temperatures fall to lower figures and the hardening of interstitial fluids gives rise to microcrystalline structures and to a large amount of glassy bodies. The whole freezing process becomes reversible.

An important part of the "free water" is bound more or less strongly and helps in the formation of amorphous rigid structures.

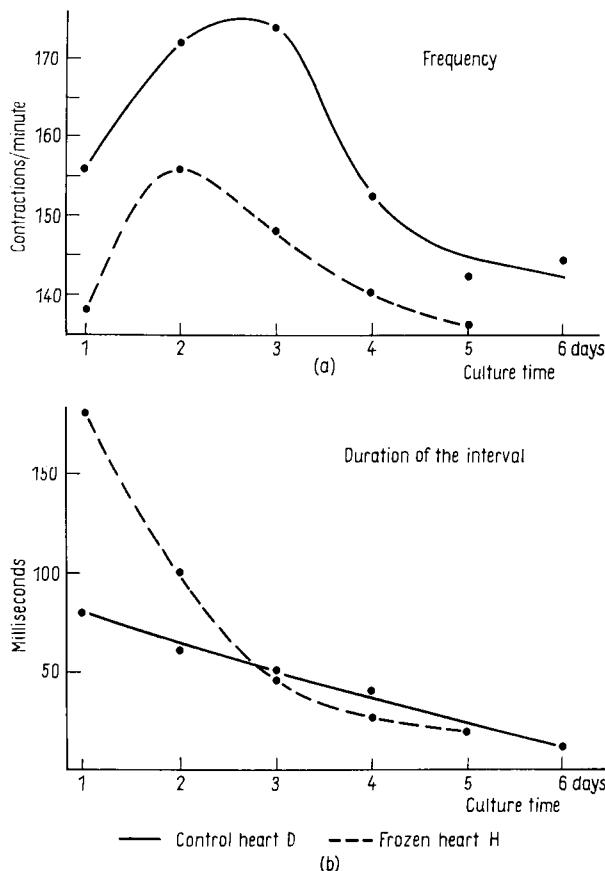


FIG. 5. Evolution of some characteristics of the electrocardiograms *in vitro* of control and frozen hearts.

Conclusion

The preservation of life by low temperature freezing and storage in the presence of glycerol can be highly significant in the medical and surgical fields and suggests the possibility of operating a tissue bank of living cells and organs.

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THE RESISTANCE OF INSECTS TO DEEP COOLING AND TO INTRACELLULAR FREEZING

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COLD-RESISTANCE in poikilothermic animals is a varying property depending on a complex combination of the conditions of the media inside and outside the organism. An increase in resistance to low temperatures in winter time is due to hereditarily conditioned metabolic rearrangements in the life cycle over which adaptive reactions and alterations of an organism arising under the direct influence of cold are superposed. Changes of the first group can be regarded as phylogenetic species adaptations expressed as alterations in the physiology of the whole organism in relation to environmental conditions (e.g. diapause of insects). The second group comprises hardening phenomena, which, especially when they take place at the temperature of about 0°C, involve changes occurring at the cellular level.

During recent years it has been shown that cells of the majority of organisms are potentially resistant to any extremely low temperature. But what the differences and fluctuations in cold resistance of cells are due to is still not clear. According to the data of V. M. Rumyantseva, from our laboratory, the resistance of yeast cells, *Endomyces magnusii* (at a constant cultivation temperature) to the temperature of liquid gases, changes in different phases of the life cycle depending on the age of the culture and the type of energy changes.

An investigation of the cold-resistance of insects shows that it depends on many factors which, acting successively and regularly, lead not only to a high resistance of physiologically active cells to extremely low temperatures, but also to retention and preservation of the organization of such complex animals. No similar examples were found in the whole animal kingdom among other multicellular organisms, and therefore a study of the reasons and factors inducing the high resistance to extreme temperatures which we observed in the corn borer (*Pyrausta nubialis* Hübn) and some other species is of general biological interest.

In the present communication we shall mention some new factors giving additional information over our previous publications. (Losina-Losinsky, 1937, 1942, 1955, 1962, 1963 a, b).

Recently we have shown that diapausing larvae of the corn borer, during hardening at low temperatures, acquire a capacity of surviving the tempera-

ture of liquid gases. The optimal temperature of hardening is about 0°C (Table 1). At a temperature of 10°C and higher the percentage of diapausing caterpillars which can survive -196°C decreases. Larvae kept at room temperature die when cooled to -196°C. Very few caterpillars kept at -9°C can stand -196°C. However, preliminary maintenance for several days at -30°C again increases resistance to -196°C.

TABLE 1. THE INFLUENCE OF INCUBATION TEMPERATURE ON SURVIVAL OF CATERPILLARS *Pyrausta nubialis* AT -196°C (FROM 10 TO 15 JANUARY, 1962)

Cooling: -30°C, 1 hr; -196°C, 24 hr.

Number of experiments	Number of caterpillars	Maintenance temp. (°C)	Percentage of mobile caterpillars	Reaction after thawing		Life duration (days)
				Percentage of caterpillars responding to electrical stimulus. Number of immobile caterpillars taken as 100%		
39	20	22	0	0		0
40	20	10	10	10		3-4
41	20	4	60	5		3-26
42	20	0	70	5		3-26
43, 46, 47, 48	82	-9	5.0	17.5		0-20
44	22	-30	27.3	13.7		4-26

TABLE 2. SURVIVAL OF CATERPILLARS *Pyrausta nubialis* AT -196°C DURING DIAPAUSING PERIOD (FROM NOVEMBER TO FEBRUARY, 1962-1963)

Cooling: -30°C, 30 min; -196°C from 1 to 4 days.

Number of experiments	Date	Number of caterpillars	Maintenance temperature (°C)	Reaction after thawing		Time of exposure to -196°C (days)	Rate of thawing	Life duration (days)
				Percentage of mobile caterpillars	Percentage of caterpillars responding to electrical stimulus. Number of immobile caterpillars taken as 100%			
1/50	10 X	20	0	0	0	1		0
2/51	10 XI	10	0	0	90	1		0-2
3/52	17 XI	10	14	0	50	1	rapid	0-2
4/53	10 XII	10	0	60	40	1		2-53
5/54	10 XII	10	14	0	30	1		2-12
6/55	17 XII	18	0	11.1	16.5	2	slow	2-3
7/56	21 XII	15	0	86.6	13.4	4		6-57
8/57	28 I	20	0	60	30	1	rapid	3-45
9/58	1 II	20	0	0	100	1		3-20

Survival of caterpillars took place following rapid and stepwise cooling, when the caterpillars were preliminarily frozen to -30°C and then rapidly cooled by solid CO_2 or liquid nitrogen. In one series of experiments we succeeded in obtaining 100 per cent survival of the larvae after freezing to -196°C . The motor reactions, the capacity of the cell to form neutral red grains and tissue respiration served as criteria of survival. The duration of hardening was not less than 2 weeks.

Table 2 represents the data on changes in resistance of caterpillars to -196°C during the period from October to January. We see that maximal resistance occurred at the end of December.

As there are neither physiological nor biochemical reactions below the temperature of liquid oxygen or nitrogen it might be expected that at the temperature near absolute zero we would obtain the same result as at -196°C . To explore the possibility of recovering insects after freezing in liquid helium a special experiment was conducted, apparently for the first time, on insects with a normal body water content during diapause.

The Experiment on Cooling Caterpillars of the Corn Borer to -269°C

Twenty diapausing larvae were kept throughout October at 0°C ; on 25 December they were transferred for 40 min to a temperature at -30°C . After the caterpillars had frozen they were exposed for 1 hr to the temperature of solid carbon dioxide (-78°C) and then placed in Dewar vessel with liquid nitrogen. After 20 hr (26 December) the insects were quickly transferred to an empty Dewar vessel which was inside the liquid nitrogen vessel. The temperature in the empty Dewar vessel was between -130 and -150°C . Under such conditions the caterpillars were kept for 1 hr 45 min after which the Dewar vessel was filled with liquid helium (-269°C).

The insects were kept in liquid helium for 6 hr 30 min, i.e. until the helium had evaporated. After the evaporation of the helium, when the temperature in the vessel rose to -210°C , the test tube containing caterpillars was immediately transferred to room temperature. In 5-6 hr observations showed that only one caterpillar retained motor reactions. On the following day seven caterpillars moved their mouth, extremities and legs, six responded to electric current by movement of their legs and the bending of their bodies, and seven did not respond to stimuli.

On 28 December, four out of six caterpillars which on the previous day had responded only to electric current began to move jaws, palps and heads spontaneously. Thus the number of caterpillars which regained motility after freezing to -269°C reached 55 per cent; the total number of caterpillars alive was 65 per cent. However, this percentage is lower than that obtained from experiments with caterpillars kept in liquid nitrogen for four days (from 21 to 25 December). In this series the percentage of motile caterpillars was as

high as 86 per cent, the rest of the animals responding to electrical stimuli. Thus a temperature decrease from -196°C to -269°C caused an additional cell injury.

The resistance to ultra-low temperatures may be increased by providing external conditions favouring recovery of the damage induce by the freezing of cells and tissues. If the larvae, thawed at room temperature after freezing to -78°C , are transferred for a month to a temperature of about 0°C , and then placed in conditions optimal for their development, metamorphosis and the flight of the mature moth can be observed.

The state of caterpillars after freezing at -196°C may be improved when they are placed on moist filter paper. Contact humidity not only prolonged the duration of life, but also favoured the reparation of motor responses in the caterpillars which did not respond to electrical stimuli after freezing.

Intracellular Freezing

The exposure of the corn borer and other investigated insects to extremely low temperatures results in the freezing of body liquids, i.e. in their crystallization. By measurements of the cooling temperature with a thermocouple, crystallization can be easily determined by the temperature jump after supercooling. In solid carbon dioxide and especially in liquid gases frozen insects become very hard and fragile. Now arises the problem which still has not been answered—whether water freezes inside or only outside the cell and whether cells can survive intracellular freezing. Till the present time it was presumed that intracellular freezing of protoplasm always led to the death of cells.

Therefore, in the cases where the cells survived at extremely low temperatures, their resistance was ascribed solely to the fact that ice crystals did not form inside the cells. This phenomenon may be: (1) due to dehydration caused by the extraction of water from cells by the ice crystals formed in the extracellular space, (2) as a result of the supercooling of intracellular liquid and the development of great viscosity, or (3) because of vitrification.

Experiments with plant cells showed that cell dehydration occurs during ice formation inside the tissue: water freezes outside the cell, the protoplast shrinks and plasmolysis occurs.

To reveal the nature of the processes which take place in the cells during the freezing of diapausing corn borer caterpillars we developed a technique to observe cellular processes at any temperature below 0°C .

Our experiments proved to be successful since the sample of organs was vitally stained with acridine orange (1 : 40000) and observations were made in the falling light of the luminescent microscope.

We froze different tissues from caterpillars but preferably samples of the salivary gland were use, since these cells possess large nuclei and a well-expressed capacity to luminescence. We also froze samples of the tracheae

and the fat body. Observations and photographs were made mostly at magnification of $\times 100$ - 250 .†

It was established the cells of different organs of caterpillars will supercool to -15 , -20°C , after which they freeze immediately.

Freezing in the medium takes place simultaneously, or a few seconds before crystallization in tissues; the freezing process in the nuclei of the salivary gland and the tracheae, stained with acridine orange, is perfectly visible. In the small nuclei, the centre usually becomes transparent, and the stained substances are gradually pressed toward the periphery. This fact is probably due to the formation of crystals. But in the large nuclei there appear some or even many centres of crystallization or perhaps monocrystals surrounded by a green thin rim of nucleoplasm. The nuclei assume the appearance of a net; crystals in the falling light first appear as small dark transparent cavities. A few minutes after freezing they grow in size and become more transparent (Losina-Losinsky, 1965, Fig. 2).

The growth in crystal size probably must be regarded as migratory recrystallization (Meryman, 1957) occurring at some constant temperature, as well as during a decrease or slow increase of temperature. Such growth of "cavities" is particularly well pronounced 10-15 min after the start of freezing.

If luminescent substances are present in the surrounding medium, the medium also acquires a net structure beginning the moment of freezing. Consequently it appears that the formation of so-called "cavities" results from the growth and enlargement of the crystals. At the moment of thawing the net structure disappears in the medium and cytoplasm of the salivary gland. In the nuclei it either remains or disappears, depending on the cold resistance of the caterpillars and on the speed of thawing (Losina-Losinsky, 1965, Fig. 5). In hardened caterpillars the net structure disappears in the nuclei after thawing. However, some changes were noticed in the luminescence of the nuclei. With a decrease in the cold resistance observed beginning in February, the net structure in the nuclei does not disappear even after thawing. Sometimes it can be made to disappear by a rapid transfer of the preparation for a few seconds from the cold to the temperature of 45 - 52°C .

Deformation of cells and their nuclei observed during drying were not found during freezing in insects or in Protozoa. Neither did we notice the growth of crystals in the extracellular space. Thus the animals which endure rapid freezing to extremely low temperatures are resistant to intracellular crystallization.

Discussion

It is well known that, independently of external conditions, the cold-resistance of insects increases during diapause and falls again in the process of

† A detailed description of the method developed in collaboration with E. P. Moroz and the results of the experiments on animal cell freezing will be presented in special articles.

reactivation. Our experiments have shown that the cold-resistance of diapausing corn borer caterpillars can be significantly increased by hardening at about 0°C. A seasonal increase in the resistance to low temperatures is accompanied by numerous biochemical processes in the organism and its cells as described more than once in the literature. However, it is difficult to determine whether these processes themselves induce in cells the high resistance to cold and freezing, or whether the resistance results from certain general alterations in the state and structure of protoplasm, nucleoproteins, proteins and lipoprotein complexes. Not all of the seasonal changes in the biochemistry of the insect fully coincide with changes in cold-resistance: either they occur before the maximum is reached or as resistance begins to decrease. This can be associated also with the accumulation of fat, reducing substances, glycerin, with the intensity of respiration and the transfer to glycolytic metabolism, etc.

Fluctuations in osmotic pressure are most closely related to changes in cold-resistance, which as mentioned earlier (Losina-Losinsky, 1937, 1942, 1955, 1963a) changes in parallel and simultaneously with cold-resistance. In December, i.e. in the period when caterpillars are most resistant to ultra-low temperatures, the freezing point of tissues (ground) is maximally lowered: on the average, it reaches -7.6°C and in some individuals it is even lower than -8°C which is only indicative of high osmotic pressure.

At the present moment it is difficult to say what causes the osmotic pressure in the insects to increase. This process seems to be related to the formation of different substances in different species (soluble sugars, amino acids), but it may be due to changes in the structure of water at temperatures near to the freezing point or to cell water binding capacity.

It is well known that a decrease in the environmental temperature favours the formation of hydrogen bonds, or the consolidation of the macromolecules.

It can be suggested hypothetically that the increase in osmotic pressure is accompanied by an increase in the resistance of the macromolecules to mechanical damage caused by the ice crystals which are being formed in the cell.

Many of the changes occurring in autumn and with cold-hardening, are similar in plants and insects. However, a further increase in the resistance to extremely low temperatures is due to different reasons.

Plants survive slow cooling because of the gradual dehydration of cells (Tumanov, 1963; Sakai, 1963). Insects, however, can tolerate rapid cooling during which they either supercool strongly or suffer intracellular freezing.

Asahina and Aoki (Asahina and Aoki, 1958; Asahina, 1959) reported another condition not directly related to the process of hardening, but necessary to enable insects to tolerate extremely low temperatures.

In order that hardened insects might withstand the temperatures of liquid gases, they must have been initially cooled to -30°C . Insects that endure such conditions then prove to be resistant to lower temperatures and further freezing.

After a short period of exposure of the corn borer caterpillars to -30°C , no processes similar to those characterizing the first stage of hardening are to be expected. It seems that the freezing of body juices and a subsequent enlargement of crystals protect cells from the effects of rapid freezing resulting from exposure to the temperatures of liquid gases, a process otherwise leading to the formation of numerous small crystals. Perhaps the rate of crystallization influences the degree of disturbances in the molecular and submolecular bonds in the protoplasm. After rapid immersion of insects in liquid gases, rupture of the cuticle, outflow of hemolymph, and tissue destruction with partial cell solvation are observed on thawing.

Contradictory data from the literature indicate the complexity of the role played by cooling and crystallization rates in the survival of cells and organisms. Most extraordinary and least studied, are the cases when in a certain temperature zone there arises a danger for the cell and organism. Thus in our experiments with caterpillars, with cooling to -30°C , the rate of cooling plays a very insignificant role. At the temperature of -50°C , caterpillars die following rapid cooling, but at a rate of cooling approximately twice as high, for instance in a medium at -78°C , caterpillars retain their full viability. With a further increase in the rate of cooling viability again decreases.

The survival of insects is also influenced by the rate of warming. Very slow warming, carried out for hours, inhibits the return of function in thawed caterpillars. These phenomena require further investigation in order to obtain conditions for optimal reparation of the alterations caused by freezing and thawing. Until recently it was supposed that cells were not able to endure intracellular ice formation. It has been shown experimentally that plant cells only survive extracellular freezing, which destroys most animal cells as soon as crystallization starts in the internal organs and tissues. Only insects are known to endure almost a complete conversion of body juices to ice, i.e. the freezing of 80 per cent of the total water (Losina-Losinsky, 1942). Salt (1962) indicated that insects tolerate intracellular ice at a temperature of about -20°C .

Our direct observations of the freezing process made with the aid of the luminiscent microscope and microphotography confirm the fact that the caterpillars of the corn borer can endure intracellular freezing. Moreover, it was discovered that the crystallization phenomena occurring at a very low supercooling, as described above, proceeds at varying cooling rates and under the same conditions which were used for the freezing of intact insects. It is well known that these conditions do not favour extracellular freezing. It should be observed that we possess no preparations or data obtained from observations made under various conditions where extracellular crystallization was not accompanied by intranuclear crystallization. We also did not observe any deformations of the cells or nuclei, which inevitably occurs with cell dehydration from extracellular crystallization.

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SOME BIOCHEMICAL MECHANISMS OF LOW TEMPERATURE ACCLIMATION IN TROPICAL POIKILOTHERMS

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IT IS now well established that most poikilotherms compensate for changes in temperature by changes in their activity and metabolism, if the change in the temperature persists for some time. Most of the earlier evidence in this regard was obtained from poikilotherms occurring in temperate or cold climates (Bullock, 1955). However, in recent times several tropical forms have also been shown to be capable of such compensation to low temperature (Pampapathi Rao, 1961; Saroja, 1962). While the evidence for the ability of poikilotherms to compensate for changes in temperature is abundant, the mechanisms involved in such compensation are not well understood. Several possibilities have been suggested by earlier workers (Precht, Christophersen and Hensel, 1955; Precht, 1958; Prosser, 1958) and it has been shown that compensation occurs at the organ and tissue levels and that quantitative changes occur in the metabolic enzymes. But the mechanisms involved in the initiation of such adaptive changes and the possible biochemical sequence of such changes have not hitherto been analysed in any great detail. During the past few years we have been studying this problem in some detail in relation to low temperature acclimation in some tropical poikilotherms (Pampapathi Rao, 1961, 1962b) and the present paper is a brief review of the results of our investigations.

Materials and Methods

The following animals are used in our studies:

Lampito mauritii, an earthworm.

Lamellidens marginalis, a freshwater mussel.

Paratelphusa hydromedus, a freshwater field crab.

Heterometrus swammerdami, a scorpion.

Etoplus maculatus, a freshwater fish.

The normal habitat temperature of these animals is around 27–32°C. The temperatures to which they were acclimated in the laboratory were 20 and 30°C.

In some cases (in scorpions only) experiments were done on 35 and 15°C acclimated animals also. In all cases the temperature was maintained within $\pm 0.05^{\circ}\text{C}$.

It might be mentioned here that in the tropics low temperature means only 15 or 20°C. Most tropical poikilotherms become inactive even at 15°C and many die if exposed to temperatures lower than 15°C for prolonged periods. Only some arthropods are capable of surviving below 10°C.

The methods used for studying the various aspects of the problem have been described in earlier papers from our laboratory and hence no detailed descriptions will be given here.

Results and Discussion

(i) Changes in the Inorganic Ions in Body Fluids

Quantitative changes in the ionic components of the blood or body fluids were observed in all the poikilotherms studied by us when they were acclimated to low temperature. It was shown earlier that in the crab, *Paratelphusa*, warm-acclimation resulted in an increase in blood calcium and magnesium (Pampapathi Rao and Ramachandra, 1961). Likewise in the fish, *Cirrhina reba*, warm-acclimation resulted in a decrease in the tissue fluid chloride. Since such changes appeared adaptive in their significance, these changes were analysed in detail in cold-acclimated poikilotherms. On cold-acclimation there was an increase in the blood calcium in the freshwater mussel, while the chloride level decreased, but the potassium and sodium tend to show some increase (Pampapathi Rao, 1963a) (Table 1). In the fish *Etroplus maculatus*, acclimation to low temperature resulted in a remarkable increase in calcium while potassium showed some increase (12 per cent over normal). Sodium, magnesium and chloride showed a decrease over normal, chloride showing the most change (Pampapathi Rao, 1962a; Parvatheswararao, 1962). Similar changes which are more pronounced, are noticed in the cold-acclimated earthworm, *Lampito mauritii*, in which there was a distinct increase in potassium, calcium and sodium, and a decrease in chloride, sulphate and magnesium (Pampapathi Rao, 1962b, 1963a) (Table 1). It is of great interest to note that exactly parallel changes occur in the natural populations in winter.

The fact that the degree and direction of change in the ionic concentrations is similar in seasonal populations and laboratory-acclimated worms indicates that the mechanisms involved and the phenomena noticed are similar in both the cases, and that the changes are adaptive in nature. This is further indicated by the recent demonstration by Pampapathi Rao and Venkatareddy (1962) that even such processes as membrane permeability and active uptake of chloride show acclimation to temperature. Therefore, it will be of interest to discuss the metabolic effects that are likely to be produced by the changes in the inorganic ion concentrations noticed above.

TABLE 1. CHANGES IN CONCENTRATION OF CERTAIN IONS IN THE BLOOD OR BODY FLUID OF TWO TROPICAL POIKILOTHERMS
ON COLD AND WARM ACCLIMATION

Species of animal	Condition of acclimation Level of significance and <i>t</i>	Na	K	Ca	Mg	Cl	SO ₄	Free amino acids
Earthworm <i>Lampito mauritii</i>	Cold (20°C) Warm (35°C) Level of significance <i>t</i>	47 36.5 1% 5.120	17.6 11.9 1% 8.531	12.65 20.52 1% 16.646	6.55 11.45 1% 10.671	31.73 21.55 1% 9.196	2.88 4.67 5% 2.594	56 252 1% 4.150
Freshwater mussel, <i>Lamellidens marginalis</i>	Cold (20°C) Warm (35°C) Level of significance <i>t</i>	22.98 19.07 1% 2.973	0.722 0.661 1% 22.59	0.434 0.274 1% 12.024	-- -- 1% --	10.06 14.68 1% 9.535	-- -- 1% --	2.28 3.99 1% 14.582

† All values for ions expressed in mm/l except calcium in freshwater mussel which is expressed in mg/ml.

Amino acids expressed in mg/100 ml.

Since the changes in the ion concentration occur in the blood and body fluids which bathe the cells and tissues, such changes would naturally influence the metabolism and activity of such cells and especially the muscle tissue. Calcium is known to play an important role in several metabolic processes. It is known to regulate membrane permeability and the relation of muscle membrane potential to temperature is regulated by calcium (Apter and Kokensu, 1960). Likewise calcium is involved in protein binding, which is in turn influenced by changes in the concentration of multivalent anions (Walser, 1960).

Potassium also is known to have a great influence on muscle metabolism. Increase in the potassium concentration of the bathing fluid results in an increase in the resting metabolism of muscle, and such effects are of relatively long duration. The activation of glycolysis of muscle by potassium is also modulated by calcium (Kaye and Mommaerts, 1960). Therefore, an increase in potassium in the body fluids of cold-acclimated forms will tend to increase the muscle metabolism and this effect is augmented by the increase in calcium concentration also.

The data presented by Robertson (1957) show a correlation between the blood magnesium level and the locomotor activity of several different crustaceans, the more active species having lower magnesium levels. It is known that magnesium acts as an anaesthetic and depresses neuromuscular transmission. Consequently a decrease in magnesium level in cold-acclimated individuals will permit greater muscular activity.

The foregoing discussion shows the important role that changes in the ionic concentrations can play in affecting metabolic compensation. The increase in the calcium level in cold-acclimated forms, accompanied by an increase in potassium and a decrease in magnesium will result in pushing up muscle metabolism against the depressing action of lowered temperature.

Similarly the increase noticed in sodium might influence nerve activity. A decrease in the chloride level during cold-acclimation has been noticed consistently (Pampapathi Rao and Ramachandra, 1961; Pampapathi Rao, 1962a, b; Pampapathi Rao, 1963a; Selvarajan, 1962; Parvateswararao, 1962; Saroja, 1962), but the metabolic significance of this decrease is not clear at present. The changes in the sulphate level might be related to amino acid metabolism.

(ii) Changes in the Free and Bound Amino Acids

Besides the changes in the level of the inorganic ions, a noteworthy change that attracted our attention was the decrease in the free amino acids in the body fluids and tissues on cold-acclimation. This decrease is quite discrete in several of the forms studied and is shown to occur in the freshwater mussel (Pampapathi Rao, 1963a), the earthworm *L. mauritii* (Pampapathi Rao, 1963a; Raghu-pathiramireddy and Pampapathi Rao, 1963; Saroja, 1962) and in the fish *E. maculatus* (Pampapathi Rao, 1962a; Parvateswararao, 1962). It is possible that

this decrease indicates an increased incorporation of amino acids into the tissues resulting from greater protein synthesis. Therefore, a detailed quantitative analysis of the amino acid levels (free and bound) was attempted in the earthworm by Raghupathiramireddy and Pampapathi Rao (1963).

It was shown that while the free amino acids decrease (by 43 per cent) the total bound amino acids increase (by 9·2 per cent) on cold-acclimation. While free arginine was found in normal worms, it was not recorded in cold-acclimated worms. The other free amino acids (cystine, lysine, aspartic acid, glutamic acid and alanine) decrease considerably (30–49 per cent) except serine (almost no change) on cold-acclimation. On the other hand all but two (ten out of twelve) of the bound amino acids increase on cold-acclimation, the two exceptions being arginine and leucine—isoleucine.

This decrease in the free amino acid level and the increase of bound amino acids on cold-acclimation indicates a shift of the amino acids from free to bound conditions, due to incorporation of free amino acids into proteins. Not all the changes indicate such a shift. While aspartic acid, serine and glutamic acid decrease in the free condition and increase in the bound condition, others such as lysine, arginine and alanine decrease in the free condition but do not show corresponding increase in the bound condition, suggesting a selective breakdown of these amino acids. This indicates the possibility of a potential arginase system operating during cold-acclimation. Yet others like phenylalanine, which are absent in the free condition, show an increase in the bound state, suggesting biosynthesis and subsequent incorporation into protein.

Jankowsky (1960) has shown an increased rate of incorporation of free amino acids during cold-acclimation. This, coupled with the present demonstration of a decrease in the total of free amino acids and an increase in the bound amino acids, indicates considerable synthesis of new proteins and a turnover of existing proteins. Such a mechanism provides scope for the alteration of the levels of specific enzyme proteins as indicated in the following discussion taken from Raghupathiramireddy and Pampapathi Rao (1963):

That the turnover of existing protein through their depolymerization to peptides or free amino acids may indirectly serve as a partial source for the possible synthesis of new enzyme proteins has been envisaged (Stanier, 1955). There is evidence for adaptive enzyme synthesis in animals (Stanier, 1955; Knox, 1958) and the incorporation of amino acids may be greater into the adaptive enzyme than into other proteins (Gros *et al.*, 1954). The release of amino acids by proteolysis and their re-incorporation on thermal acclimation probably results in altered levels of specific enzyme proteins. The patterns of release and re-incorporation, however, are not identical in cold and warm adapted earthworms, thereby indicating different extents of alteration in the levels of the same or different enzymes on acclimation to cold and warm temperatures.

(iii) *Changes in the Protein Content of Cells and Tissues*

Such a movement of amino acids from the free to the bound condition (into the proteins) would result in an increase in the total protein within the

cells and tissues. Measurement of the dry matter in the cells, using the interference microscope, showed that the protein content of the cells increases on cold-acclimation both in the mussel (22 per cent increase above normal) and in the earthworm (31 per cent) (Pampapathi Rao, 1963 b). Analysis of the protein content of the tissues in the earthworm (using micro-Kjeldahl technique) also showed an increase in tissue protein nitrogen, and there was a parallel increase in the dry weight of the tissues (Saroja, 1962).

These results indicate increased protein synthesis and since RNA levels are closely associated with and are considered to be indices of protein synthetic activity, this possibility was studied.

(iv) *Changes in the RNA Content of Tissues*

If there were to be increased protein synthesis during cold-acclimation, tissues of cold-acclimated individuals should show an increase in the RNA concentration. Measurements of the RNA content revealed that the RNA level increased in the cold-acclimated mussel, *L. marginalis* (Pampapathi Rao, 1963 c), in the earthworm (Saroja, 1962) (Fig. 1) and in the fish, *E. maculatus* (Parvateswararao, 1962). Such increases were particularly conspicuous (82 per cent above normal) in metabolically active tissues such as the hepatopancreas of the mussel.

The increase in RNA may be related to the operation of the hexose monophosphate shunt which is believed to operate at a higher capacity in cold-acclimated poikilotherms (Ekberg, 1958; Kanungo and Prosser, 1959). According to Glock and McClean (1954) an increase in such a shunt activity might be correlated with increased protein synthesis. This is possible since the HMP pathway could contribute pentoses to the nucleic acid (Burma, 1960; Fruton and Simmonds, 1961). This is all the more probable since "so far attempts to demonstrate the formation of pentoses by other pathways have failed" (Burma, 1960). That the HMP pathway might be active (and thus contribute to the formation of RNA) in cold-acclimation is further indicated by the demonstrated decrease (Raghupathiramireddy and Pampapathi Rao, 1963) in the glucogenic amino acids like glutamic acid, aspartic acid and alanine which might be due to their utilization via the HMP pathway.

This increased RNA content indicates an increased protein synthesis, including enzyme protein, in cold-acclimation.

(v) *Changes in the Activity of Metabolic Enzymes*

The above-mentioned facts indicate that there should be an increase in enzyme protein on cold-acclimation and that such enzyme activity should be greater in cold-acclimated poikilotherms. Previous studies by Precht and his collaborators (Precht *et al.*, 1955) have amply demonstrated the fact that several Krebs' cycle enzymes show increased activities during cold-acclimation.

In our laboratory it has also been shown that succinic dehydrogenase increased in activity in cold-acclimated earthworms. In the scorpion, *H. swammerdami*, cold-acclimation results in the increased activity of several dehydrogenases.

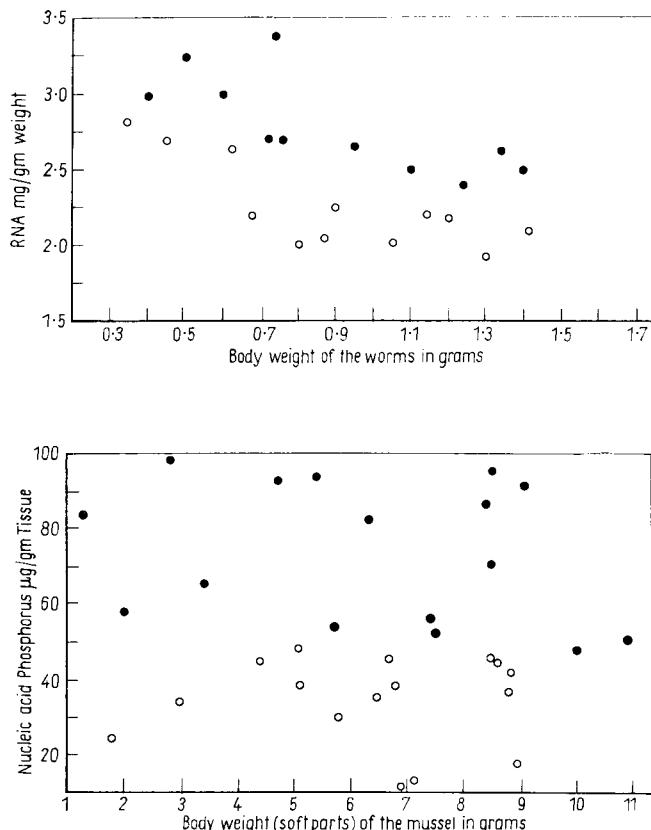


FIG. 1. Comparison of the nucleic acid level in cold- and warm-acclimated earthworm (top: level of significance = 1 per cent; $t = 4.493$) and freshwater mussel (bottom: level of significance = 1 per cent; $t = 7.04$). Closed circles cold (20°C) acclimated individuals. In the case of earthworms whole animals were taken and in the case of mussel the hepatopancreas was taken for purposes of estimation. (Figure for earthworms taken from Saroja, 1962; for mussel taken from Pampapathi Rao, 1963c.)

It is to be expected that not only the metabolic enzymes but other enzymes also show increased activity on cold-acclimation. Thus it has been shown in our laboratory that the cholinesterase activity in the nerve cord of the earthworm increases on cold acclimation.

(vi) *Changes in Nerve Activity*

Since acclimation to low temperatures not only results in compensation of the metabolic level but also results in a change in the whole activity of the organism and since it is well known that poikilotherms acclimated to low temperature habitats show the same degree of activity and speed of reflexes (Bullock, 1955) involving the nervous system, it was natural for us to examine the possibility of a compensation occurring in the nervous system so as to change the level of activity of the nervous system. While higher nervous functions (such as reflex activity) could not be investigated, the changes noticed in the cholinesterase content in the nerve cord of the earthworm, suggested to us the measurement of the speed of conduction in the giant fibres in the ventral nerve cord as one measure of any compensation that might occur.

Along with an increase in the cholinesterase content, there was also an increase in the acetylcholine content of the nerve cord in the cold-acclimated earthworms. The degree of increase of acetylcholine was somewhat greater than the degree of increase in acetylcholine esterase. The speed of conduction in the giant fibres in the ventral nerve cord is greater in the cold (20°C) acclimated worms (3·6 to 4 m/sec) as compared to the warm (35°C) acclimated worms (0·6 to 1·0 m/sec) when both were measured at the intermediate normal (room) temperature of 28°C (Pampapathi Rao and Saroja, 1963 b).

Recently Roots and Prosser (1962) have demonstrated compensation to temperature in the activity of the central nervous system (involving certain reflexes) in fish.

Viewing these changes in the nervous system in relation to the fact that reverse acclimation of oxygen consumption in the earthworm takes a much shorter time, leads us to ask the question whether acclimation to low temperature includes a process of "learning" also. Would subsequent exposures to low temperature result in faster or fuller acclimation compared to the first experience of a similar temperature change?

(vii) *Changes in the Neurosecretory and Endocrine Tissues*

The above-noted changes in body fluids, in RNA levels, in protein synthesis and in enzyme activities remind one of similar changes in insects before moulting. Since in insects it is known that these changes are under hormonal control, involving changes in the activity of the neurosecretory system, the possibility of similar hormonal control with associated neurosecretory or endocrine changes, being present in cold-acclimation of poikilotherms was investigated. Hence, changes in the neurosecretory cells in the supra- and sub-oesophageal ganglia of the cold- and warm-acclimated earthworms and the changes in the activity of the thyroid gland of cold- and warm-acclimated fish, *E. maculatus*, were studied.

In the earthworm there are very few neurosecretory cells in the supra-oesophageal ganglion and activity in this part of the nervous system is

TABLE 2. CHANGES IN THE ACTIVITY OF THE THYROID GLAND OF THE FRESHWATER FISH, *Etroplus maculatus* ON ACCLIMATION TO HIGH AND LOW TEMPERATURES

(Figures in brackets indicate number of determinations.)

State of acclimation	Cell height			Mean height of cellular area (e) as percentage of mean height of total follicular area (f)		
	Mean height in micra	S.D.	Level of significance of difference	(e/f) 100	S.D.	Level of significance of the difference
Cold (20°C)	17.00 (50)	0.4316	At 1% level	52.66 (50)	0.04	At 1% level
Warm (35°C)	14.875 (49)	0.3285		43.69 (50)	0.1766	

negligible. In the sub-oesophageal ganglion several neurosecretory cells are seen and in general the same neurosecretory cells which were noticed in normal animals were also noticeable in cold-acclimated animals, but their activity was greatly increased on cold-acclimation and in some cases even the number of active cells was greater in cold worms compared to normal worms (Pampapathi Rao and Saroja, 1963a). On the other hand the neurosecretory cells exhibiting secretory activity in warm (35°C) acclimated worms are different in position.

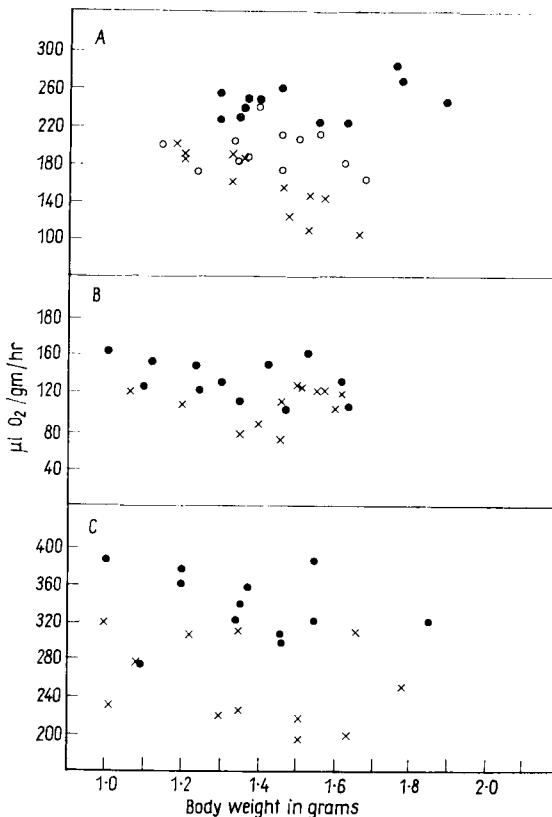


FIG. 2. Effect of body fluid of cold- and warm-acclimated earthworms on the tissue respiration of cold, warm and normal earthworms. A. Oxygen consumption of tissue from "normal" worms with body fluid from "cold" (closed circles); from "warm" (crosses) and from "normal" (open circles) animals. Level of significance between "cold" and "normal" fluid effect is 1 per cent; $t = 3.085$. B. Oxygen consumption of tissue from "cold" worms with body fluid from "cold" (closed circles) and "warm" (crosses) animals. Significant at 1 per cent level; $t = 3.2$. C. Oxygen consumption of tissue from "warm" worms with body fluid from "cold" (closed circles) and from "warm" (crosses) animals. Significant at 1 per cent level; $t = 4.628$.
(Taken from Saroja, 1962.)

Likewise in the fish it was found that the activity of the thyroid gland (as measured by the increase in cell height and increase in the percentage of the cellular epithelial area in the total follicular area) shows a marked increase on cold-acclimation, the increase being highly significant statistically (Table 2). These changes in neurosecretory cells and endocrine organs indicate that hormonal factors might be involved in bringing about the metabolic changes noticed in cold-acclimation.

(viii) *Evidence for Hormonal Factors Influencing Metabolism*

In view of the noticeable change in the endocrine organs (viz., neurosecretory cells and the thyroid) it was considered possible that there might be a direct hormonal effect on tissue respiration in cold-acclimation. Therefore, this possibility was checked by the addition of small quantities of cold-worm body fluids to the tissues of the normal worm contained in Warburg flasks and the tissue respiration was studied. It was found that the addition of body fluids of cold-acclimated worms to the perfusion fluid increased (by 25 per cent) the oxygen consumption of the tissues from normal worms or of the tissues from warm (35°C) acclimated worms (by 31 per cent). On the contrary addition of body fluids from warm-acclimated worms depressed the metabolism of the tissues from normal- and cold-acclimated worms (Pampapathi Rao and Saroja, 1963 a) (Fig. 2).

Likewise in the fish similar results were obtained, using body fluids extracted from warm- and cold-acclimated fish and studying their influences on muscle tissue metabolism.

These effects of body fluids from cold-acclimated worms (or fish) and warm-acclimated worms (or fish) indicate respectively the presence of activating or depressing substances in these fluids. These active principles are in all probability hormonal products of the neurosecretory or endocrine systems, released into the body fluids.

General Conclusion

The above results indicate the following sequence of events in cold-acclimation in poikilotherms, and a general summary is given in Fig. 3.

The decrease in the oxygen consumption when the animal is exposed to low temperature may itself act as a stress, which stimulates the neurosecretory system or the endocrine system. This results in the production of hormones which affect the metabolism in many ways initiating a series of changes in different parts of the organism as follows:

1. They may act directly on the tissues respiration increasing the oxygen consumption.
2. They may change the permeability properties of cells and tissues resulting in changes in the concentration of ions in the body fluids, thus producing metabolic effects which are of long duration.

3. They may act on the coenzyme systems of different metabolic pathways, which may lead on to an increased RNA level and hence to an increased enzyme protein synthesis resulting in higher metabolism.
4. They may directly stimulate increases in RNA synthesis leading to increased protein synthesis.

The question would arise whether hormonal action could cover such a variety of actions. The available information on hormone action in invertebrates and vertebrates permits such possibilities. In vertebrates it is well known

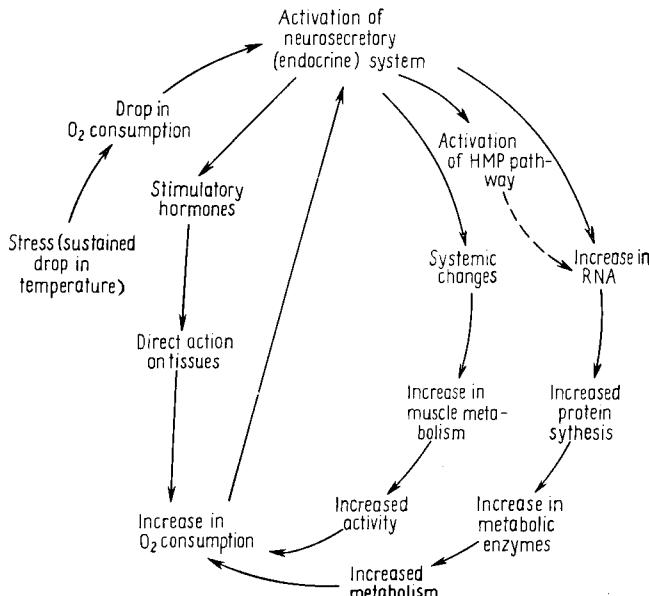


FIG. 3. Diagrammatic summary of the likely sequence of events occurring during acclimation to low temperature. (Modified from Pampapathi Rao and Saroja, 1963.)

that homeostasis of the internal medium, involving changes in permeability and active transport, is under the control of hormones. In insects, similar endocrine control of such processes has been demonstrated in some cases. Stolkowski and Reinberg (1956) showed that the cellular potassium in the snail, *Helix pomatia*, is influenced by corticosteroid hormones. Therefore, it is possible that in cold-acclimation, the ionic changes noticed, are under hormonal control.

Likewise in mammals it has been shown (Glock and McClean, 1955) that the levels of activity of the enzymes of HMP pathway are under hormonal control, and that the levels of oxidized and reduced coenzymes are influenced by thyroxine, growth hormone and the like. Steroid hormones, in low concentrations, inhibit DPNH oxidation (Yielding and Tompkins, 1959). It has

been pointed out earlier that some of these pathways are related to RNA production and protein synthesis. Therefore, through such affects, these hormones might influence RNA and protein synthesis.

While there is some argument about an immediate *in vitro* effect of hormones on tissues (Buddenbrock, 1950) there is considerable evidence pointing to such an effect. Administration of thyroxine stimulates the metabolism of intact animals (Pritchard and Gorbman, 1960; Madanmohanrao, 1962). Isolated tissues also show an increase in activity or metabolism on the addition of thyroxine (Davis *et al.*, 1934; Haarman, 1936, 1954; Barch, 1953). In insects, immediate metabolic changes are known to occur on the administration of hormones or on the transplantation of endocrine tissues. Therefore it is quite conceivable that in cold-acclimation hormonal agents directly affect tissue metabolism and this is what is seen when "cold" or "warm" fluids are added to normal tissues.

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MECHANISMS OF RESISTANCE OF POIKILOTHERMIC ANIMALS TO SUBFREEZING TEMPERATURES

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ALL organisms naturally capable of withstanding tissue freezing appear to be poikilotherms. There are no reports of intact homeotherms withstanding complete freezing although certain cells or tissues in isolation may do so. Two important examples of non-lethal freezing of homeotherm tissues are found in the experiments of Smith (1961) who was able to achieve freezing of about 50 per cent of the body water in hamsters and the experimental freezing of animal extremities in the course of frostbite experimentation Meryman (1957). In both situations the survival of the tissues following brief exposures to extracellular ice has been repeatedly demonstrated.

Among the poikilotherms, the most familiar organisms of the animal kingdom which show a natural resistance to freezing injury are nematodes, insects and the intertidal mollusks. Asahina (1959) has demonstrated extracellular freezing in the nematode, *Aphelenchoides ritzema-bosi*. The resistance, however, is not uniform, for the percentage of survivors falls off with decreasing temperature although a substantial percentage survived temperatures as low as -183°C . In our laboratory, observations by Burns (1964) have shown that with the nematode, *Panagrellus redivivus*, there is a difference in susceptibility to freezing as a function of age; the adult nematodes being extremely sensitive to freezing injury while the young worm and particularly the ova, are quite resistant.

Studies on the resistance to freezing in insects suggest that there are at least two mechanisms by which injury is avoided. Salt (1936) showed that very extensive supercooling is possible in many insects. In these species, survival results from the avoidance of freezing rather than immunity to its effects.

It has been shown by Wyatt and Kalf (1958) and later by Salt (1957) that free glycerol exists in certain pupae and larvae which are tolerant to freezing. The concentrations were such that one might reasonably expect this to exert a protective influence just as it does in artificial laboratory situations. Since then, a number of other insects tolerant to freezing have been shown to contain glycerol. Unfortunately for the uniformity of the theory Salt also reported

free glycerol in a non-resistant larva, *Glooxostege sticticalis*. The probability that protective compounds other than glycerol may be elaborated by resistant organisms should be expected.

Most measurements of freezing in resistant insects show fairly modest percentages of water freezing out at non-lethal temperature. A notable exception is reported by Scholander *et al.* (1953) who found that, at -15°C , over 90 per cent of the body water in the *Chironomid* larva was converted to ice.

The intertidal mollusks are among the most interesting animals resistant to freezing, being quite large and resistant to repeated freezing and thawing at very low ambient temperatures. Kanwisher (1966) has found that all of the mollusks which leave the intertidal area, either by moving offshore or by burrowing into the sand, which he has subjected to freezing, show no resistance. All of those which remain in the intertidal area during freezing weather are clearly resistant. Studies by Kanwisher (1955) showed that the internal temperature of these mollusks was within one degree or less of ambient and that complete freezing occurs. Studies with the mussels, *Modiolus modiolus* and *Mytilus edulis* and the snail *Littorina littorea*, showed from 59 to 65 per cent of the body water frozen at -15°C ; at -22°C , *modiolus* and *littorea* had, respectively, 71 and 76 per cent of their body water frozen. Both of these organisms are known to exist in areas where the ambient temperatures commonly reach -30°C and lower; at which temperatures there may be an even further freezing out of body water. However, there do not appear to be any reports of the freezing of more than 80 per cent of the body water with survival in mollusks.

Theories of Freezing Injury

Several alternative theories have been proposed to explain the mechanism of freezing injury and resistance in animals. Perhaps the most widely quoted is the so-called salt concentration theory. When water is removed from a cell or tissue to form ice, the remaining solutes are progressively concentrated. When approximately 80 per cent of the water has been removed, the concentration of the solutes, particularly of sodium chloride, can become sufficient to cause the denaturation of the proteins. Lovelock (1953) has attributed the action of glycerol in protecting erythrocytes to its ability to prevent water from freezing, thereby reducing the amount of ice formed and holding the ultimate electrolyte concentration to less than the lethal level.

If the salt denaturation theory were applicable in all situations, we should expect to find that the freezing out of more than about 80 per cent of the body water would inevitably lead to cell death. This appears to be the case in most situations but there are a few notable exceptions, such as the above-mentioned *Chironomid* larva. The fact that many isolated tissues survive freezing followed by rapid thawing but do not survive when slowly thawed also indicates that other factors are involved. The evidence that salt denaturation can be an important factor in freezing injury is undeniable. It is also evident, however,

that it is not the sole cause. The effects of dehydration need not be limited to salt concentration. Levitt (1962) has proposed a very elegant model for protein denaturation resulting from the formation of S—S bonds during protein compression from the dehydration of freezing. On thawing, distortion or rupture of protein chains may occur with rehydration because of the strength of the S—S bonds. It is also possible that denaturation may not in itself be irreversibly lethal, but simply set the stage for a subsequent irreversible process.

A second theory attributes injury to the mechanical effects of ice crystals. This has been applied in particular to plant tissues where rigid structures are insufficiently flexible to withstand the deformation caused by ice crystals. This theory has also been applied to animal tissues where intracellular freezing has occurred. The fact that intracellular ice formation is almost invariably associated with cell death has been offered as evidence that the complex internal structure of the cell is unable to accommodate the dislocation caused by internal crystallization. It should be noted, however, that no direct evidence of such a physical effect has been presented. The report by Salt (1962) of non-lethal intracellular ice formation within the fat body and labial gland cells of the larvae of *Cephus cinctus* and *Eurosta solidaginis* can be cited as contradictory to the inevitability of intracellular ice damage although the extent of intracellular structures in these cells should be investigated. One must conclude that, whereas intracellular crystallization probably results in cell death in most cases, this may not be universally true.

Mazur (1963) has theorized that to prevent the formation of intracellular ice crystals during extracellular freezing it is necessary that the intracellular water pass through the membrane to form extracellular ice with sufficient rapidity so that the freezing point of the intracellular solution does not lag behind the cell temperature. He has calculated theoretical curves which show a reasonable relationship to experimental data. The cooling rate possible without the formation of intracellular ice will depend upon the water permeability of the cell membrane thus providing one possible explanation for the limitations in freezing rate commonly observed. A similar theory has been proposed by Asahina (1961).

Merryman (1962a) has proposed that extreme dehydration could result in the removal of intracellular water to such an extent that structures normally separated from each other make contact resulting in the formation of unnatural chemical linkages or the transfer of components from one surface to another. In the mitochondria, for example, packed with membranes upon which enzymes are presumably located in highly specific sequence, the direct contact of membrane surface could be understandably damaging. Levitt's theory of protein linkage might apply equally well to contact between opposing membranes. The protective action of penetrating additives such as glycerol can be explained by this theory. Their water binding capability can serve simply to maintain a separating liquid medium between structures and prevent contact.

Meryman *et al.* (1962b) have proposed still another means by which freezing injury may occur. It has been observed during experiments on the rapid freezing of human erythrocytes that the protective effect of polyvinylpyrrolidone is exerted almost as well when cells frozen in isotonic saline suspension are thawed by immersion in 20 per cent PVP as when they are both frozen and thawed in the presence of the polymer. It was proposed that the protective effect exerted following thawing is a purely mechanical one in which the large polymer molecules wrap themselves around the red cell immediately following thawing. This provides mechanical stability and preserves an otherwise weakened membrane from being ruptured by the extreme concentration gradients existing momentarily during the redistribution of water released from thawing ice crystals. There is little doubt that these concentration gradients can be of sufficient magnitude to rupture a membrane which has not fully recovered its integrity following freezing and thawing. Electron microscope studies by Saacke and Almquist (1961) of frozen bovine spermatozoa show a disruption and partial loss of the external membrane following lethal freezing. Leibo (1963) has found a direct correlation between the freezing death of the red algae, *Porphyridium cruentum*, and the presence of the internal pigment in the suspending medium. The possibility that the final irreversible injury may often be membrane rupture following thawing cannot be overlooked.

Metabolism in the frozen state is of major importance to organisms naturally resistant to freezing. Kanwisher (1959) and Scholander *et al.* (1953) have investigated the metabolism of organisms in the frozen state and found it to be low but measurable. Since the removal of from 40-90 per cent of the body water will produce a concentration of enzymes and enzyme substrates in some cases approaching saturation, it is clear that the organism must be equipped with processes specifically designed to function under these extreme conditions.

Although the avoidance of freezing through supercooling does not strictly constitute a resistance to freezing injury it is none-the-less a very effective mechanism for survival in the presence of freezing temperatures. The degree to which many organisms can supercool is quite extraordinary. This adaptation is presumably achieved through the absence of ice crystal nuclei within the body of the organism. At temperatures above -40°C , ice crystal nuclei are believed to consist of particles with surface configuration suitable for the support of ice crystal growth. Organisms adapted to low temperature through their ability to supercool may, on a genetic basis, have achieved an absence of suitable nuclei or have elaborated a protective material which, much in the manner of antibody, binds to potential ice-supporting surfaces, effectively blocking them.

It is apparent that the several theories of freezing injury summarized above provide attractive explanations for many observed experimental results. It is equally apparent that no single theory can be applied to all observations.

However, they are at least not mutually exclusive, and there is no reason why we should not be prepared to find any or several of these phenomena operative in particular situations.

It is also of interest that several commonly observed phenomena appear to be unexplained by any of these theories. It has been frequently observed, for example, that survival following rapid thawing is substantially better than that following slow thawing. Another commonly observed phenomenon is the preliminary modest dehydration which can markedly increase the freezing-resistance of an organism. No adequate explanation for these observations appears to have been proposed.

In conclusion, it is evident that among the rather large number of ways in which freezing injury may be effected no proposed mechanism appears to be compatible with all experimental observations and we can only conclude that there are many ways in which freezing can injure living systems. Which consequence of freezing delivers the final blow to a living system will depend upon the system itself. Systems that are resistant to freezing can achieve this through a variety of means and we cannot at this stage look with any optimism for a single common mechanism.

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THE COMPARATIVE RESISTANCE OF TISSUES OF SOME HIBERNATING AND NON-HIBERNATING RODENTS TO COOLING AND SUPERCOOLING

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IT IS well known that hibernating heterothermal animals can endure very sharp fluctuations of body temperature without disturbances in the vital functions of the organism. The body temperature of many hibernating rodents may vary considerably in the active period, but during hibernation it decreases to 0°C and even lower.

The maximum low temperatures at which viability has been observed for hibernating hamsters are +5 (Adolf, 1951), +6, +2°C (Smith, 1956) and even -5, -6°C (supercooling) (Smith, 1956), and for hibernating ground squirrels, +7, +1.5 (Kalabukhov, 1956), -1.7, -1.9 (Ipatyeva, 1962), -1.1°C (Losina-Losinsky, 1943).

These temperatures do not affect the tissues and cells of such hibernating animals directly, but through a very complicated system of neurohumoral regulations of the organism (Slonim, 1961). However, there is evidence indicating that some organs and tissues of heterothermal mammals (active as well as hibernating) are more resistant to low temperatures than those of homeothermal ones.

Thus, temperatures near 0°C can be regarded as maximal for the survival of the isolated heart of hamsters, marmots, ground squirrels and chipmunks, while the heart of rats, squirrels and other non-hibernating animals stops when the temperature falls below 11–12°C (Smith, 1957; Lyman and Blinks, 1959). Sciatic (Chatfield *et al.*, 1948) and phrenic (South, 1961) nerves of the hamster proved to be more resistant to lower temperatures than those of the rat.

In the above studies the authors compared the tissue resistance of hibernating and non-hibernating rodents in the thermal range exceeding (often considerably) 0°C (from 38 to +5, +4°C). It also seems interesting to study the tissue resistance of heterothermal animals to the effect of positive and

especially negative temperatures near 0°C which hibernating rodents encounter under natural conditions. Therefore, the purpose of the present investigation was to compare the resistance of some isolated cells and tissues of the ground squirrel (*Citellus pygmaeus* Pall) and the hamster (*Cricetus auratus* Waterhaus) with the resistance of similar tissues in rats to cooling in the range from 5 to 0°C and supercooling at -4°C. The organism of a hibernating animal adapts itself to tolerate a sharp decrease in temperature during hibernation, and it was interesting to compare tissue resistance of these animals in different seasons of the year. The tissues of the ground squirrel were investigated both in summer (June–July) in the active period and at the time of hibernation (January–February). The experiments were performed *in vitro* on the ciliated epithelium, diaphragm muscles and erythrocytes. The measure of susceptibility of ciliated epithelium to injury was the survival time of ciliary movement. Muscle resistance was determined by the retention time of excitability and the steepness of increase in the threshold of excitability. The susceptibility of erythrocytes to injury was determined by the amount of haemoglobin outflux into the surrounding medium.

Figure 1 a shows curves indicating the relationship of the survival time of ciliated epithelium of rats, hamsters and ground squirrels (active and hibernating) to temperature. One can see that upon transfer of the animals from temperatures of 20–22°C to positive temperatures about 0°C, the survival time of the rat ciliated epithelium first rises, attaining the maximum in the thermal range of +5°C and, below this zone (0–4°C), decreases again. Similar relationships were observed for the ciliated epithelium of hamsters and also of active and hibernating ground squirrels.

However, although the curves of the three investigated species of rodents are similar, the absolute survival time of their ciliated epithelium differs considerably, increasing in the order: rat < hamster < ground squirrel. The difference in the survival time proved to be statistically significant for all the temperatures studied with the exception of room temperature at which the survival time of all these tissues tends to become similar.

A comparison of the survival time of the diaphragm muscles of these animals (Fig. 1 b), gives the same relationship. One can see from the graph that with the transfer of animals from room temperature to near 0°C the survival time first increases, then shortens again with further decrease in temperature.

A comparison of the absolute survival time of the diaphragm muscles of active and hibernating ground squirrels has shown that with supercooling (-4°C) the survival time of this tissue in hibernating ground squirrels (Fig. 1 b) considerably exceeds that for active animals. A slight increase in the survival time of the diaphragm muscles was noted at 0°C but not observed at +5°C. At this temperature, the survival time of the diaphragm muscles of the hibernating ground squirrel is less than in the active animal. Thus the curve indicating the survival time of this tissue in hibernating ground squirrel is shifted toward lower temperatures.

Experiments with ciliated epithelium carried out simultaneously on the same animals have revealed a considerable increase in the survival time of this tissue at -4°C (Fig. 1a) and also at $+5^{\circ}\text{C}$. At 0°C the survival time decreased.

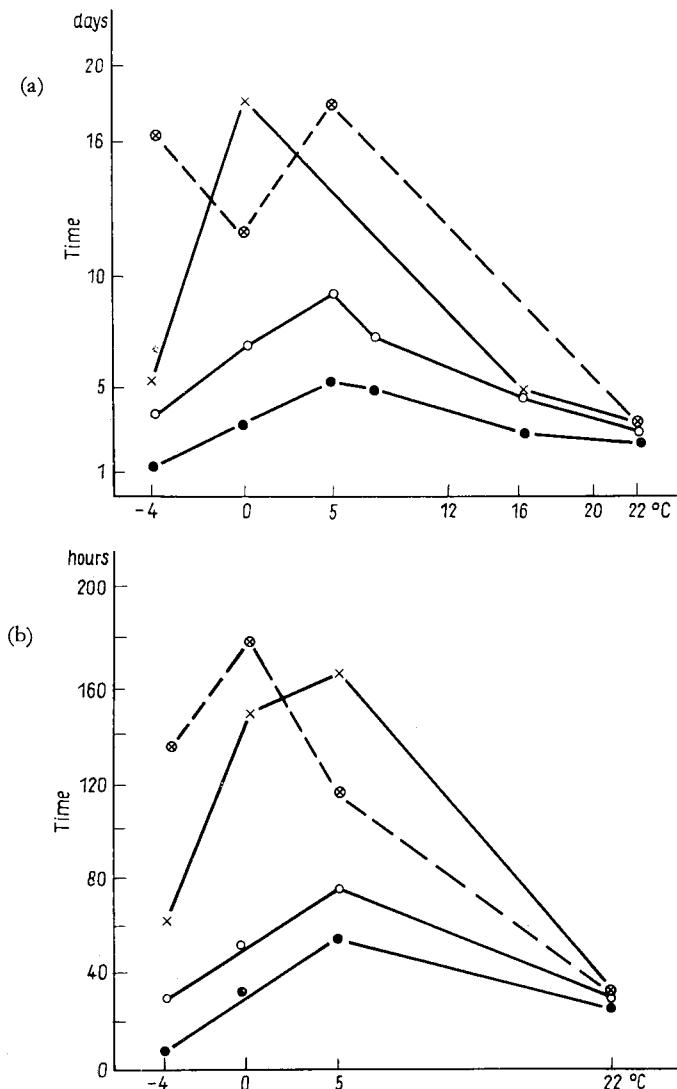


FIG. 1. Tissue resistance of rats, hamsters and ground squirrels (active and hibernating) to cooling and supercooling. The abscissa gives the temperature (in $^{\circ}\text{C}$), the ordinate, the survival time (in days and hr). Points—rat; circles—hamster; crosses—ground squirrel (active); dotted lines—ground squirrel (hibernating) a—ciliated epithelium; b—diaphragm muscle.

It should be pointed out that differences in the survival time of the tissues investigated increase with the decrease of temperature. This phenomenon is most strongly pronounced in the diaphragm muscle. Figure 2 represents relative survival time (in per cent) of the diaphragm muscle for the three species of rodents at the investigated temperatures. The survival time of the rat muscle under the same temperatures was taken as 100 per cent (straight line). As can be seen from the graph, differences in the resistance of the diaphragm muscle of hamsters and ground squirrels increase with fall in tem-

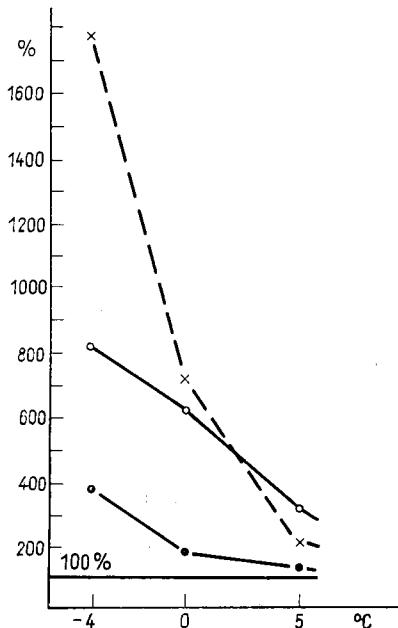


FIG. 2. Survival time of diaphragm muscle in hamster, active and hibernating ground squirrel in percentage of the survival time of the rat muscle. The abscissa gives the survival temperature. Points—hamster; circles—active ground squirrel; crosses—hibernating ground squirrel.

perature and are most evident with supercooling. As has been mentioned above the diaphragm muscle of the hibernating ground squirrel proved to be the most resistant. The resistance of muscle tissue to cooling and supercooling was determined not only by the retention time of excitability, but also by the character of excitability time changes.

As one can see from Fig. 3, the threshold of excitability of all the three species of animals increases with time. However, the steepness of the threshold is different in each species. It rises most steeply in rats whose curve begins to ascend just after the tissue has been transferred to low temperature. In hamsters, and especially in ground squirrels, the rate of increase in the thresh-

old of excitability is even less within the first 10–15 hr of incubation. This fact suggests a greater resistance to low temperatures in the muscle tissue of these animals.

The results obtained from a study of the resistance of erythrocytes in rats and ground squirrels to the effects of reduced temperatures are shown in

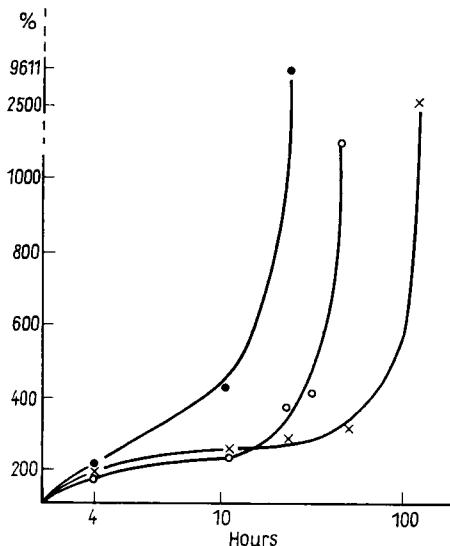


FIG. 3. The rate of changes in the threshold of excitability of the diaphragm muscle in rat, hamster, ground squirrel at 0°C. The abscissa gives the survival time of muscles at 0°C (logarithmic scale), the ordinate, the magnitude of the threshold of excitability (in per cent of the initial value). Points—rat; circles—hamster; crosses—ground squirrel.

Fig. 4, which indicates the outflux of haemoglobin from erythrocytes after a 25-hr incubation at different temperatures. Changes in the resistance of these cells at low temperatures are similar to those observed in the ciliated epithelium and diaphragm muscles. In both species of rodents, the susceptibility of erythrocytes to injury first decreases and becomes minimal at +5°C. With a further decrease of temperature (0–4°C) the susceptibility of erythrocytes to injury increases again. However, at any temperature, the erythrocytes of rats were found to be 2–3 times more susceptible to damage than those of ground squirrels. This fact is not due to a greater initial amount of haemoglobin in the erythrocytes of the rat, for measurements of the haemoglobin content per ml erythrocytic mass (after haemolysis) revealed about the same amount of haemoglobin for ground squirrels and rats.

Thus, by applying different indices of cell viability (survival time, character of excitability changes and the rate of haemolysis) we reach the conclusion that

some tissues of hibernating rodents (ground squirrel and hamster) are more resistant to cooling and supercooling than the same tissues of non-hibernating rats. The differences are insignificant at room temperature but gradually increase with a decrease of temperature and become well pronounced in the range of positive temperatures about 0°C and continue to increase below this temperature.

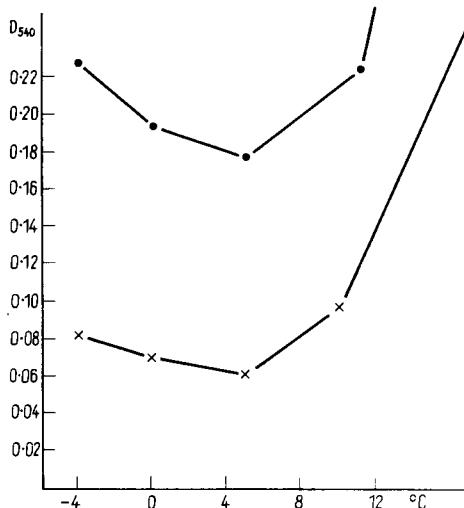


FIG. 4. Haemoglobin outflux from erythrocytes of rat and ground squirrel after 25-hr incubation at different temperatures. The abscissa gives the temperature (in °C), the ordinate, the optical density in haemoglobin solution at 540 mm. Points—rat; crosses—ground squirrels.

Our experimental data have also shown that the tissue resistance of heterothermal rodents changes with season. The tissues of hibernating ground squirrels proved to be significantly more resistant to supercooling than those of non-hibernating animals.

Moreover, it has been demonstrated that the tissues of ground squirrels are more tolerant than those of hamsters; this is probably related to some peculiarities in the biology of the ground squirrel whose seasonal fluctuations are more strongly pronounced. A comparison of the survival time of tissues of active hamsters and ground squirrels with fluctuations of their body temperature within 24 hr confirmed that there is a correlation between the degree of heterothermia of the rodents studied and the cold-resistance of their tissues. In ground squirrels, whose tissues proved to be more cold-resistant, the range of fluctuations within 24 hr is wider than in hamsters (Slonim, 1961; our own experimental data).

Our experimental results have shown that there is an adaptation of tissues in heterothermal rodents (ground squirrel and hamster) to cooling and supercooling.

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DISCUSSION

Discussion of H. T. Meryman's Paper

O. A. KRASAVTSEV: How can you explain the difference between a slow and a rapid freezing of cells? The final degree of denaturation and hence the attachment of active groups in the two cases is the same.

H. T. MERYMAN: First I must re-emphasize the conclusion of my paper: neither of the theories on freezing is universal, neither of them can explain all the phenomena observed.

The type of water crystallization during slow freezing differs from that during rapid freezing which is of great significance even if a slight or no denaturation occurs.

U. HEBER: Is there any direct evidence of protein denaturation upon freezing? I am aware it is difficult to obtain evidence of this kind. To my mind the only reliable method is to investigate the specific activity of enzymes. We carried out our experiments in this direction, but were not able to get any denaturation. I mean the phenomenon for which chemists used the term "denaturation".

H. T. MERYMAN: There is much evidence of such changes as a decrease of solubility or of biological activity of enzymes etc. We performed experiments of this kind on muscle proteins in Cambridge 25 years ago. Changes in salt concentrations in the course of freezing lead to protein denaturation *in vitro*. However, purified protein seems to be very stable not only to freezing but to extreme drying as well. This difference between purified proteins and protein complexes is to be taken into consideration.

J. LEVITT: I should like to point out that the role of cell permeability in preventing intracellular freezing was first proposed by Scarth† in 1936 and the first evidence in favour of this idea was supplied by Siminovitch and Scarth‡ in 1938. They should be given credit for this, and not the later workers who confirmed their results.

É. ASAHLINA: In connection with communications of Dr. Simatos and Dr. Meryman I would like to report some results of my own experiments dealing with mechanisms of rapid freezing. A cell suspension was frozen in different solutions (KCl , $NaCl$, $MgCl_2$, $CaCl_2$, NH_4Cl , $NaNO_3$ and sugars). The cell suspensions were exposed to low temperature for one minute. The

† SCARTH, G. W. (1963) *Trans. Roy. Soc. Can. (Sect. V)* **30**, 1-10.

‡ SIMINOVITCH, D. and SCARTH, G. W. (1938) *Can. J. Res., C* **18**, 467-481.

temperature of death was found to be roughly at the eutectic point. At higher temperatures, even fast freezing did not cause the formation of intracellular crystals and the cell remained viable. Thus the lethal temperatures are determined by the eutectic point of the solution. The addition of protective substances prevents eutectic crystallization and thus prevents freezing injury.

Discussion of K. Pampapathi Rao's Paper

S. S. HOVANESSIAN: What is the role played by acetylcholine accumulation in changes in cold-resistance with cold-acclimation?

K. P. RAO: We have shown an acceleration of nerve conduction with cold-acclimation. Therefore it is reasonable to assume that the concentration of substances which are connected with the conduction of nerve impulses, acetylcholine and cholinesterase, change too. I do not know exactly whether there is a relationship between acetylcholine and cold-resistance, but I suspect that acetylcholine may influence the cold-resistance by changing the cell membrane permeability.

N. L. GERBILSKY: Do you have any data on morphological changes occurring in the thyroid gland or in the neurosecretory system during acclimation?

K. P. RAO: The staining of secretory granules is more intensive in neurosecretory cells of cold-acclimated animals and this can serve as an indication of a higher secretory activity. The staining intensity was estimated by a photometrical measurement of the stained slides with the aid of the microphotometer. With cold-acclimation, cells of one kind were found to become more active, while quite another kind of cell revealed the same picture upon heat-acclimation. For the evaluation of thyroid gland activity on slides we measured the total area of the secretory epithelium and the area of the secretory follicle on the microphotographs. The activity was evaluated by the ratio of the two areas measured. It was found that the activity of the thyroid gland increased during the development of cold-acclimation and decreased with heat-acclimation.

H. PRECHT: What conditions the change in O_2 consumption occurring with acclimation? I wonder whether it is either hormonal or salt action? If you suspect a direct hormonal effect, you could support your assumption by experiments with the addition of hormones to the tissues *in vitro*.

K. P. RAO: I presume that a hormonal effect exists since it is seen when most insignificant amounts of body fluid from the acclimated animal are added to the control samples. Only a hormone could induce changes in such minute amounts. I do not insist on the supposition that it is thyroid hormone. Some other enzyme may be a trigger in this case. We have not performed experiments on thyroxine action *in vitro*. However, our experiments on feeding or injection of the thyroid hormone produced a considerable increase in O_2 consumption.

C. L. PROSSER: What do you mean by "body fluids"? Are they tissue extracts or blood?

K. P. RAO: We dealt with mixture of blood and tissue fluids squeezed from animals which had been cut into pieces.

C. L. PROSSER: Did you use brain extracts?

K. P. RAO: No, we did not perform experiments of this kind.

H. PRECHT: The experiments of Dr. Rao which show the effect of the thyroid hormone on the process of cold-acclimation are of great interest. Many investigators, including ourselves, have tried to demonstrate this effect but unsuccessfully. The hormonal mechanism discovered by Prof. Rao may account for a lot of phenomena in the capacity adaptation and resistance-adaptation.

Discussion of Rey and Simato's Paper

K. P. RAO: What index of cell growth in the culture did you measure?

D. SIMATOS: The growth of cells was measured by the area occupied by the culture (to be exact, by the area of the culture image), or, less often, by the weight of the culture.

O. A. KRASAVTSEV: Did you observe recrystallization after the rapid freezing of tissues, during the increase in temperature from -195 to -85°C ?

D. SIMATOS: During rapid freezing to -195°C vitreous bodies and micro-crystals form. With the increase of temperature to -85°C we observed recrystallization, fusion of microcrystals into larger crystals, and devitrification. These changes occur when the temperature rises above -130°C .

INVESTIGATIONS ON THE VARIABILITY OF HEAT-RESISTANCE IN PLANTS

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THE capability of plants to withstand short periods of high temperatures, i.e. their protoplasmic or primary heat-resistance† (see Lange, 1959) is not constant. Within a certain range, which is characteristic for a species (Alexandrov, 1962; Alexandrov, Ushakov and Poljansky, 1961) heat-hardiness may show variations in the same organism, depending on endogenous influences or induced by external factors (see reviews on heat-resistance in plants by: Bělehrádek, 1935; Precht, Christophersen and Hensel, 1955; Levitt, 1956a, 1956b, 1958; Fuchs and v. Rosenstiel, 1958; Biebl, 1962). These variations can have special ecological significance and therefore a study is necessary in order to understand the relationship between plants and their surroundings and from this to find out the possibility of existence of the species in different places. Furthermore, the analysis of the variability of heat-resistance may give information about the possible mechanism of heat-hardiness—especially by comparing the changes of the plants' resistance against heat injury with their resistance against other injuries, for instance those caused by frost or desiccation. Keeping these points in mind we carried out investigations on the variability of heat-resistance in lichens, mosses, ferns and phanerogams. The results are summarized in this report.

A. Methods

In order to measure heat-resistance, generally the whole plant, parts of shoots, cuttings of branches or single leaves were heated for a period of half an hour in a water bath at a controlled temperature. Fully turgid terrestrial plants were directly immersed in the heated water. Poikilohydric organisms and phanerogams, which may maintain a certain water saturation deficit during heating were enclosed in watertight plastic bags, to prevent direct contact between the plant body and the water. Besides this method, plants were also heated in laboratory ovens, with thermostatic control or in climatic chambers. In these cases the temperature of the plant tissues was controlled and registered by employing thermocouples or thermistors.

In order to determine the resulting heat injury, the plants (branches or leaves respectively) were recultivated after completing the heating procedure.

† This protoplasmic heat-resistance is identical to "heat-hardiness" or "heat-tolerance" as defined by Levitt (1956 a, b).

The damage caused by heat was examined macroscopically. The injury was recognizable after a period of time (in some cases hours, in others weeks) after the treatment and became apparent through changes in colour, dying and drying of the tissues. The dead areas of leaves, moss stems, etc. were measured or estimated and the percentage of damage was calculated. In the case of lichens the algae were isolated from the thallus and cultivated to determine their vitality.

B. Variabilities

1. Heat-resistance and Ageing Processes

The heat-resistance of a plant organ may change due to its natural ageing processes. Several authors (viz. Sachs, 1864; Sapper, 1935; Gorban, 1961, 1962, etc.) found differences in heat-hardiness of the tissues of the same plant, due to differences in age. Many higher plants show an increase in heat-toler-

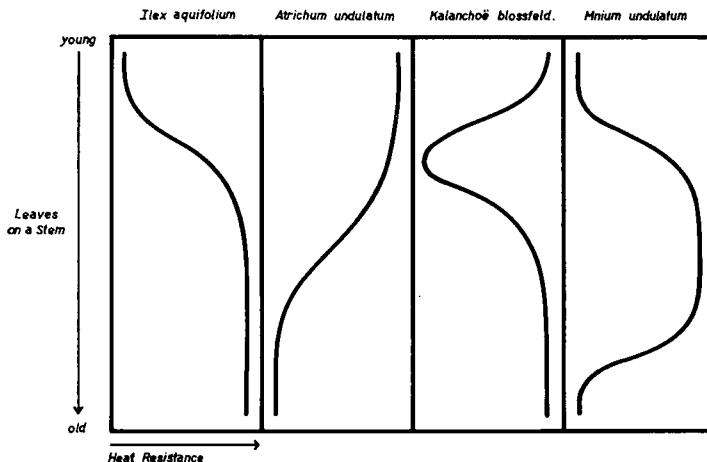


FIG. 1. Heat-resistance of the leaves of the phanerogams *Ilex aquifolium*, *Kalanchoë blossfeldiana* and the mosses *Atrichum undulatum*, *Mnium undulatum* in relation to their age. Abscissas: heat-resistance. Ordinate: position of the leaves on the stem (youngest leaf on the top). (After Dirksen, Schwemmle and Lange).

ance from younger tissues to older ones, the younger leaves being the more sensitive to heat and the older ones the more resistant. This was apparent in a great number of mediterranean evergreen and herbaceous plants (Lange and R. Lange, 1963) and also in some evergreen and winter-green species, growing in the surroundings of Göttingen (Lange, 1961). In these plants the increase of heat-resistance is distinct, particularly during the growing phase of the leaves. After hibernating there are only small or no differences in the hardiness of leaves at different ages (see *Ilex aquifolium*, Fig. 1). These species show a similar behaviour in their frost-resistance (see Till, 1956). The

leaves of some other higher plants do not show a continuous increase in their heat-tolerance during growth. Experiments carried out with *Kalanchoë blossfeldiana* (Crassulaceae) have revealed results suggesting that conditions could be more complex, than in the case of e.g. *Ilex*. In *Kalanchoë* (Fig. 1) heat-resistance first of all decreases during development of the leaves and reaches a minimum value and later on increases again. In most of the specimens examined (Schwemmle and Lange, 1959b; Lange and Schwemmle, 1960: 413 plants analysed) the leaves reached a minimum value for heat-hardiness at the third or the fourth vertical (counted from the bud above). In this growth stage there exists a correlation between the lowest heat-resistance and the highest photoperiodic sensitivity, i.e. a short day treatment of these leaves causes the strongest flower formation. It becomes obvious that in such a stage of development, the increased heat-sensitivity of the leaves is due to the particularly active and at the same time labile metabolism of the protoplasm.

Further possibilities for the relationship between heat-hardiness of leaves and their age, can be found in several mosses, investigated by Dircksen (unpublished).† In contrast to most of the phanerogams, the youngest leaves on a stem of some moss species have the highest and the older ones a lower resistance (for example *Atrichum undulatum*, Fig. 1). There are also species (for example *Mnium undulatum*, Fig. 1) where the younger and older leaves are very sensitive to brief actions of high temperatures. Middle-aged leaves are the highest in heat-hardiness (see also Scheibmair, 1938). Parallel to their heat-resistance, frost-hardiness of these mosses was also determined. No relation could be found between the resistance against high and low temperature during growth and ageing.

These examples obviously indicate that a general rule for the relation between heat-tolerance and age of the organs in the plants cannot be established. Further research will prove, through analysis of each case mentioned, what cytological changes cause the increase or decrease of hardness during the ageing processes (compare Fischer, 1950; Paech and Eberhardt, 1956).

2. Stage of Development and Heat-resistance

Furthermore, the stage of development of the whole plant influences the heat-tolerance of its organs. The degree of hardness in the leaves of seedlings for example, may differ from that in older ones of the same species (Laude and Chaugule, 1953). Variations of heat-resistance may also occur within short periods in the leaves of plants, changing from their vegetative to the reproductive phase of development. This effect can be clearly demonstrated by the short day plant *K. blossfeldiana* (Lange and Schwemmle, 1960; compare also Henkel and Margolina, 1948). Leaves of flowering specimens of this species have a significantly higher heat-hardiness than those of vegetative

† A. Dircksen: Vergleichende Untersuchungen zur Frost-, Hitze- und Austrocknungsresistenz einheimischer Laub- und Lebermoose unter besonderer Berücksichtigung jahreszeitlicher Veränderungen. Dissertation, Göttingen 1964.

specimens of the same age. The temperatures which cause 50 per cent damage to the leaves after 30-min treatment (this being the standard "value of heat-resistance") differ from each other by 2°C. The increase of resistance already begins during the photoperiodic induction, that is, long before the flowers appear. This was proved by the following experiment. Vegetative plants of *Kalanchoë*, cultivated under long day conditions, were exposed for different periods (1–21 days) to flower-inducing, short day conditions. Immediately after this treatment heat-resistance of the leaves was measured. A period of 10 light-dark cycles was already sufficient to cause a considerable increase in heat-hardiness. Thus, heat-resistance proves to be a sensitive indicator of the metabolic processes in the leaves of the plants. The ecological significance of these phenomena is apparent.

3. Actual State of Water and Heat-resistance

The heat-resistance of poikilohydric organisms is mainly dependent on their actual water content. In a series of investigated air-dried lichens, for example (Lange, 1953), some are damaged after heating for half an hour at 71–100°C, while the same species, in a water-saturated condition, are already damaged at temperatures in the range of 35–46.5°C. In the same manner the heat-resistance increases in homoiohydric phanerogams while water saturation deficit increases. Sapper (1935) already indicated that wilted leaves have a great capability to resist the influences of high temperatures. The problem was systematically investigated by Hammouda and Lange (1962). We found that the leaves of *Commelina africana* after being experimentally dried for a short period to a water saturation deficit of 22 per cent, were damaged 50 per cent, after heating at 49.5°C for 30 min, while this "value of resistance" in the water-saturated conditions was at 46.5°C. Similar results were obtained with experiments on *Hedera helix* and *Phoenix reclinata*. We stressed, that such variations of heat-resistance can become important to the plants in xerothermic habitats, where the periods of highest temperatures as a rule occur together with periods of extreme natural water deficit.

4. "Heat-resistance Adaptation"

The history of a plant may furthermore influence the heat-resistance of its organs. Thus the nutrition of a plant can be of importance to its heat-hardiness (Illert, 1924; Sapper, 1935; Carroll, 1943; Julander, 1945). The previous water and temperature conditions may also affect it strongly: periods of drought generally lead to heat-hardening, while cultivating under ample water supply may result in sensitivity (Sapper, 1935; Lange, 1955). The possibility of increasing heat-hardiness through treatment at raised temperatures is of particular interest. The investigations of Alexandrov (1956), Alexandrov and Feldman (1958), Lyutova (1958), Lomagin (1961), Yarwood (1961) proved that the cells of higher plants react with a reversible increase of their heat-resistance, on short exposure to high temperatures, which are supraoptimal

but not yet fatal. Alexandrov (1962) concludes that this heat-hardening is caused by the damaging influence of temperature during the preceding treatment. Apart from this type of heat-hardening, Lange (1962) also found a "heat-resistance adaptation" during extended cultivation at raised temperatures, still within the optimal range, in the cases of *Commelina africana*, *Phoenicurus dactylifera* and *Veronica persica*. These plants were cultivated in controlled climate chambers for periods of 5–10 weeks under identical conditions at different temperatures of 20 and 28°C. At both temperatures the growth of *Commelina* was optimal. The newly developed leaves of the plants in the two series differed considerably in their heat-resistance. The "value of resistance" of the plants cultivated at 28°C was 4°C higher than that of the plants cultivated at 20°C. After treatment at raised temperatures, a resistance adaptation also occurred in *Veronica* and in the sclerophyllous leaves of *Phoenix*.

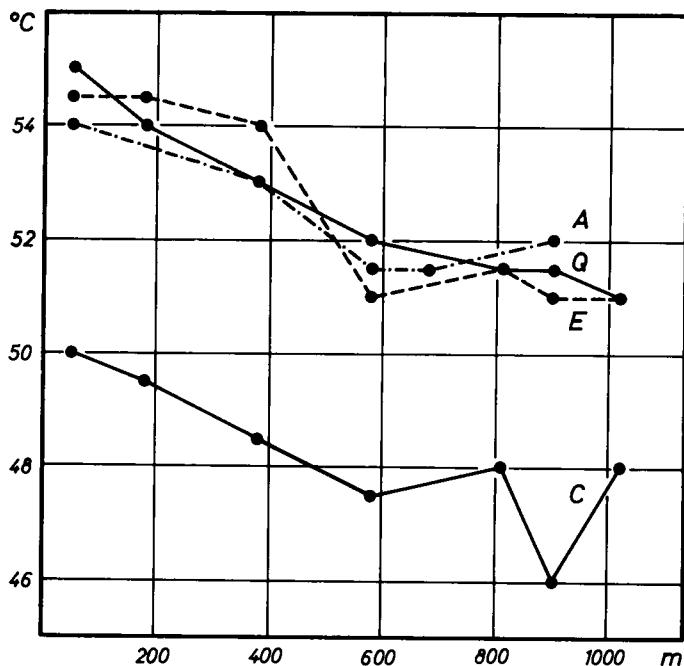


FIG. 2. Heat-resistance of the leaves (water-saturated) of *Cistus salviaefolius* (C), *Erica arborea* (E), *Quercus ilex* (Q), *Arbutus unedo* (A) in relation to the altitude of their habitats (Sierra Montseny, Spain). Abscissa: altitude. Ordinate: value of heat-resistance, i.e. the temperature which causes 50 per cent killing of the leaves, 30-min treatment. (According to Lange and R. Lange, 1962).

Differences in heat-hardiness, occurring in samples of the same species growing in different localities in the field, may probably be caused also by the above-mentioned adaptations and hardenings. We (Lange and R. Lange, 1962)

investigated the heat-tolerance of some evergreen mediterranean species (*Quercus ilex*, *Erica arborea*, *Cistus salviaefolius*, *Arbutus unedo*) in relation to the altitude of their habitats in Spain (Fig. 2). The specimens grown in hot dry valleys showed the highest heat-hardiness; while heat-resistance mostly decreases at increasing altitude of habitats, that is with lowering environmental temperatures and increasing precipitation. These measurements indicate to what extent the heat-resistance in higher plants may be influenced by external environmental factors.

5. Seasonal Changes in Heat-resistance

Large fluctuations of heat-resistance may occur in evergreen and winter-green plants during their seasonal variations of activity. Investigations concerning this problem have been carried out by Alexandrov, Lyutova and Feldman (1959), Alexandrov and Jaskuliev (1961), Lange (1961), and Shukhtina (1962) with phanerogams, by Kappen (1964), with ferns, by Lange (1955) and Dircksen (unpublished) with mosses. The comparison between the annual course of heat-resistance and frost-resistance is of special significance.

All treated plants from the surroundings of Göttingen (Southern Niedersachsen) showed a low frost-resistance during summer time. In autumn there already appeared a frost-hardening leading to a winter maximum of frost-resistance corresponding to the environmental temperatures. During spring, frost-hardiness decreased again to the summer minimum. The annual course of heat-resistance not only differed from that of frost-resistance but is also different for single species. The most important types of behaviour are shown in Fig. 3. In *Erica tetralix* the winter maximum of frost-resistance corresponds with a similar maximum in heat-resistance (Till, 1956; Lange, 1961). In summer, however, there exists a second maximum of heat-hardiness corresponding with a minimum of frost-resistance at the same time. Summer and winter peaks are separated from each other by minimum values in autumn and spring. Similar courses of heat-resistance were found by Kappen (1964) in ferns grown in the same area. In these cases the winter maximum is often higher than the summer maximum (for example *Dryopteris spinulosa*, Fig. 3). Some mosses according to Dircksen behave in a similar way (for example *Conocephalum conicum*, Fig. 3); the annual fluctuations of heat-resistance in fully hydrated mosses, however, are low. Higher seasonal changes in tolerance of heating were found in dried mosses by Lange (1955). Three species (*Ctenidium molluscum*, *Fissidens taxifolius*, *Syntrichia montana*) showed a maximum resistance in summer and a minimum in winter. The fern *Asplenium septentrionale* behaved similarly (Fig. 3), reaching a maximum resistance in summer but zero in winter. Still smaller fluctuations are indicated (Fig. 3) by the moss *Mnium punctatum* and the fern *Asplenium trichomanes*. The heat-resistance increases only in spring and early summer during the period of shooting forth and remains more or less constant for the rest of the year. No annual differences in heat-resistance are apparent in the water moss *Fontinalis antipyretica* (Fig. 3), its

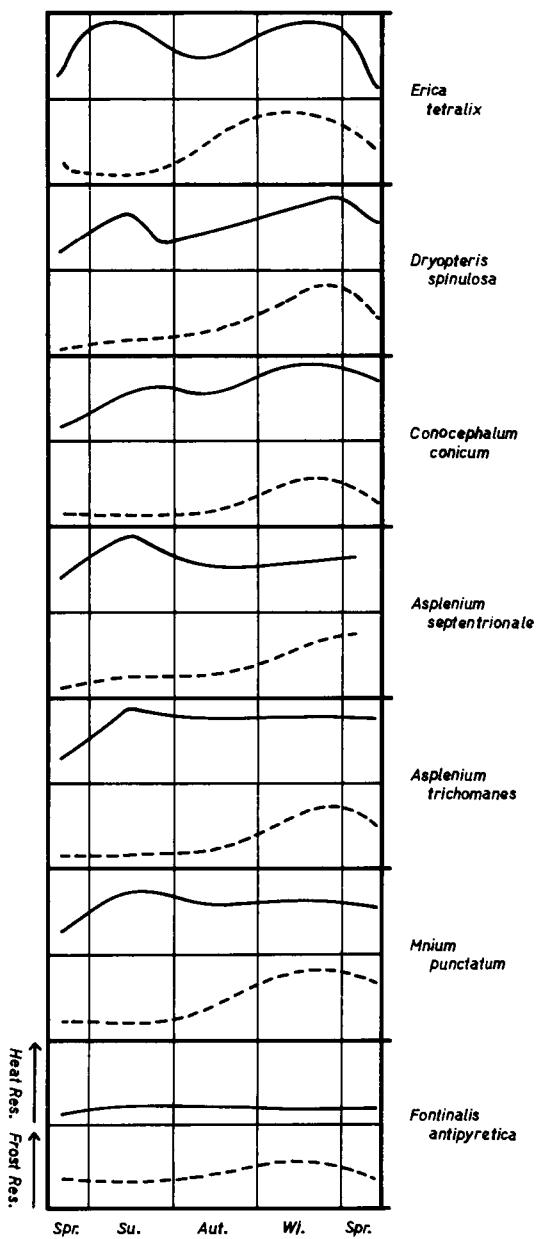


FIG. 3. Seasonal changes of heat- and frost-resistance in the leaves of hibernating plants (surroundings of Göttingen, Germany); *Erica tetralix* (phanerogam), *Dryopteris spinulosa*, *Asplenium septentrionale*, *Asplenium trichomanes* (ferns), *Conocephalum conicum*, *Mnium punctatum*, *Fontinalis antipyretica* (mosses). Abscissa: seasons (time). Ordinates: heat-resistance (—), and frost-resistance (---). (After Dircksen, Kappen, Lange, Till.)

heat-resistance remaining low in all seasons. In this species, nevertheless, a slight rise in frost-resistance is perceptible in winter. Between all the types of curves described above, transitional forms have been found.

In comparison with frost-resistance, heat-resistance appears significantly more variable, the latter depending to a greater extent on the particular constitution of the individual objects and on their special environmental conditions.

6. Diurnal Variations of Heat-resistance

Not only seasonal variations of heat-resistance are found in plants, but also oscillations of resistance during their diurnal fluctuation in activity. This was proved by Schwemmle and Lange (1959a, b) in the short day plant *K. blossfeldiana*. Experimental material was exposed to 12:12 hr light-dark cycles and the resistance of the leaves against heating, for 30 min at 46°C, measured

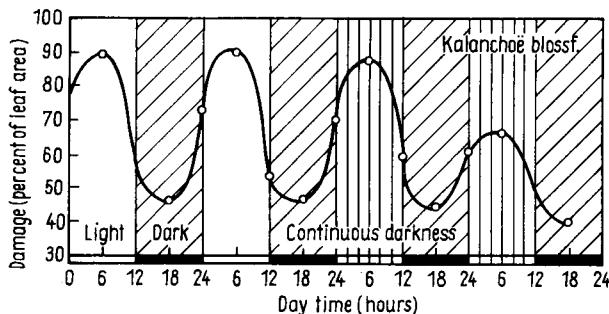


FIG. 4. Endogenous daily fluctuations of heat-resistance in the leaves of *Kalanchoe blossfeldiana*. Abscissa: time of day. Ordinate: degree of leaf damage caused by 30-min heating in water bath of 46.0°C, a rising curve means decreasing heat-resistance, a sinking curve means increasing resistance. (According to Schwemmle, 1960, after Schwemmle and Lange.)

at different hours. The damage by heat during this treatment may be seen in Fig. 4. During the 24-hr cycle, heat sensitivity of the leaves is considerable in the first half of the light period (heat-resistance being low). It decreases after about 6 hr and is very slight in the first half of the dark period, the heat-resistance in this phase reaching a high degree. Starting from about the middle of the dark period the sensitivity rises again. This progress also takes place with increasing attenuation under constant conditions (continuous darkness) for about 2 days. Such variations of heat-resistance therefore, prove to be of endogenous character (see Schwemmle, 1960). The amplitude of this kind of fluctuations, however, is small.

C. Discussion

As becomes apparent through the above-mentioned investigations, the heat-resistance of plants, which is characteristic of a species, is very variable in value, within a certain range. It depends very markedly on single changes

in the cells and on the whole organism. The conditions causing changes in heat-resistance may be very different.

It appears that plants have generally an increased heat-resistance during phases of inactive metabolism. This is evident in the diurnal oscillations of resistance in *K. blossfeldiana*. The leaves of this plant show the greatest hardness during the dark period, when their enzyme activity (Ehrenberg, 1954) and their auxin content (Becker, 1953) are lowest. Accordingly most of the investigated evergreen and winter-green species show high values of heat-hardiness during the inactive resting period in winter. This maximum heat-resistance is connected with a high frost-resistance occurring at the same time, a high drought-resistance and—according to Alexandrov, Lyutova and Feldman (1959)—also with increased tolerance of several other factors. The rule stated by Levitt (1958), namely that a “clearcut parallel between frost-, drought-, and heat-tolerance” exists, is confirmed within this state of the plants during winter. Probably this non-specific, general increase of resistance is caused by a single protoplasmic factor, which can possibly be interpreted by means of the “sulphydryl-disulphide hypothesis” (Levitt, 1962). This, however, can definitely not be considered to be the only possibility for the changes of heat-resistance in plants. A relation between heat- and frost-resistance occurs in most of the investigated species only periodically and is even totally absent from others. The heat-resistance for example in *Asplenium septentrionale*, *Asplenium trichomanes*, *Mnium punctatum* and *Fontinalis antipyretica* (see Fig. 3) remains unchanged in autumn and winter, whereas at the same time, their frost-resistance increases and reaches a maximum in winter. Still clearer are the differences between the courses of heat- and frost-resistance in summer. In many species the minimum of frost-resistance at the same time is connected with the second maximum of heat-hardiness (see *Erica tetralix*, *Dryopteris spinulosa*, *Conocephalum conicum*, *Asplenium septentrionale*, Fig. 3). If Levitt's theory for the winter conditions of the plants applies, this second increase of hardness in the metabolically active phase of the plants during the summer months, must be based upon other causes than the non-specific increase in resistance during winter. Probably during summer the ability of the cells to resynthesize the heat-damaged proteins is increased, as discussed by Allen (1950) for explanation of the thermophily of bacteria (see also Levitt, 1956a; Alexandrov, 1962). Similar processes may bring about the experimental heat-resistance adaptations and the differences in the plants' heat-resistance during varying temperature conditions in the field.

The present data lead to the conclusion, that there are at least two different mechanisms for the increase of protoplasmic resistance in plants. It is, however, plausible that further processes in the protoplasm are contributing too to the actual heat-hardiness of a plant organ. Future research should be conducted in order to examine these facts, in experiments and in the field, in more detail, to discover their protoplasmic relations and to understand their ecological importance for the existence of the plants in their natural habitats as well as in culture.

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A STUDY OF THE CHANGES IN RESISTANCE OF PLANT CELLS TO THE ACTION OF VARIOUS AGENTS IN THE LIGHT OF CYTOECOLOGICAL CONSIDERATIONS

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THE aim of cytoecology is to study those forms of adaptation of organisms to environmental factors in which the adaptive effect may be already achieved at the cellular or molecular levels of the living organism. The most important type of adaptation which not infrequently limits the distribution of a species is the establishment of a relationship between the resistance of organisms to a given environmental factor and its intensity in the medium. The level of an organism's resistance to a given environmental factor may be determined by the stability of its cell and protoplasmic molecular components. Therefore, the study of the mechanisms underlying cell resistance and its possible changes is the essential basis of cytoecological research.

The degree of resistance of cells to one or another agent is determined in accordance with the results of its action, and the result depends on a number of circumstances which are as follows:

1. The character of primary disturbances caused by an agent.
2. Destructive after-action (this is especially developed as a result of radiation injury).
3. Ability of a cell to neutralize the injurious agent.
4. Reparatory activity of a cell during and after the action of an agent.
5. Ability of a cell to increase its level of resistance in response to the action of an agent (Alexandrov, 1952, 1956, 1963).

Therefore, resistance is a complex characteristic of a cell. The aim of the Laboratory of Cytophysiology and Cytoecology of the Komarov Botanical Institute is to study separate elements of resistance and to elucidate their ecological significance. The main results of the laboratory, chiefly on epidermal and parenchymal cells of leaves of many species of plants, are as follows.

1. As the temperature of a short-term (5-min) heating is raised the injury to the various functions of a cell takes place gradually and in strict

succession: first of all the streaming of protoplasm† and photosynthesis are inhibited and the viscosity of the protoplasm is increased; the changed adsorption of vital dyes, the disturbance of selective permeability of a protoplast, and suppression of respiration appear only after considerably more extensive damage (Alexandrov, 1955, 1963; Kiknadze, 1960; Lyutova, 1962 b; Melnikova, 1960; Zavadskaya, 1963 a).

2. Immediately after a heating of 40–80 min or less, the effect of the damage is determined by a denaturating action of the heating on the proteins of protoplasm (Alexandrov, 1956, 1962b; Lyutova, 1963 b). In a long period of time after the heating (hours, days) the degree of damage will depend not only on the thermoresistance of proteins but on the reparatory ability of cells as well (Alexandrov, 1956, 1963, 1964; Alexandrov, Khachaturov and Shukhtina, 1963; Gorban, 1963; Lyutova, 1962b; Shkolnikova and Shterman, 1963).
3. During prolonged (above 80–160 min) and moderate heating the process of injury proceeds side by side with the reparatory activity of cells and an adaptative increase in the level of heat-resistance–heat-hardening (Alexandrov, 1956; Alexandrov and Feldman, 1958; Alexandrov and Jaskuliev, 1961; Denko, 1963; Feldman and Lyutova, 1962; Kiknadze, 1960; Kislyuk, 1962; Lomagin, 1961, 1963; Lomagin, Antropova and Ilmete, 1963; Lyutova, 1958, 1962a, 1963 a, 1963b; Shkolnikova and Shterman, 1963; Zavadskaya, 1963 a, b).
4. The heat-resistance curve of plant cells (in which the temperature is on the abscissa and the logarithm of the time of preservation of protoplasmic streaming on the ordinate) is a straight line for the short-time intensive heatings that cause damage by protein denaturation. Its slope corresponds to the temperature coefficient of the damage (Q_{10}) and is equal to hundreds and thousands. In the area of prolonged and moderate heating the straight line breaks upward; this indicates that mechanisms counteracting heat injury (reparation, heat-hardening) are involved.
5. Primary injury from heating in growing cells appears to be greater than that in cells which have ceased growing (Gorban, 1962; Feldman and Kamentzeva, 1963).
6. The rate of reparation in young cells is higher than that in old ones (Gorban, 1963).
7. The heat-resistance of cells of various tissues is different (Alexandrov, 1956).

† For microscopic investigations *in vivo* plant tissues were infiltrated by a simplified method in a syringe (Alexandrov, 1954). In a number of cases to improve visibility the surface of the material was moistened with silicone oil the coefficient of refraction of which is similar to the coefficient of refraction of a cell cuticle (Alexandrov, 1962a).

8. In cereals (Fig. 1), seaweeds, early-spring bulbs, and brown algae, a correspondence has been found between the average temperature conditions in which a species lives and the heat-resistance of its protoplasmic proteins (as determined by the thermoresistance of their cells to short-term intensive heatings), (Alexandrov, 1956, 1963; Alexandrov, Ushakov and Polyansky, 1961; Feldman and Lyutova, 1962; Feldman, Zavadskaya and Lyutova, 1963).
9. The heat-resistance of mature cells of higher plants which have completed growth shows constancy during changes in the environmental temperature in a wide range of thermal optima (Alexandrov, 1963; Alexandrov and Feldman, 1958; Alexandrov, Ushakov and Polyansky, 1961; Feldman and Lyutova, 1962).

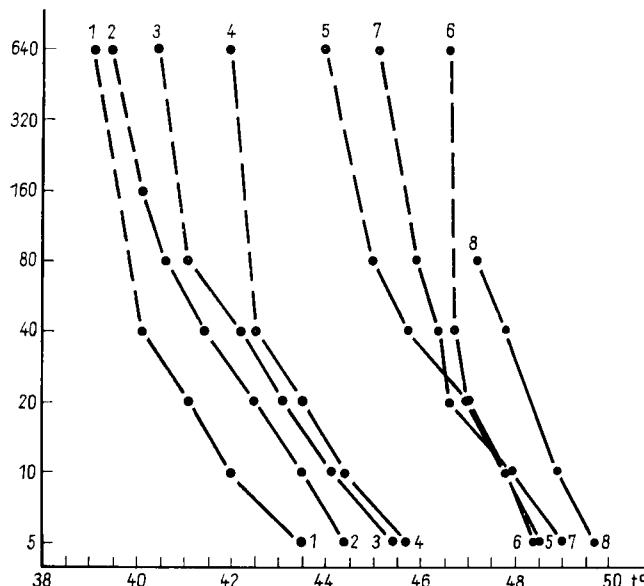


FIG. 1. The heat-resistance of the epidermal cells of a leaf sheath in a number of *Gramineae*. Abscissa—the temperature of heating. Ordinate—the duration of retention of protoplasmic streaming (in min logarithmic scale). 1—*Poa alpina* L. 2—*Dactylis glomerata* L. 3—*Elymus arenarius* L. 4—*Phragmites communis* Trin. 5—*Panicum miliaceum* L. 6—*Imperata cylindrica* (L.) Beaw. 7—*Eleusine indica* (L.) Gaerth. 8—*Aristida karelinae* (Trin. et Rupr.) Rosev.

10. Only in response to super-optimal heatings do the cells of higher plants increase their resistance to heat and other agents—the heat hardening (Alexandrov, 1956; Alexandrov and Feldman, 1958; Kiknadze, 1960; Lomagin, 1961; Lomagin, Antropova and Ilmete, 1963; Lyutova, 1962a).
11. In contrast to higher plants, unicellular and multicellular algae change their heat-resistance with changes in temperature over the whole range

- of biokinetical temperatures—this is called temperature adjustment (Feldman and Lyutova, 1963; Feldman, Zavadskaya and Lyutova, 1963; Luknitzkaya, 1963; Lyutova and Feldman, 1960).
12. Short-time cooling apparently damages various functions almost synchronously at the moment of ice-formation within the cell.
 13. The damage to cells of cold-sensitive plants (cucumber, maize) by low positive temperatures gives rise to a gradual disturbance of functions but in a sequence different from that caused by heating: photosynthesis appears to be much more vulnerable than the movement of protoplasm, which ceases only with extensive damage to the cell (Kislyuk, 1964).
 14. Cold-hardening of some plants leads to resistance to agents of a different kind as well as to an increase in frost-resistance (Alexandrov, Lyutova and Feldman, 1959; Kislyuk, 1962; Shukhtina, 1962).

Some of the above-mentioned principles are embodied in the reports of our laboratory. Let us consider the question of biological significance of resistance to cold or heat in detail. The level of thermoresistance of cells may change during phylogenesis in the process of adaptation of a species to new temperature conditions. In the life of an individual organism it takes place in connexion, with the growth of cells, and also under temperature hardening, and adjustment. Changes in the level of temperature resistance may be seasonal or diurnal. In studying mechanisms that alter thermoresistance it is essential to elucidate whether they are specific or non-specific, i.e. to define to what extent the resistance of cells to cold or heat correlates with their resistance to other agents. We have ascertained that in response to the action of super-optimal temperatures the resistance of cells increases not only to heating but to alcohol, ether, acetic acid, potassium rhodanide, cadmium chloride, as well as to high hydrostatic pressure.

On the basis of the data given above and from data given in Feldman's and other papers as well as in the abstracts of reports presented by Lomagin, Antropova and Ilmete, arguments have been advanced to the effect that the non-specific increase in resistance under heat-hardening is the result of the general increase in the stability of protoplasmic proteins. An increase in the stability of the macrostructure of protein molecules must decrease their biochemical reactivity. Evidently this is reflected in the decrease in photosynthesis and growth observed under heat-hardening (Lyutova, 1958, 1963a; Kislyuk, 1962).

From this point of view heat-hardening should be acknowledged as disadvantageous for the maintenance of active cellular metabolism. It is of advantage only during danger of thermic injury and is lost by the cells when they return to optimal conditions.

The changes leading to heat-adaptation during phylogenesis are different. When comparing the plant cells of various species the differences in heat-

resistance may not be related to the differences in the resistance of the cells to other agents. Thus, the cells of millet are much more heat-resistant than the corresponding cells of wheat; but they do not differ in resistance to alcohol and acetic acid. The cells of the grains of *Aristida karelini* (Trin. et Rupr.) Roshev. differ from those of *Arundo donax* L. in that they show higher heat-resistance but much less resistance to hydrostatic pressure. Biebl and his collaborators have tested a wide range of various agents on a great number of species of plants and found that the high level of resistance to a certain environmental factor is in no way related to the resistance to non-ecological agents. In connexion with this fact Biebl (1962) draws a distinction between "ecological resistance" and "non-environmental resistance". In other words, the increase in heat-resistance of protoplasmic proteins of a species in phylo-

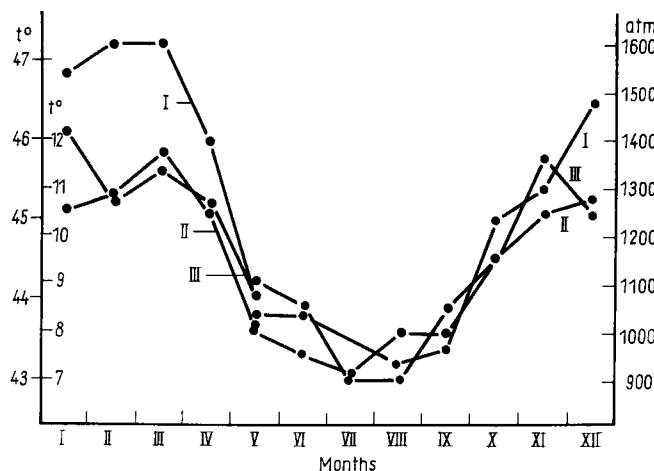


FIG. 2. Seasonal changes in the resistance of epidermal cells of *Geum rivale* L. to low and high temperature and high hydrostatic pressure. Abscissa—the months. Left ordinate—positive and low temperature. Right ordinate—the pressure in atmospheres, under which a 5-min exposure stops protoplasmic streaming. I—Resistance to high pressure. II—Cold resistance. III—Heat resistance.

genesis is more specific in contrast to the heat-hardening of individuals which may be non-specific. Evidently, this means of phylogenetic adaptation is more economical.

We have shown that in nature under cold-hardening in grains, sedges and representatives of other families (*Geum rivale* L. (Fig. 2), *Calluna vulgaris* (L.) Hill., *Vinca minor* L., *Leucanthemum vulgare* L.) an increase in the resistance to sudden short-term cooling (5 min) takes place in parallel with increases in the resistance to heating, high hydrostatic pressure, alcohol and probably to some other agents. The increase in heat-resistance under frost-hardening has been confirmed in a number of plant species (Jameson, 1961; Lange, 1961). The

increases in resistance to various agents in autumn are approximately the converse to the decreases in spring. The same phenomenon is observed under artificial dehardening of plants which have been taken into the room from under the snow in winter.[†]

In the case of cold-hardening as in the case of heat-hardening, there is, to a great extent a non-specific increase in the resistance of cells. These states are similar but not identical: heat-resistance increases under frost-hardening, but frost-resistance does not increase under heat-hardening (experiments on *Dactylis glomerata* and *Leucanthemum vulgare*). One may think that non-specific frost-hardening is connected with the limitation of cellactivity. The well-known connexion of frost-hardening with the inhibition of growth processes speaks in favour of this suggestion.

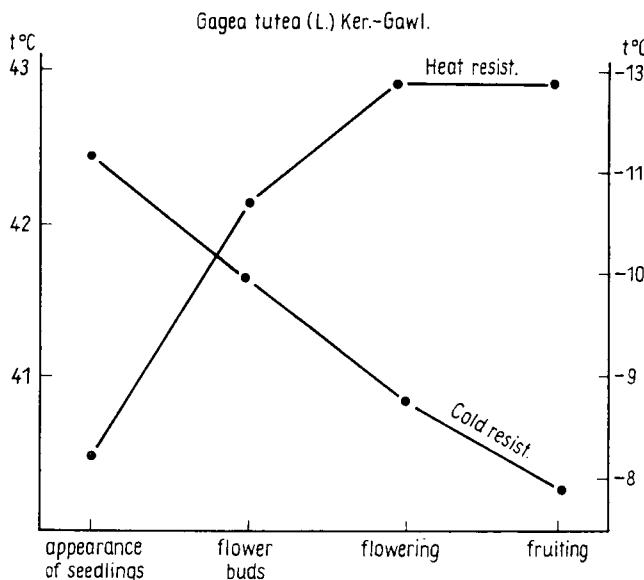


FIG. 3. Changes in heat- and frost-resistance of leaf epidermal cells of *Gagea lutea* (L.) Ker.-Cawl. at different phases of plant development. Abscissa—phases of development, left ordinate—high temperature, right ordinate—low temperatures at which a 5-min exposure stops protoplasmic streaming.

High resistance to cold and in particular to a sudden cooling is not always determined by a high level of general non-specific resistance of cells. At the same time high cold-resistance does not necessarily accompany the cessation of growth processes (Tjurina, 1957; Zalensky, 1955). In the early-spring plants *Gagea lutea* (Fig. 3), *Leucojum aestivum*, *Leucojum vernum* (experiments

[†] But this is not typical of all plants. In some winter-green plants (for example *Hepatica nobilis* Schreb.) we did not observe any increase in resistance either to short-term freezing or heating by the time winter had set in.

by Feldman) the leaves appear in March–April. They are often exposed to night and morning frosts; their cells accordingly are of high cold-resistance. Nevertheless the leaves grow intensively during the intervals between the frosts at relatively low positive temperatures. Experiments have shown that in contrast to the phenomena occurring during cold-hardening in the autumn in the above-mentioned winter-green plants, high cold-resistance of cells combines with low heat-resistance in early-spring plants. The cold-resistance of a plant decreases, with its further development, whereas the heat-resistance increases with the gradual retardation in the growth of its cells. Its resistance to alcohol also increases. The behaviour of the cells of young leaves of some winter-green plants is similar to that of early-spring plants. In *Saxifraga cuneifolia*, *Saxifraga umbrosa*, *Viola cornuta*, *V. minor* and *Leocanthemum vulgare* the cold-resistance of the cells of newly grown leaves in April–May is equal to or exceeds the cold-resistance of the winter leaves found on the same plant. However, the heat-resistance of the cells of young leaves is much lower than that of the old leaves. In due course the cells of young leaves decrease in cold-resistance but increase in heat-resistance. Resistance to high hydrostatic pressure usually changes simultaneously with heat-resistance (Fig. 4). There-

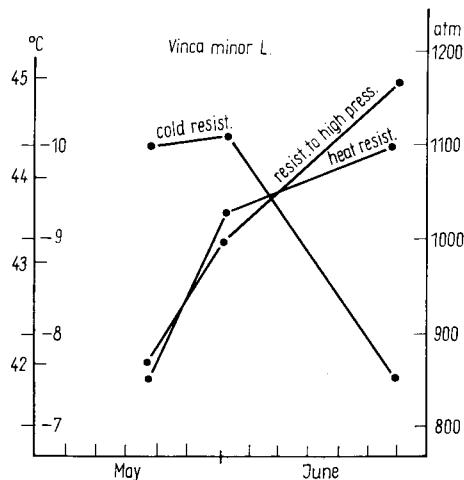


FIG. 4. Changes in the resistance of epidermal cells of young leaves in the process of their development to high and low temperatures and to high hydrostatic pressure. Abscissa—time of year, right ordinate—the pressure in atmospheres under which a 5-min exposure stops the streaming, left ordinate—high and low temperature which during 5 min stops streaming.

fore, it is not only in ephemerals that the young leaves that begin developing in the spring combine active growth with a high level of resistance to cold. This resistance, however, is not connected with the general non-specific stabilization of the protoplasm.

Thus, comparing the levels of resistance of cells to high and low temperatures with their resistance to other agents we have been able to distinguish two ways of changing temperature resistance: specific and non-specific. They must be based on essentially different biochemical mechanisms. The following facts show the evidence for the presence of different mechanisms increasing cell-resistance to one and the same factor, namely to a high temperature. Investigations of the thermostability of proteins of thermophilic bacteria, and thermolabile mutants of a *Neurospora* species together with numerous researches in Ushakov's laboratory on enzymes of animals that vary in heat-resistance and our indirect data on plant cells and so forth, all show that phylogenetic adaptations to temperature conditions at the molecular level are connected with genetically fixed changes in the synthesis of proteins and, therefore, with their primary structure (in the sense of Linderstrom-Lang). Furthermore, heat-hardening may be realized within seconds and cannot be connected with the synthesis of new proteins (Lomagin, 1961; Zavadskaya, 1963 b). Evidently, it is conditioned by the stabilization of the secondary and tertiary structures of protein macromolecules of the protoplasm. The method of determining the degree of specificity will enable us to find various means by which the level of resistance of cells to ecological factors is regulated and to direct the analysis of the biochemical basis of resistance along rational lines.

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HEAT-HARDENING OF PLANT CELLS UNDER NATURAL AND EXPERIMENTAL CONDITIONS

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IT HAS long been known that plant cells increase their frost resistance when subjected to low temperatures. This ability of plants to frost-harden is of great theoretical and practical importance.

The capacity of plant cells to alter their heat-resistance while exposed to high temperatures has not been previously studied. Unsystematic and contradictory data found in the literature were as a rule obtained without applying cytological and cytophysiological methods (Sapper, 1935; Laude and Chaugule, 1953).

The experiments of Alexandrov in 1956 showed that plant cells exposed to high temperatures show a pronounced increase in their heat-resistance. In order to determine heat-resistance pieces of leaves were subjected to heating for a standard length of time. The temperature at which protoplasmic streaming stopped was taken as a criterion of heat-hardiness. By analogy with frost-hardening this phenomenon was called heat-hardening. To produce the phenomenon of heat-hardening the temperature must be high enough. For instance leaves of *Tradescantia fluminensis* Vell. exposed to +1 to +28°C did not change their heat-resistance (Alexandrov and Feldman, 1958). But at +30°C the increase in heat-resistance was already observed. The higher the temperature, the greater the heat-resistance of the cells (Fig. 1a). However, the temperature above +38°C killed the cells. In our laboratory we have observed heat-hardening on isolated leaves of 34 species belonging to 18 different families and so far there were no exceptions to the rule. Similarly it was observed that whole plants (such as seedlings of cereals) when exposed to high temperatures increase their heat-resistance (Laude and Chaugule, 1953; Coffman, 1957; Kislyuk, 1962; Lange, 1962). Thus it may be concluded that the ability of plant cells to change their heat-hardiness under the influence of high temperatures is typical for all plants.

In the state of heat-hardening the heat-resistance of different cell structures increases. Therefore, the state of heat-hardening can be established not only by the character of protoplasmic streaming but also by changes in other

functions of cells. Heat-hardening has been found to increase the thermo-resistance of photosynthesis (Fig. 1b) and respiration (Lyutova, 1958; 1962). Quenching of chlorophyll fluorescence, a loss of capacity for plasmolysis, anthocyanin and electrolyte loss, occur at higher temperatures (Kiknadze, 1960; Lomagin, 1961; Oleinikova and Uglov, 1962; Alexandrov, 1963; Zavadskaya, 1963). It should be noted that the rise of photosynthetic resistance during heat-hardening is accompanied by a decrease in its intensity (Fig. 1c).

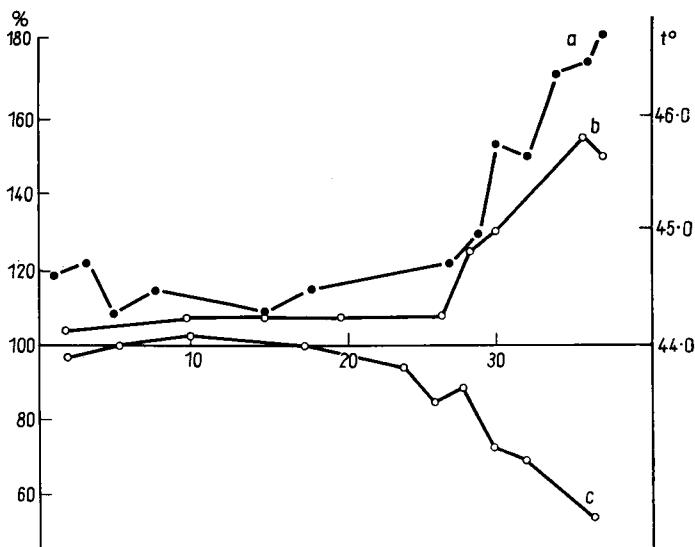


FIG. 1. The dependence of the thermoresistance of the protoplasmic movement (a), the thermoresistance of photosynthesis (b), and the amount of photosynthetic activity (c) of the leaves of *Tradescantia fluminensis* on 18-hr incubation at different temperatures. Abscissa shows the temperature in °C at which the leaves were kept. Upper left ordinate shows the intensity of photosynthesis after a 5-min heating at 45°C in per cent compared to the control level (control level—100 per cent). Lower left ordinate shows the degree of decrease of photosynthesis in per cent of control level. Right ordinate shows the temperature of the cessation of protoplasmic streaming after 5-min heating.

As has been mentioned above, heat-hardening is produced in the plant under the influence of super-optimal temperatures. It may be concluded that heat-hardening is the reaction of plant cells to the injurious effect of temperature and not its adjustment to any temperature of the environment. The following facts support this conclusion:

1. Heat-hardening temperatures suppress different aspects of plant cell activity. Prolonged hardening at moderate temperatures (about +36°C) causes a slowing down of protoplasmic streaming (Shkolnikova and Shterman,

1963) while brief hardening at high temperatures (about +50°C) causes its cessation (Lomagin, 1961; Zavadskaya, 1963). In both cases, however, the cellular heat-resistance is high. Heat-hardening lowers the intensity of photosynthesis, a fact proved by observations of six species of plants: *Tradescantia fluminensis* Vell., *Podophyllum peltatum* L., *Phaseolus vulgaris* Wall., *Campanula persicifolia* L., *Polygonum sachalinense* Schm. and *Hepatica nobilis* Schreb. (Lyutova, 1962). The degree of suppression of photosynthesis in the plant is determined by its thermostability developed in the process of hardening. The higher the thermostability level, the lower is the intensity of photosynthesis. Also, the growth of the plant is inhibited during heat-hardening. Hardy seedlings of winter wheat, spring rye and barley lagged behind in their growth by as much as 25–30 per cent (Kislyuk, 1962).

All the above-mentioned facts prove the lowering of physiological activity in hardy cells; this may be regarded as compensation for the increased molecular stability.

2. Heat-hardening may be attained by means of different time and temperature combinations, i.e. as a result of the effect of certain doses. After hardening for 18 hr the maximum resistance in the epidermis of *T. fluminensis* and *C. persicifolia* is observed at +36 to +37°C. Increasing the temperature made it possible to reduce the time required for a heat-resistance change (Lomagin, 1961; Yarwood, 1961; Zavadskaya, 1963). Heating for 10 sec at +50 to +51°C was enough for attaining a certain degree of heat-hardening. At +59°C heating for 1 sec was sufficient. Consequently heat-hardening may be regarded as a responding change and not a gradual habituation to a given temperature.

3. The greater the initial levels of cell heat-hardiness, the higher the critical temperatures bringing forth the heat-hardening. For example, in four species of cereals, *Dactylis glomerata* L., *Phragmites communis* (L.) Trin., *Panicum miliaceum* L. and *Eleusina indica* (L.) Caertn. the protoplasmic streaming in the leaf epidermis stopped after heating for 5 min at +44·0, 46·1, 49·0 and 49·5°C respectively. A similar effect of heat-hardening is observed in *D. glomerata* at +30°C, in *P. communis* at +36°C, in *P. miliaceum* and *E. indica* at +40·0°C (Alexandrov and Feldman, 1958).

The high level of heat-resistance attained in the process of heat-hardening is reversible. Under normal temperature conditions cell heat-hardiness drops. This is confirmed by the observation of *P. sachalinense* and *C. persicifolia* (Lyutova, 1962). The decrease in the resistance of protoplasmic streaming and that of photosynthesis is not absolutely synchronous (Fig. 2). It depends on the species of plants and the conditions of dehardening. The leaf recovers its intensity of photosynthesis more quickly than it regains its normal level of resistance. Thermoresistance of protoplasmic streaming is the last to reach its control level.

This gives rise to the following question: why does hardening increase the heat-resistance of the cell? Thermal protein denaturation is responsible for the killing of the cell by high temperatures. Therefore, one can assume that

the increase in thermal-resistance during heat-hardening is due to native protein stabilization.

Lyutova (1963) has found much evidence in favour of this point of view. She studied the reasons for the growth of photosynthesis resistance under heat-hardening. The chlorophyll in chloroplasts is known to be in a complex linkage with lipoid and not to dissolve in non-polar solvents (such as petroleum ether). The chlorophyll-protein linkage is broken under the influence of different denaturing factors (high temperature, ultrasound, etc.) and chlorophyll begins to dissolve in petroleum ether (Sapozhnikov *et al.*, 1962). After heat-hardening, more heating is required to extract chlorophyll from leaves

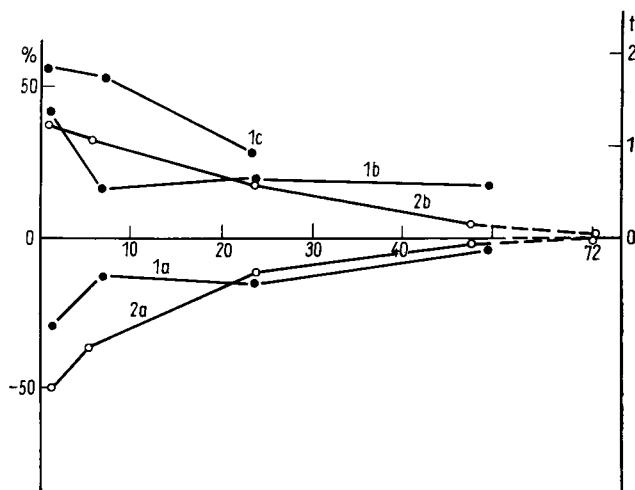


FIG. 2. The intensity of photosynthesis (1a, 2a), the thermoresistance of photosynthesis (1b, 2b) and protoplasmic movement (1c) in *Campanula persicifolia* (1) and *Polygonum sachalinense* (2) at different times after the hardening. The abscissa shows the time after the hardening; the right ordinate shows the temperature for cessation of protoplasmic movement after 5-min heating (in °C) in comparison with the control; the left upper and lower ordinates are the same as in Fig. 1.

of *T. fluminensis* and *Taraxacum officinale* than in the case of non-hardy leaves. This may be explained by the marked rise in the resistance of the protein part of the chlorophyll-protein complexes to the effect of heat denaturation.

In case of injuries—thermal injuries in particular—a complex of non-specific changes is observed in animal and plant cells (Nassonov and Alexandrov, 1940; Nassonov, 1960; Alexandrov, 1960; Alexandrov, 1962). According to Nassonov and Alexandrov's denaturation theory these non-specific changes are caused by denaturation of protoplasmic proteins. If heat-hardening is due to the rise in structural stability of native proteins, it could be expected that the increase in heat-resistance should be accompanied by an increased

resistance to a number of denaturing factors. The experiments proved that the rise in heat-resistance is non-specific (Alexandrov and Feldman, 1958; Lomagin, Antropova and Ilmete, 1963, etc.). After the heat-hardening of *T. fluminensis* leaves, the resistance to alcohol, ether, acetic acid, cadmium chloride, potassium rhodanate (Fig. 3) and high hydrostatic pressure becomes

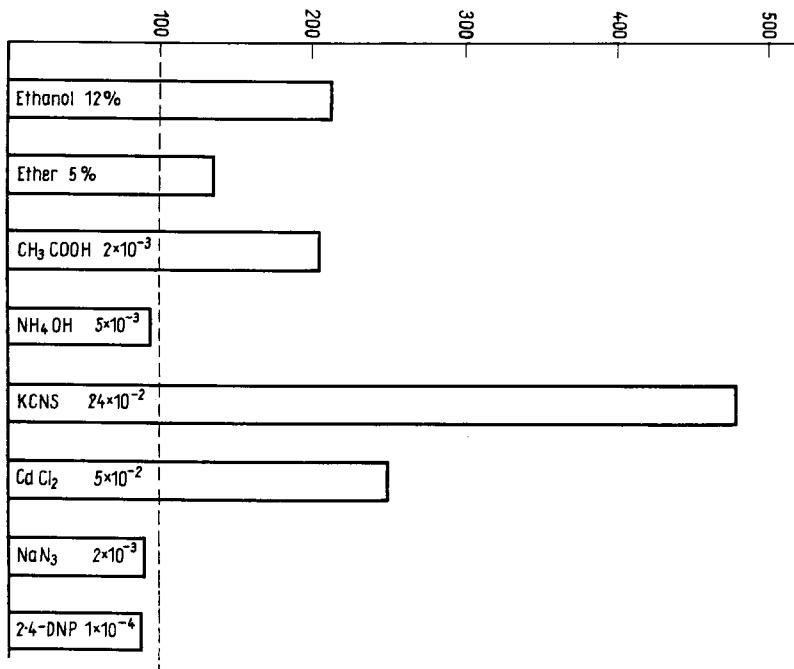


FIG. 3. Cellular heat-resistance of the leaves of *Tradescantia fluminensis* to different agents after heat-hardening. Ordinate shows the time of cessation of protoplasmic streaming in per cent compared to control level (control level — 100 per cent).

greater, while the resistance to ammonia does not change. Moreover, the sensitivity of hardy and non-hardy cells to metabolic poisons (2,4-dinitrophenol, sodium azide) which do not denature the protoplasmic proteins, is the same (Lomagin, Antropova and Ilmete, 1963). It should be noted that hardy cells are less sensitive to large doses of sodium azide than non-hardy ones. An analysis of the phenomenon proved that the high concentration of sodium azide is not a specific inhibitor of energetic metabolism but it does denature cell proteins (Lomagin, 1962). Thus, the denaturation theory of cell injuries is capable of explaining the most important features characteristic of heat-hardening. The course of protein stabilization during heat-hardening should be discussed in the light of the same theory. Protein stabilization may result from:

1. A change in structure of protein molecules bringing forth an increase in their stability.
2. The appearance of antidenaturants (sugar, aminoacids) stabilizing the protein molecular macrostructure as well.
3. The acceleration of protein resynthesis and renaturation.

The second supposition seems to be more probable. Our investigations and some data stated by other authors (Feldman, 1962) show that sugar and certain other anti-denaturants increase cell thermoresistance. However, direct determinations of sugar contents of hardy and non-hardy leaves carried out by Zavadskaya by means of quantitative paper chromatography methods failed to reveal the expected difference. The sugar changes produced by high

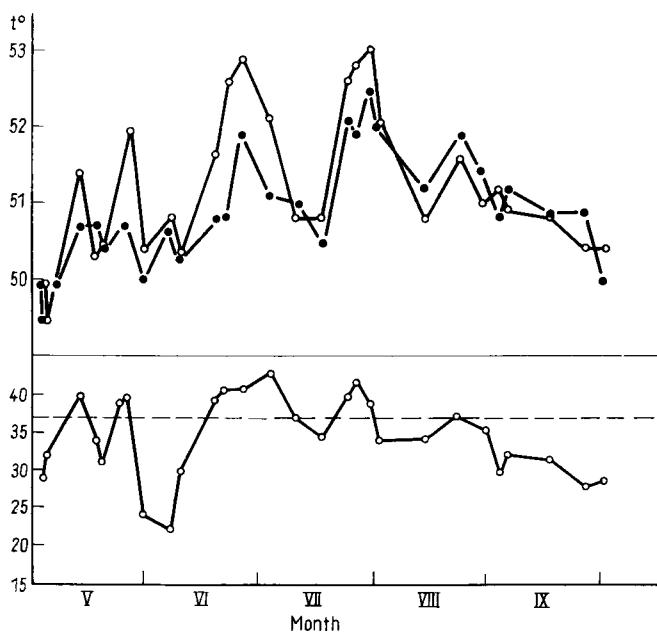


FIG. 4. The dependence of cellular heat-resistance of *Aristida karelini* leaves on maximal air temperature: ·—· heat-resistance in the morning, ○—○ heat-resistance in the afternoon. Abscissa shows the months. Upper ordinate shows the temperature of the cessation of protoplasmic streaming after a 5-min heating. Lower ordinate shows the maximal air temperature.

temperatures are manifold. Therefore their linkage with protein—probably leading to stabilization of macrostructure—is hidden behind other processes such as the intensification of respiration in the course of which a loss of sugar occurs.

The above-mentioned experimental results refer to the changes in thermostability under laboratory conditions. It is important to find out if this ability is significant in plants under natural conditions. For this purpose a number of investigations were carried out under the conditions of the hot climate of Central Asia. The grasses *Aristida karelini* (Trin. et Rupr.) Roshev. and *Arundo donax* L. were studied thoroughly (Alexandrov and Jaskuliev, 1961). Observation during the whole period of vegetative activity showed that in the hot summer months heat-hardiness is 3·0°C higher than that during the spring and autumn seasons.

A 24-hr study of the dynamics of cell heat-hardiness revealed most interesting regularities (Fig. 4). *A. karelini* vegetating during the whole summer in the sands of the desert of Central Asia at maximal air temperatures ranging from +1 to +28°C, proved to have a constant daily heat-resistance of the leaf cells. But if the air temperature became higher than +38°C, the heat-resistance increased in the afternoon. Sometimes the increase of thermostability was no less than 2°. This rise in thermostability was reversible. After the nightly lowering of the temperature the thermoresistance fell and next morning was lower than on the day before (Table 1). On those days when the air temperature

TABLE 1. CELLULAR HEAT-RESISTANCE OF THE LEAVES IN THE AFTERNOON AND THE NEXT MORNING

Species	Air temp. (°C)	Number of experiments	Temperatures for cessation of streaming		
			In the afternoon	In the morning	Difference
<i>Aristida karelini</i>	hardy (38)	18	52·0	50·1	+1·9
	non-hardy (20-37)	39	50·4	50·2	+0·2
<i>Arundo donax</i>	hardy (32)	24	49·0	47·9	+1·1
	non-hardy (20-31)	15	47·1	46·7	+0·4

failed to rise above some critical value, thermostability did not vary within the 24 hr. Consequently the observed daily variations of thermosensitivity are not due to the day and night changes, and the rise of the heat-resistance in *A. karelini* must be regarded as heat-hardening under natural conditions.

Heat-hardening was also observed in the leaf cells of another grass, *A. donax*, the critical temperature of which was +32°C. The fact that the initial heat-resistance of *A. donax* is lower than that of *A. karelini* may account for this difference. The increase of heat-resistance was observed in plants belonging

to different families (*Catalpa speciosa* Warden and *Alhagi persarum* Boiss. et Buhse) on the days when the air temperature was above some critical level.

In such cases heat-resistance fell at night too. However, this length of time was not sufficient for the disappearance of the heat-hardening effect. Next day if the plant was again influenced by high temperatures a new rise in thermostability was added to the remaining level of the previous heat-hardening. This is likely to be the cause of the rise in thermostability of Central Asian plants during the summer season.

A reactive increase in cellular heat-resistance occurs not only in hot climates. Lange (1961) described the increase in heat-resistance of some evergreen and winter-green plants from the neighbourhood of Göttingen in a hot and dry summer season.

On the basis of all this material we come to the conclusion that the rise of environmental temperature to a certain critical level increases cellular heat-resistance. In the process of evolution a number of adaptations has been evolved due to which the species can endure critical temperatures. In spite of this an individual plant has a rather limited means in its struggle against overheating. Since the plant is immovable, these means are limited to transpiration and the movement of leaves. Therefore, cellular heat-resistance may be an essential factor in plant adaptation to external temperature conditions.

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THE INFLUENCE OF CULTIVATION TEMPERATURE ON CELLULAR RESISTANCE OF *CABOMBA AQUATICA* AUBL. TO VARIOUS AGENTS

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TEMPERATURE alterations in the wide range of the optimal zone do not change cellular heat-resistance of higher plants. Super-optimal temperatures causing heat-hardening increase resistance of plant cells not only to heating but also to numerous agents different in nature (Alexandrov, 1956; Alexandrov and Feldman, 1958; Lomagin, Antropova and Ilmete, 1963 and others). Resistance to cold does not increase in this case. In many plants cold-hardening results in an increase of plant tolerance to different agents, heating included (Alexandrov, Lyutova and Feldman, 1959; Lange, 1961; Shukhtina, 1962).

Unlike higher plants, cellular heat-resistance in lower plants (fresh water and sea algae) changes adequately with temperature shifts in the whole biokinetic temperature range—so-called temperature adjustability (Lyutova and Feldman, 1960; Feldman, Zavadskaya and Lyutova, 1963; Luknitskaya, 1963). In this instance a rise in the environmental temperature leads to an increase in heat-resistance and a decrease in cold-resistance of cells. The converse occurs in the case of a decrease of temperature. Such phenomena were observed under both experimental and natural conditions. The changes in cellular heat-resistance of algae with temperature alterations were detectable in a few hours.

It was important to know whether this capacity of temperature adjustability in lower plants is the result of their aquatic mode of life or is related to their place in the phylogenetic series. For this purpose we had to study the influence of cultivation temperature on the cellular resistance of higher aquatic plants. Experiments were made on the water plant *Cabomba aquatica* Aubl. fam. Nymphaeaceae with the leaves completely immersed in water. In summer each shoot (sprout) was transplanted to a small aquarium. Plants were grown at a temperature about 20°C. The experiments were carried out in winter and autumn of 1961 and 1962. Lower epidermal cells were used as an object of investigation. Changes in the resistance of cells to the effects of high and low temperatures, ethanol and high hydrostatic pressure were studied. Heat-resistance was determined by the minimum temperature the action of which

stops protoplasmic streaming. The time of exposure was 5 min with intervals of 0·4°C. Cold-resistance of cells was determined by cooling them for 5 min in silicone oil and by measuring the minimum temperature at which protoplasmic streaming completely stopped. Cooling was carried out in a small refrigerator equipped with a semiconductor thermo-battery (Alexandrov, Lyutova and Feldman, 1959). Resistance to ethanol was characterized by the survival time of protoplasmic streaming in ethanol-infiltrated leaf discs. Resistance to hydrostatic pressure was determined by the cessation of protoplasmic streaming after treatment for 5 min (for technique see Alexandrov and Feldman, 1958) and measured in atm/cm².

At the initial stage of the investigation heat-resistance was determined after a short (17-hr) incubation period of leaves at different temperatures. It has been discovered (Fig. 1) that heat-resistance remains unchanged after 17 hr

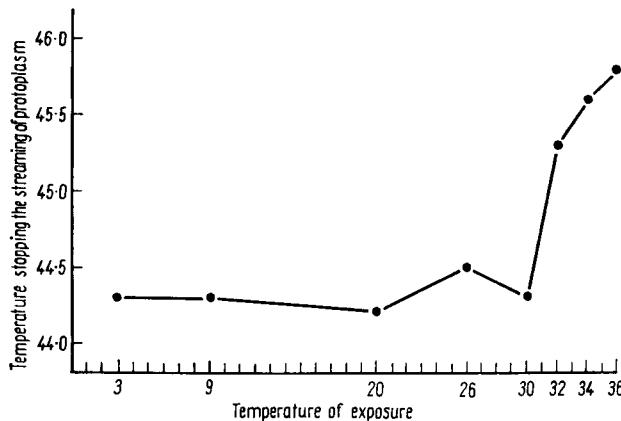


FIG. 1. Heat-resistance of cells of *Cabomba aquatica* after a short (17 hr) exposure to different temperatures.

in the temperature range from 3 to 30°C and the temperature for cessation of streaming is about 44·5°C. The same result was obtained by Alexandrov and Feldman (1958) from analogous experiments with *Tradescantia fluminensis*. However, the effect of hardening which results in an increase of heat-resistance is observed already at 32°C. It rises after exposure to higher temperatures and attains the maximum at 36°C when the heat-resistance increases by 1·6°C as compared with the initial resistance. A temperature of 37°C causes partial death of the cells while 38°C results in a complete stoppage of protoplasmic streaming. Thus it was established that the cells of *C. aquatica*, as well as the cells of all investigated higher plants respond to a short incubation by an increase of heat-resistance only under super-optimal temperatures. Determination of resistance to 12 per cent ethanol before and after exposure to 36°C indicates that in comparison with control cells cessation of protoplasmic streaming occurs 2·5 times more slowly: in 225 min instead of 105 (mean quantity from 9 experiments).

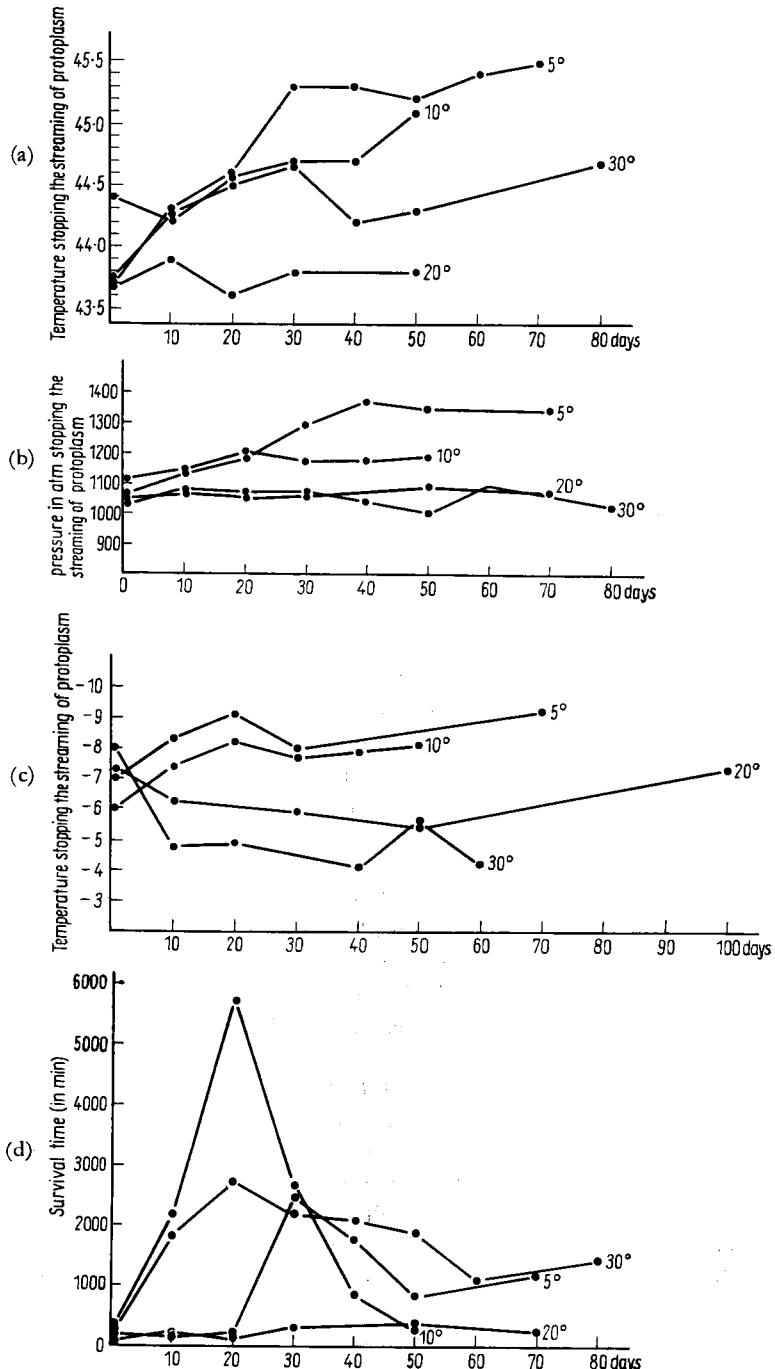


FIG. 2. The resistance of cells of *Cabomba aquatica* to different agents under continuous cultivation at different temperatures: a. heat-resistance, b. the resistance to hydrostatic pressure, c. cold-resistance, d. the resistance to ethanol.

Unlike some other objects *C. aquatica* exhibited no increase in resistance to high hydrostatic pressure. However, the cells of *Arundo donax* in the experiments of Jaskuliev behaved similarly.

The temperatures of 5, 10, 20 and 30°C which do not cause any shift in heat-resistance during a short exposure were chosen for a longer incubation of plants. The aquaria with plants were placed in thermostats with a corresponding temperature and with the same illumination by means of a fluorescent lamp (1400 lux). Initial resistance of the cells to all the given agents was determined. Despite the fact that plants before the experiment were always kept under the same temperature conditions, the initial heat-resistance of the cells in the experiments begun at different times was not the same. The cause of this variation is not known. The experiment lasted from 30 to 100 days. The resistance was determined every 10 days. The results obtained are presented in Fig. 2 (a, b, c, d). Each point on the graph corresponds to the mean quantity of 6–9 experiments.

The resistance to all the factors investigated during the whole experiment of plants kept at 20°C does not undergo any considerable change.

A continuous incubation of plants at 30°C, unlike a short thermal effect, in 10 days causes an increase in the heat-resistance, on the average by 0.6°. It continues to increase so that by the end of the experiment (by the 70th day) the heat-resistance exceeds the initial value by 1°. The resistance to ethanol increased very sharply (10–15 times). In some series of experiments it fluctuated markedly in the course of further incubation but at the end of the experiment it considerably exceeded the initial value. The initial cold-resistance of the cells was about -8°C. In 10 days it decreased approximately by 3–3.5° and kept this level until the end of the experiment. The resistance of cells to high hydrostatic pressure (determined by cessation of protoplasmic streaming due to a 5-min treatment and by the reversibility of protoplasmic damage) does not change throughout the experiment. An increase of heat-resistance accompanied by a non-specific increase in ethanol resistance can be considered as a reaction similar to heat-hardening.

In plants kept in the aquarium under 5°C, heat-resistance increases by 0.6° in 10 days. Under further incubation it continues to increase and by the end of the experiment (the 70th day) it exceeds the initial value by 1.8°. The resistance to hydrostatic pressure also increases in the process of incubation. In 20 days, cold-resistance exceeds the initial value by 2° and remains at this level to the end of the experiments. The resistance to ethanol rises almost as markedly as at 30°C, but this occurs only up to the 30th day and after that it decreases somewhat.

A continuous incubation of plants at 10°C produces the same effect on cellular resistance as maintenance at 5°C. But in this case the effect obtained is a little less and occurs later.

Thus cold-resistance of *C. aquatica* increases as a result of continuous incubation under low temperatures. However, unlike lower plants whose heat-

resistance simultaneously decreases, the heat-resistance of *C. aquatica* rises. Resistance to ethanol and high hydrostatic pressure also increases. Hence the response of *C. aquatica* to low temperature is similar to the reaction observed in grasses, sedges and some other plants during cold-hardening. The fact that this reaction in *C. aquatica* takes place already at 10°C may be due to its tropical origin.

A capacity to increase its heat-resistance under the influence of low temperatures, the absence of changes in heat-resistance in a wide temperature range after an exposure for 17 hr, a capacity to respond by heat-hardening to super-optimal heating which increases not only its heat-resistance but also its ethanol-resistance, all these facts show that the reaction of *C. aquatica* to thermal changes is similar to the way higher land plants respond to temperature alterations and distinguishes their behaviour from that of algae. This conclusion is in good agreement with the results previously obtained by Feldman and Lyutova (1962) in our laboratory which indicate a high constancy of the heat-resistance level in two species of sea grasses in the physiologically normal temperature range and a capacity of heat-hardening during a short exposure to super-optimal temperatures.

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TEMPERATURE ADAPTATION OF CELLS OF MARINE AND FRESHWATER ALGAE

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DATA in the literature on the lability of the heat-resistance level of water algae are rather contradictory. The results obtained by Tikhovskaya (1960) and Parker (1960) show that the heat-resistance of *Fucus vesiculosus* can change under natural conditions in conformity with temperature alterations within a year. In the experiments of Marrè and Servettaz (1956) performed on thermophilic algae, samples of the algae cultivated at high temperatures proved to be the most resistant. However, Schwenke (1960) detected only slight differences in the heat-resistance of two species of deep-water algae kept in the laboratory for 2–4 months.

The experimental results obtained by Montfort, Ried and Ried (1957) enabled these investigators to suggest that the heat-resistance of algae is a hereditarily fixed characteristic and only a few species are able to alter the level of their heat-resistance. But this comparison was made on objects taken from different thermal conditions and cultivated for a long period of time under the same temperature. Thus the statement of Montfort is yet to be confirmed under more strict experimental conditions.

Numerous investigations indicate that photosynthesis and respiration of algae can adapt to new temperature conditions (Harder, 1924; Ehrke, 1934; Lampe, 1935).

Considering the fact that the literature data are few and contradictory, we thought it necessary to investigate the capacity of algae to endure shifts in cellular heat-resistance.

The investigations were carried out on littoral and sublittoral algae of the Barentz and White Seas and unicellular freshwater algae grown in the Leningrad Botanical Garden.

The first series of experiments (Lyutova and Feldman, 1960) was performed in summer, when the temperature of the sea water was 10°C. Vegetative thalli taken from natural conditions were placed in sea water of different temperatures and kept there for 24 hr.

The heat-resistance was characterized by the maximal temperature after which the cells retained normal distribution of vital stains (new methyl blue).

The thalli were each time exposed to heating for 5 min. It was established that the higher the cultivation temperature, the greater the cell-resistance of algae (Fig. 1). In our experiments the most strongly pronounced increase in heat-resistance due to the rise of cultivation temperature was observed in *Enteromorpha compressa* and *Porphyra* sp.

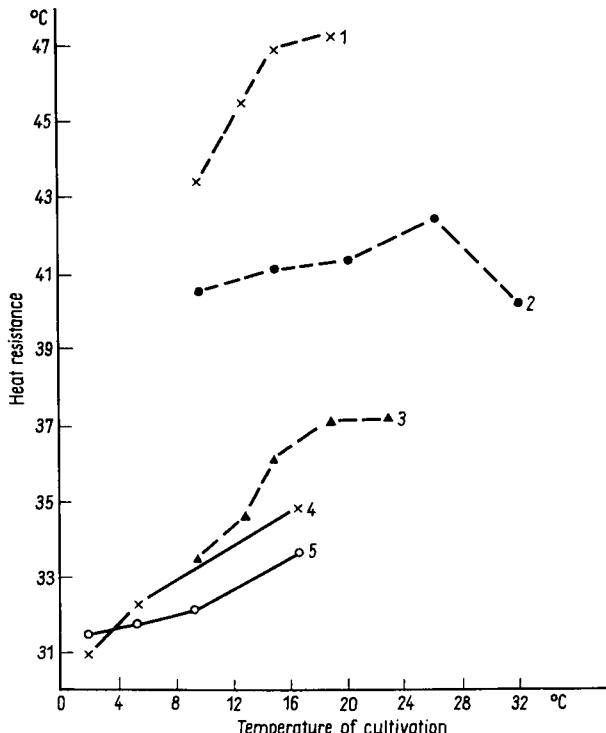


FIG. 1. The heat-resistance level of the algae in relation to the cultivation temperature. 1. *Enteromorpha compressa* (L.) Grev. 2. *Fucus inflatus* Vahl.
3. *Porphyra* sp. 4. *Peridinium bipes* Stein. 5. *Chlamidomonas eugametos* Moewus.

Studies of photosynthesis and respiration showed that a preliminary cultivation of *Fucus inflatus* at different temperatures (10, 15, 20, 26.6 and 29°C) during 24 hr does not influence the rate of these processes determined at 20°C. However, the injurious effect of heating as measured by the decrease in the photosynthetic rate was considerably less in the algae kept under higher temperature. Thus an increase in cultivation temperature is accompanied by an increase in resistance of the photosynthetic apparatus of algae.

Next, the rate of change in heat-resistance was studied. Under our experimental conditions the maximal heat-resistance of *Porphyra* sp. was attained at 30°C in 1 hr, at 24°C in 5 hr, at 19°C in 24 hr, at 13°C in 48 hr. Thus, the higher the temperature in which the algae were placed, the more rapid the shift of heat-resistance.

During our experiments with marine algae we failed to establish whether a lengthening of the exposure is necessarily accompanied by a further increase of heat-resistance.

The cultivation of excised thalli of algae in stagnant waters leads to a decrease in viability of algae. The higher the temperature, the earlier this process takes place.

The second series of experiments (Feldman, Zavadskaya and Lyutova, 1963) was carried out in the White Sea on brown algae taken in winter, when the temperature of the water was about 0°C. The cultivation of thalli of *Ascophyllum nodosum*, *F. vesiculosus* and *Fucus serratus* in the temperature range 16–20°C for 24 hr led to an increase in their heat-resistance as compared with the algae kept at sea water temperature.

A certain shift of resistance was noted not only when the algae were transferred from low to high temperature, but also on the reverse transfer. Thus the heat-resistance of the algae collected in warm seasons at 16–20°C and maintained at low temperatures (about 0°C) decreases as compared to the algae kept at sea water temperature. In both cases the degree of the change in heat-resistance is approximately the same (1°).

On temperature alteration not only the heat-resistance of algae but also their resistance to low temperatures undergoes certain changes. When the cold-resistance of cells was measured, the damaging temperature was constant and the resistance was determined by the time after which a normal distribution of stains was retained, even if only in a few cells. The cold-resistance of winter algae maintained under conditions of high temperature for 24 hr is considerably less than that of the algae kept under low temperatures (Table 1).

Thus a change in the cultivation temperature of marine algae under laboratory conditions is accompanied by a corresponding shift of their temperature-resistance. The capacity for reactive changes of heat-resistance was also studied on fresh water unicellular algae (Luknitskaya, 1963). In this case the conditions of algae cultivation were in conformity with their natural environmental conditions. *Peridinium bipes* and *Chlamydomonas eugametos* taken from the water temperature of 15–18°C were cultivated for a long time at lower temperatures (2–3, 5–6 and 9–10°C). A partial decrease in heat-resistance was noted after 6–8 hr; a stable level was reached in 5–10 days and maintained throughout the experiment till the 46th day. Figure 1 shows the values of heat-resistance on the 10th day of cultivation (heat-resistance is represented by the maximal temperature after 5-min action of which the algae still retain mobility).

The transfer of these algae to the original high temperature (15–18°C) gives a reliable increase in heat-resistance in 2–4 hr, and in 24 hr it attains the control level.

Thus, on transfer of algae from warm marine water into cold water, alterations in heat-resistance occur more slowly than in the opposite case. Moreover, these experiments show the instability of the level of heat-resistance which was observed at high temperatures.

TABLE 1. CHANGES IN CELLULAR HEAT-AND COLD-RESISTANCE OF BROWN ALGAE IN RESPECT TO ENVIRONMENTAL TEMPERATURE

Species†	In laboratory conditions			In nature			Temperature Adaptation
	Cultivation temp. of algae within 24 hr (°C)	Heat- resistance (°C)	Cold- resistance (min)	Season	Heat- resistance (°C)	Cold- resistance (min)	
<i>Ascophyllum nodosum</i>	0·2	39·5	375	Winter	39·3	293	
	23·0	40·3	154	Summer	41·5	79	
<i>Fucus vesiculosus</i>	0·6	41·9	341	Winter	41·6	268	
	22·0	42·5	277	Summer	42·5	184	
<i>Fucus serratus</i>	0·8	39·1	56	Winter	39·1	36	
	16·5	40·3	26	Summer	40·7	32	
<i>Fucus distichus</i>	—	—	—	Winter	41·0	—	
	—	—	—	Summer	42·3	—	

† Cold-resistance of *Ascophyllum nodosum* and *Fucus vesiculosus* was determined at -29 to -30°C, cold-resistance of *Fucus serratus*, at -20°C.

Under natural conditions marine as well as freshwater algae are able to change their heat- and cold-resistance. To determine the heat-resistance we used a series of thalli of the same age.

In summer, when the air temperature rose to 25°C, the heat-resistance of algae proved to be higher than in winter at temperatures below 0°C (Table 1).

The cold-resistance of these algae in winter exceeded the values obtained in July and August. However, the difference in the cold-resistance of winter and summer specimens was not significant in one species—*F. serratus*. The range of change in heat- and cold-resistance is several times higher under natural conditions than in the laboratory—a fact which might be connected with the duration of exposure.

Under natural conditions we also recorded shifts in heat-resistance due to short-time changes of temperature. In *Pelvetia canaliculata* and *F. vesiculosus* collected on a hot day at low tide, the heat-resistance increased by 0·6–0·7°C.

As the water temperature in the pond rose, the heat-resistance of the freshwater alga *P. bipes* increased. The heat-resistance of the algae taken from the water at a temperature of 20°C was 17° higher than in the algae taken at 10°C.

The results obtained and a critical survey of the literature led to the conclusion that the heat-resistance of algae shows a considerable lability. Changes in the resistance of algae are due to certain influences of temperature. These alterations are sometimes found in natural conditions and can also be induced by artificial cultivation (Feldman and Lyutova, 1963).

Attention should be given to the peculiarities of the reaction of algae in comparison with higher plants.

In the laboratory as well as under natural conditions, shifts in the temperature-resistance of algae occur as a result of alterations within the whole temperature range. In higher plants under similar conditions, an increase in heat-resistance was observed only after the exposure of plants to super-optimal temperatures (Alexandrov, 1956, 1963). Similarly a rise in heat-resistance of higher plants in nature occurs under the influence of extreme overheating (Alexandrov and Jaskuliev, 1961; Lange, 1961; Shukhtina, 1962).

In algae, shifts in heat-resistance are always in conformity with the environmental temperature. In higher plants, an increase in heat-resistance is also observed under the action of low temperatures (Alexandrov, Lyutova and Feldman, 1959). Moreover when the cultivation temperature is increased, a rise of heat-resistance is not always accompanied by a change in cold-resistance. The difference in the character of the response to shifting of the environmental temperature in algae and higher plants is not related to the aquatic mode of life of the algae. Special investigations of the temperature-resistance of aquatic higher plants have shown that alterations in their resistance are subject to the same laws as terrestrial higher plants (Feldman and Lyutova, 1963; Denko, 1963).

A similar difference is observed in animals as well. In Protozoa (Poljansky, 1959) and some lower multicellular animals (Kamshilov, 1960; Gorodilov,

1961; Dregolskaya, 1963) cellular heat-resistance may also change according to changes in the environmental temperature. However, the cellular heat-resistance of most multicellular animals does not depend on the temperature conditions of the environment (Ushakov, 1963).

Taking into consideration the above-mentioned facts we can conclude that the degree of lability of heat-resistance for the whole organic world depends on the level of organization.

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EFFECTS OF HIGH TEMPERATURE AND LIGHT ON THE THERMOSTABILITY OF CELLS OF DIFFERENT CROP VARIETIES

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MODERN ideas that plant resistance is closely related to colloid and chemical changes in the cytoplasm have been widely developed in many studies. Protoplasmic permeability and viscosity have been shown to increase and protoplasmic movement to slow down under the influence of high temperature and wilting. When injured tissues are immersed in water, the loss from the cell of electrolytes and soluble substances increases (Maximov, 1939; Vasilyeva, 1953; Alexandrov, 1956; Genkel and Bodanova, 1956; Belikov and Kirillova, 1959; Levitt, 1958).

The critical temperature, at which the protoplasmic permeability sharply increases, is different for various plant species and is defined by their heat-resistance (Vasilyeva, 1957). The thermoresistance of plants is determined by the resistance of proteins and by the ability of cells to increase their resistance by hardening (Alexandrov and Feldman, 1958). Some authors stress the positive role of light in the increase of plant resistance (Laude, 1939; Heyne and Laude, 1940; Coffman, 1957). It should be observed that the reaction of some varieties to high temperatures is insufficiently known. Also, few data have been obtained on the effect of light during hardening on the increase of plant resistance to high temperatures.

The aim of this study is to find out the reaction of diverse crop varieties to the injurious action of high temperatures and the effects of light and the length of the hardening period on cell heat-resistance as well.

Different spring-wheat, pea, bean, lentil and chick-pea varieties were studied. The degree of cellular permeability was defined by electrolyte exudation (specific electroconductivity change) and total exudation of the substances of leaves into the surrounding solution (recorded by a refractometer) as a result of exposure to a temperature of 40°C for 2–4 hr (Oleinikova, 1962).

To define the degree of cytoplasmic heat-resistance of certain crop varieties and species, the leaves of the same tier were exposed to temperatures of: 25, 30, 38, 43, and 45°C for 2–4 hr and total exudation of electrolytes and soluble substances was determined.

Data for two spring-wheat varieties differing as to their drought-resistance show that their reaction to high temperature is different (Fig. 1). A temperature

increase up to 38–43°C had a slight injurious effect on the cytoplasm of a drought-resistant wheat variety *Erythrospermum* 841, the electrolyte exudation into the solution increased slightly (up to 98 mho at 43°C). A similar temperature rise markedly injured the leaf cytoplasm of the non-drought resistant variety *Diamant*, a large increase of the electrolyte exudation resulting (up to 345 mho at 43°C). A higher temperature had a severely injurious effect on the protoplasm of all the wheat varieties under trial. Among the varieties differing in heat-tolerance, the greatest differences in the degree of cytoplasmic permeability are observed at temperatures of 40–43°C.

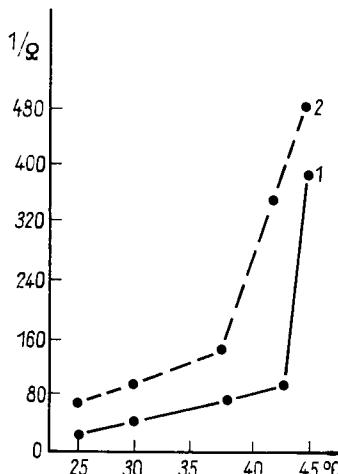


FIG. 1. High temperature influence on electrolyte loss during 2 hr (in units of specific electroconductivity) in spring-wheat varieties at the stage of final leaf development. 1—*Erythrospermum* 841. 2—*Diamant*.

Similar trials with lentils showed obvious differences among varieties as to the degree of cytoplasmic heat-resistance (Fig. 2). The variety *Dnepropetrovskaya* 10 proved to be more susceptible to temperature increase when compared to the lentils from *Ethiopia* (cat. 958) and *Iran* (cat. 6). The electrolyte exudation of the former variety was 206 mho at a temperature of 43°C for 4 hr, whereas in the latter two it amounted to 141 and 120 mho respectively.

The bean variety "Russkiye cernye" ("Russian Black"), commonly found in the Moscow district, is less heat-resistant as compared to the beans of Spain (cat. 792). Evidently, the leaf cytoplasm of varieties growing in more northern regions is less resistant to effects of high temperature.

Pea, lentil and chick-pea varieties of the same origin (from *Ethiopia*) respond differently when exposed to high temperatures. The pea plants are less tolerant of the injurious action of high temperature as compared to lentil and chick-pea, probably, due to the pea being more common in alpine regions, and consequently, growing under different ecological conditions.

The data presented show not only species but also varietal differences as to the degree of cytoplasmic resistance to the injurious action of high temperatures. This fact has a direct practical meaning, contributing to a definition of varietal differences as regards high temperature resistance.

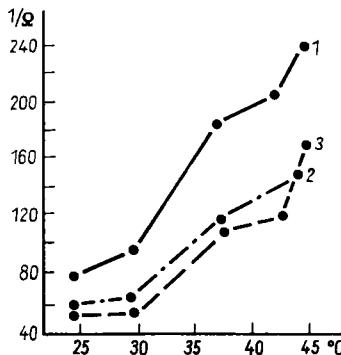


FIG. 2. High temperature effect on electrolyte loss from leaves during 4 hr (in units of specific electroconductivity) in lentil varieties of diverse geographical origin at the beginning of the flowering period. 1—Dnepetrovskaya 10. 2—A sample from Ethiopia cat. 958. 3—A sample from Iran cat. 6.

To induce an effect of light during crop-hardening on the increase of cytoplasmic heat-resistance the following experiment was conducted. The plants of two spring-wheat varieties (at the stooling stage) were placed for hardening into a light chamber at a temperature of 35°C for 5 hr, one group of plants being exposed at that time to the direct sunlight, the other one being kept under reduced light (covered by one layer of coarse calico) and the third in darkness. After 5 hr the leaves of the same tier were taken from the plants of all treatments and exposed to a high temperature of 40°C for 4 hr, the total exudation of electrolytes and soluble substances being determined in the solutions.

The results of the experiments (Table 1) showed that the cytoplasm of the plants exposed to light during hardening was more heat-resistant as compared to the plants which were kept in darkness for the same time. The reduced intensity of light decreased cytoplasmic heat-resistance. The lowest exudation of electrolytes and soluble substance was from the plants, the leaves of which had been subjected to a high temperature of 40°C and exposed during hardening to direct sunlight; observations indicate their high resistance.

With the aim of determining the effect of length of hardening period, plants of three wheat varieties were placed for hardening into a light chamber at a temperature of 35°C for 1, 2, 4, 6, 16 and 24 hr. The plants were removed at different times and were tested for difference in heat-resistance. The leaves of the test and the control (without hardening) plants were subjected to a

TABLE 1. LIGHT EFFECT DURING HARDENING ON CYTOPLASMIC HEAT-RESISTANCE OF LEAVES EXPOSED TO A HIGH TEMPERATURE OF 40°C FOR 4 hr
(PLANTS AT STOOLING STAGE)

Light conditions during hardening at 35°C	Time (hr)	Electrolyte exudation (in units of specific electroconductivity) in varieties		Exudation of soluble substances (by refractometer) in varieties	
		Erythrosperm. 841	Diamant	Erythrosperm. 841	Diamant
Sunlight	5	45	77	104	165
Reduced light (under one layer of coarse calico)	5	57	89	112	173
Darkness	5	68	113	123	245

TABLE 2. EFFECT OF LENGTH OF HARDENING PERIOD AT 35°C ON ELECTROLYTE EXUDATION OF PLANTS AFTER EXPOSURE TO A HIGH TEMPERATURE OF 40°C FOR 4 hr

Variety	Electrolyte exudation (in units of specific electroconductivity) of plant leaves subjected to hardening for:						
	24	16	6	4	2	1 hr	Check without hardening
Erythrospermum 841	46	40	60	58	80	167	185
Caesium 111	52	43	48	43	102	106	201
Thatcher	46	53	88	106	122	147	213

temperature of 40°C for 4 hr with the aim of determining the degree of cytoplasmic-hardening in individual treatments of the test.

Records of electrolyte exudation demonstrate (Table 2) that hardening of plants for 1-2 hr increases cytoplasmic tolerance. Longer periods of hardening (4-6 and more hours) result in a further decrease of electrolyte exudation, pointing to the fact that these plants are more hardened than the former.

The recorded data show that the conditions, under which the hardening of plants proceeds (light intensity, length of hardening period) have a definite effect on the degree of cytoplasmic heat-resistance.

Summary

1. Crop varieties differ as to the degree of cytoplasmic heat-resistance, this being related to their heat-tolerance. Exudation of electrolytes and soluble substances gives a basis for observing varietal differences in relation to their resistance to the injurious action of high temperatures.

2. The conditions under which the hardening of plants proceeds (light, time length) influence the degree of cytoplasmic hardening.

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ON REPARATION OF HEAT INJURY BY PLANT CELLS

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1. A cytophysiological analysis of the cellular response to the injurious action of heating shows that the thermal effect by which we can judge cellular heat-resistance is defined by the following factors: (a) resistance of protoplasmic proteins to the denaturing action of heating or, at lower temperatures, the resistance of metabolic processes; (b) capacity of cells to increase their resistance in response to heating, i.e. heat-hardening; (c) capacity of cells to eliminate the injury caused by heating, i.e. reparation (Alexandrov, 1956).

2. Reparation is observed after heating and in the course of heating at moderately high temperatures. Reparation is connected with the activity of a cell.

3. The capacity of cells to restore the repressed function is more pronounced in respect to the most sensitive functions, which are affected before damage of the entire cellular system. In the case of high temperatures, protoplasmic streaming and photosynthesis can be found among such functions (Lyutova, 1962).

4. The capacity of cells to repair disturbances of a function can be estimated by the difference between the minimum dose of an agent repressing the given function and the maximum dose allowing its reparation. In the case of a constant heating period the difference is expressed in degrees; the interval between these doses we denote as the reparation zone.

5. The reparation zones for the cessation of protoplasmic streaming were obtained for a 5-min heating on epidermal cells of leaves (*Tradescantia fluminensis* Vell.—7·1°C, *Campanula persicifolia* L.—7·4°C, *Dactylis glomerata* L.—7·5°C, *Catabrosa aquatica* (L.) P.B.—3·8°C, *Chlorophytum elatum* R.Br.—9·0°C, etc.). Reparation zones in plant cells are more extensive than in animal cells (Alexandrov, 1963; Arronet, 1963).

6. It was demonstrated on epidermal cells of leaves of *T. fluminensis*, *C. persicifolia*, *D. glomerata* that the extent of a reparation zone during intensive heating for a short time is greater than during prolonged action of more

moderate temperatures. At temperatures repressing protoplasmic streaming by heat denaturation of cell proteins (Alexandrov, 1956) the reparation zone shortens insignificantly with a decrease of heating and a lengthening of the heating time. With still less intensive heating the shortening of the reparation zone is more precipitate, and upon prolonged weak heating it may approach zero. Under such conditions it was impossible to obtain reversible stopping of protoplasmic streaming (Alexandrov, Khachaturov and Shukhtina, 1963).

7. The difference between the thermostability of the function of movement and other vital cellular functions is less under such conditions than in the case of intensive but short heating, which is the principal cause of shortening of the reparation zone under a decrease of temperature and a lengthening of heating time. Owing to this, the decrease of temperature and lengthening of time of heating result in the fact that the moment of cessation of protoplasmic streaming becomes identical with the moment of death of the cell.

8. The described change in the extent of the reparation zone as a function of the relation between intensity and time of heating is to be taken into consideration when estimating the ecological role of cellular reparation in plants subjected to heat under natural conditions. The experimentally determined capacity of plant cells to repair injuries caused by an intensive short period of heating may not correspond to phenomena observed in nature where there is a different intensity and time of heating.

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THE INFLUENCE OF HEAT-HARDENING ON THE RESISTANCE OF PLANT CELLS TO DIFFERENT INJURIOUS AGENTS

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EPIDERMAL cells of leaves of *Tradescantia fluminensis* Vell. and *Campanula persicifolia* L. after heat-hardening for 18 hr in a moist chamber at 36.5°C (*T. fluminensis*) or at 37°C (*C. persicifolia*) were exposed to the action of cadmium chloride, potassium rhodanate, ethyl ether, sodium azide, 2,4-dinitrophenol and sucrose. Survival time of protoplasmic streaming in these chemical solutions was taken as a criterion of cellular resistance to injury. In cases when cellular resistance to larger doses of the injurious agents was being tested, the degree of the resistance of cells was determined through repression of plasmolysis or through anthocyanin exit from vacuoles.

It was found that heat-hardening increased cellular resistance of the epidermis of *T. fluminensis* not only to heating, ethyl alcohol, acetic acid and high hydrostatic pressure (Alexandrov and Feldman, 1958; Kiknadze, 1960; Lomagin, 1961) but also to the injurious action of ethyl ether, potassium rhodanate and cadmium chloride. All these factors are denaturants of native proteins. Heat-hardened cells of *T. fluminensis* do not change their resistance to the injury induced by the plasmolytic effect of sucrose. Heat-hardening does not increase the resistance of cells of *C. persicifolia* and *T. fluminensis* to 2,4-dinitrophenol and sodium azide applied in doses which repress protoplasmic streaming. Under conditions providing the photoreduction of 2,4-dinitrophenol (light without aeration) the hardened cells of *C. persicifolia* and *T. fluminensis* show greater sensitivity to this poison. Heat-hardening increases the cellular resistance of *C. persicifolia* and *T. fluminensis* to doses of sodium azide which produce irreversible cellular injury (anthocyanin exit, repression of plasmolysis). In these experiments sodium azide seems to act not as a specific inhibitor but as a denaturing agent.

The results of the experiments suggest that the heat-hardening stabilizes the native structure of cell proteins in respect to the denaturing action of different agents.

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CHANGES IN CARBOHYDRATE CONTENT OF PLANTS UNDER HEAT-HARDENING

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OUR laboratory studies have shown that plant cells increase their resistance to high temperature and to various agents in response to injurious heating (Alexandrov and Feldman, 1958; Lomagin, Antropova and Ilmete, 1963, etc.).

To obtain evidence on the biochemical mechanisms underlying the non-specific increase in cellular resistance, Feldman (1962) carried out an investigation which showed that the incubation of leaf discs in solutions of dextrose, fructose, sucrose and raffinose caused a considerable increase of their resistance to heat and to high hydrostatic pressure. These results may be ascribed to the anti-denaturing action of sugars on the cytoplasmic proteins. In connexion with this a question arises whether an increase in the concentration of soluble sugars in cells is a cause of heat-hardening. Our purpose was to study this question.

Isolated leaves of *Campanula persicifolia* L., *Dactylis glomerata* L., *Leucanthemum vulgare* L., *Hepatica nobilis* L. were hardened in a moist chamber for 18 hr at 37.5°C. After hardening, the heat-resistance of epidermal cells and the carbohydrate content of the entire leaf were measured. Fresh leaves (K_1) and leaves kept for 18 hr at room temperature (K_2) were taken as controls. The heat-resistance of K_2 leaves of *D. glomerata* and *C. persicifolia* was found to be about 0.4° higher than that of K_1 leaves. As a result of hardening, heat-tolerance increased by 1.5–2.0°C in comparison with that of K_2 leaves. Determination of the amount of soluble carbohydrates made by means of quantitative paper chromatography have shown that in K_2 leaves of *D. glomerata* and *C. persicifolia* the amount of fructose and dextrose increased compared with K_1 leaves while the amount of sucrose decreased. The increase in monosaccharides seems to result from hydrolysis of starch and other polysaccharides. Measurements by Ilijin's method indicate a 50–80 per cent decrease of starch. Perhaps leaves of *Leucanthemum vulgare* contain the non-labile form of starch, and thus their starch content remains unchanged. However, the dextrose content dropped.

In the course of hardening at 37.5°C hydrolytic processes and respiration evidently become more intense. That is why the starch in the leaves of

D. glomerata and *C. persicifolia* disappears completely; the dextrose, fructose and sucrose content in leaves of *D. glomerata* and *L. vulgare* drops; *C. persicifolia* shows a decrease in the sucrose content, the amount of dextrose remaining unchanged. Alterations in the quantitative content of sugars, analogous to those observed in leaves of *D. glomerata*, have been found in leaves of *H. nobilis*. Besides, there is a considerable increase of raffinose in the latter plant.

Thus heat-resistance of hardened cells rises sharply whereas the mono- and disaccharide content either falls or does not change.

The effect of heat-hardening of epidermal cells can be obtained by heating the leaves for 1–10 sec at 49–50°C (Lomagin, 1961; Yarwood, 1961; Zavadskaya, 1963). As in the case of prolonged exposure to more moderate temperatures the hardened cells also show a non-specific increase in their resistance. In our experiments, cellular thermostability of *D. glomerata* leaves heated for 10 sec at 50°C and afterwards cooled for 20 sec at 20°C showed a 1·1° increase. At the same time a considerable increase of the sucrose content and a certain increase in the fructose content was observed. The starch content decreased by 43 per cent due to hydrolysis.

Thus after a short period of hardening, the sugar content rises, after prolonged hardening it falls, whereas heat-resistance is increased in both cases.

We do not see the increase in soluble sugar content as an explanation for the increase in cellular resistance of isolated leaves under heat-hardening.

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DISCUSSION

T. M. BUSHUYEVA (question to D. Lange): Your data show that the heat-resistance of cells depends on environmental conditions. I mean, for example, the mediterranean plants which differ in heat-resistance depending on the altitude of the place where they grow. On the contrary Prof. Alexandrov established in his experiments that the cell heat-resistance of higher plants does not change in different environmental conditions. How do you explain this contradiction?

O. L. LANGE: I think that the difference in the results of the school of Prof. Alexandrov and my own experiments is caused by the difference in the methods applied. Prof. Alexandrov and his co-workers use as an indicator of the degree of heat-resistance the temperature at which protoplasm streaming is suppressed. This is a very sensitive cytological criterion. In my investigations I am not so interested in the special cytological changes after heating, as I am in studying heat-resistance from a more ecological point of view. I am rather interested in the overall effect of heating, i.e. in the question whether a plant is capable of surviving high temperature. Therefore, I recultivate the heated plants to be able to judge the heat damage after it has become manifest. Maybe the plants in my experiment just after heating would show the same effect on protoplasmic streaming as the objects of Prof. Alexandrov. The "heat-adjustment" which was apparent in my objects may be based on the capability of plants to repair an injury induced by heating.

I will repeat my experiments also using the method of Prof. Alexandrov to find out whether our different experimental results can be explained satisfactorily by this possibility.

A. G. LOMAGIN: How do you explain the influence of diurnal cycles on the heat-resistance of *Kalanchoë*?

O. L. LANGE: I have up to now no idea what might be the explanation. There are some data showing metabolic changes in the leaves of *Kalanchoë* when the flowering was induced by photoperiod. But I do not know what is the cause of changes in heat-resistance.

A. G. LOMAGIN: The short-day treatment of long-day plants stimulates leaf growth. Such an effect can be observed in short-day plants after a long-day treatment. In your experiments the increase in heat-resistance of short-day plants of *Kalanchoë* was established in the dark period. On the contrary the long-day plants have the maximal heat-resistance in the day time as was established by Laude. Oleinikova also showed the increase in heat-resistance

of long-day plants after long-day treatment. Is it possible to explain the influence of light-dark cycles on the heat-resistance by its influence on the intensity of growth, taking into consideration the fact that you and Dr. Gorban have shown the relationship between these two processes?

O. L. LANGE: The growth of leaves cannot be the reason of the increase in heat-resistance after a short-day treatment of *Kalanchoë blossfeldiana*. We cultivated *Kalanchoë* for ten days ("short-day treatment") and it was sufficient to increase the heat-resistance. But this ten-day cultivation did not change the size of leaves, their dry weight, and so on. We tried to repeat this experiment with long-day plants but we failed to find any changes in day time heat-resistance.

V. Y. ALEXANDROV: I have some ideas about Dr. Lange's most interesting work. I should like to express them. Comparing his and our data we in some cases observed the same results while in other cases the results were quite different. For instance Dr. Lange believes that the cell heat-resistance of a number of plants corresponds to their environmental temperature—just the same as we do. Both Dr. Lange and we observed the increase of heat-resistance in winter. However, Dr. Lange found out that sometimes there exists a summer increase of heat-resistance. Dr. Lange considers that winter and summer increase in heat-resistance are caused by different factors. In winter it is accompanied by an increase of cold-resistance, while in summer it is not.

Our experiments prove that cold-hardening of some plants both in nature and under experimental conditions greatly increases their heat-resistance but we failed in all attempts to increase cold-hardiness after heat-hardening. So in this respect our data correspond to Dr. Lange's data. Some discrepancy appeared after Dr. Lange published his data referring to the corresponding change of heat-resistance of plants cultivated at different temperatures in the optimal range. These results of Dr. Lange which appeared not long before the Symposium stimulated us to make a series of special experiments. They gave the following results. *Tradescantia*, wheat and peas were cultivated for 35, 26, and 14 days at 10 and 20°C. Then their heat-resistance was studied and its criterion was the temperature stopping protoplasmic streaming after 5-min heating. All 10° plants had slightly increased heat-resistance while 20° plants did not. These results and some other data obtained in our laboratory prove that by increasing the cultivation temperature in its tolerant range one does not change cell heat-resistance. Heat-hardiness is a complicated notion and by applying different methods we recorded different sites for it. In our experiments we established a primary injurability of cells. Dr. Lange observed macroscopic changes and his criteria included the reparatory ability of plants. Dr. Lange wants to use our methods in his experiments and we shall make experiments in order to study the influence of the cultivation temperature on the various sites of heat-resistance, for instance on the reparation ability. I think that such cross methods will enable us to solve the important problem

of the influence of cultivation temperature on the cell heat-resistance of higher plants.

And now some words about daily fluctuation in cell heat-resistance. In our experiments heat-resistance was the same in the day time and at night when the temperature was lower than the hardening temperature. Maybe in that case the diversity between Dr. Lange's and our data can also be explained by the different methods we applied.

O. L. LANGE: How do you explain the conclusion that lower plants have the ability for adjustment while the higher ones have none? Do you know in what systematic group lies the borderline of adjustment ability and whether the mosses belong to the group capable of adjustment or not? Such data could be of great importance for systematics.

V. Y. ALEXANDROV: We do not as yet know where the borderline occurs and we do not know to what group mosses and mushrooms belong as concerns their adjustability. We can only speculate why at a certain phylogenetic level the adjustment ability is lost. As this loss occurs in phylogensis of both plants and animals, one can suggest that this phenomenon is connected with some significant peculiarities of the progressive development of organisms. The loss of adjustment ability may reflect the increased constancy of protein synthesis in the course of phylogensis.

G. I. POLJANSKY: I cannot agree with the hypothesis of Prof. Alexandrov concerning the loss of adjustment ability in the course of phylogensis. I prefer another explanation although I am not sure that it is more correct as in this problem we find ourselves in the realm of imagination. I believe that the explanation should be looked for in the appearance of new, more convenient, overall adaptation mechanisms in the process of phylogensis.

V. Y. ALEXANDROV: I think that Prof. Poljansky in his hypothesis may meet certain difficulties. I cannot imagine new overall mechanisms for withstanding temperature influence that brown algae posses while the higher water plant *Cabomba* (mentioned in the report of Denko) does not possess it.

O. L. LANGE: Dr. Lyutova, have you any idea in what state of development the unicellular algae were when you determined their heat-resistance. According to Lorensen the resistance of *Chlorella* mostly depends on their stage of development. This was established in the experiments made on synchronized cultures.

M. I. LYUTOVA: Our experiments were made on non-synchronized cultures hence I cannot answer your question.

P. P. RUMYANTSEV: There probably is a certain discrepancy between plant and animal cells in respect to the relationship between their hardiness and differentiation. Dedifferentiation of frog myocardial fibres in tissue cultures increases heat-resistance instead of lowering it. This was observed when we

took as a criterion primary injurability by heating and not its after-action. In the case of after-action the reparation ability of cells could be of importance.

I. S. GORBAN: We studied the cells in the period of elongation when they stopped dividing. We have not yet studied dividing meristematic cells. Therefore our results should not be compared with the results obtained on the experiments of animal cells cultured *in vitro*.

O. L. LANGE: I have a question for Prof. Alexandrov concerning the stability of cell thermoresistance of plants, belonging to one species and living in different environmental conditions. You have given us examples covering several species in which the coincidence of all thermoresistance within one species was demonstrated.

I have found a considerable difference in cell thermostability of plants from different temperature and water supply conditions. You have described 24-hr fluctuations in thermostability. If the plant displays such fluctuations during the year and during one day you must have discovered some changes in thermostability in the plants which grow in different environmental conditions. How could two plants which grew as far as 3000 km from each other be in the same state of the daily rhythm of their thermostability?

V. Y. ALEXANDROV: I would like to remind you that in the case you mentioned, *Morus alba* and *Dactylus glomerata* did not display great fluctuations in cell thermostability when examined at high temperature ranges.

Our experiments on natural "hardening" of plants carried out in the Middle Asia allowed us to see that daily fluctuations are characteristic of many species of plants, while some species did not display such an ability. For instance in case of *Morus alba* from Ashkhabad we failed to observe either the thermal "hardening" on hot days, or any daily fluctuations of the thermostability. I draw your attention to the fact because it was not mentioned in the paper of Dr. Feldman.

CELLULAR ECOLOGICAL ADAPTATIONS AND REACTIONS, DEMONSTRATED BY SURVIVING ISOLATED GILL TISSUES OF BIVALVES

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FIRST, I would like to express my deepest gratitude for the invitation to report the results of my special research field at this famous institution. Being merely a "marine zoologist" I am nevertheless deeply convinced that every research concerning ecological problems needs to be complemented by physiological observations and especially by cell physiological studies in the laboratory. Only in this way we can completely analyse and understand what happens biologically in the sea and why it happens.

For many years we have been investigating the various horizontal and vertical distributions of marine species. The method which we used was to study experimentally the cell physiological behaviour of selected species as a function of alterations of single physical and chemical qualities of the external medium. In many cases we obtained very good results by working with surviving gill tissue, especially of bivalves. During the short time I have here at my disposal I would like to present some examples of these experiments.

The gills of bivalves are covered with a layer of ciliated epithelium. Isolated gill pieces can easily be kept alive for a week and the following cellular functions may be observed without difficulty:

1. The survival time by direct observation of the ciliary movements.
2. The mechanical activity by measuring the ciliary frequency or the transportation speed of the ciliated epithelium.
3. The metabolic rate by manometric measurements of the oxygen consumption of gill pieces or of tissue homogenates.
4. Some metabolic values by determination of the corresponding particular enzymatic activities (e.g. the dehydrogenase activity).

With the aid of the above-mentioned criteria it is possible to analyse cellular reactions as well as genetically and environmentally induced adaptations to the following ecological factors:

Temperature, salinity, hydrostatic pressure.

Oxygen and carbon dioxide tension.

Dissolved organic substances.

Cellular resistance to various external physico-chemical factors seems to be based on different mechanisms. There are species which are very eurythermic but not euryhalinic to the same degree, e.g. *Tapes decussatus* (Table 1). But there are also euryvalent species which are simultaneously eurythermic, euryhalinic and eurybathic at the cellular level without occurring at deeper layers (e.g. *Mytilus edulis*, Fig. 4).

TABLE 1. LETHAL TEMPERATURES (SHORT-TIME EXPERIMENTS AT INCREASING TEMPERATURES) AND LETHAL SALINITIES (48 HR DILUTION EXPERIMENTS) OF ISOLATED GILL TISSUE OF MARINE BIVALVES FROM DIFFERENT DEPTHS

(After Schlieper, Flügel and Rudolf, 1960)

Species	Habitat	Lethal temperature upper limit °C	Lethal salinity lower limit ‰ S
<i>Avicula birundo</i>	Sublittoral	35	20
<i>Pinna pectinata</i>	Sublittoral	35	20
<i>Pinna nobilis</i>	Subtidal	38	15
<i>Mytilus galloprovincialis</i>	Intertidal	38	10
<i>Cardium edule</i>	Intertidal	39	10
<i>Tapes decussatus</i>	Intertidal	41	15

(a) Temperature

The cellular thermal resistance of bivalve species is genetically established. It is easy to demonstrate distinct correlations of the cellular thermal resistance with the habitat of the species. We are able to differentiate: (1) types with a narrow cellular thermal resistance and adaptation range (e.g. cold stenothermic species from polar latitudes, deep water and mountain waters; and warm stenothermic species from tropical latitudes), (2) types with a broader cellular thermal resistance and adaptation range (e.g. eurythermic species from habitats with different or changing temperatures). For example, the cells of intertidal species of the temperate zones are more resistant to temperature stress than the cells of subtidal species. The intertidal forms also exhibit a greater cellular phenotypic plasticity in response to thermal acclimation (Fig. 1, Table 2).

Not only the cellular thermal resistance range but also the cellular temperature-related capacities (mechanical activity, metabolic rate) may be different according to genetic adaptation as well as according to individual acclimation. Only types with a broad phenotypic resistance range may also show individual cellular thermally induced capacity adaptations.

The necessary time of thermal adaptation at the cellular level was observed to be at least two days, but in some forms a complete adaptation seems to require 14–20 days (Table 3). The time required for a compensatory adaptation of the oxygen consumption to an altered temperature is usually longer than the time needed for a simple resistance adaptation.

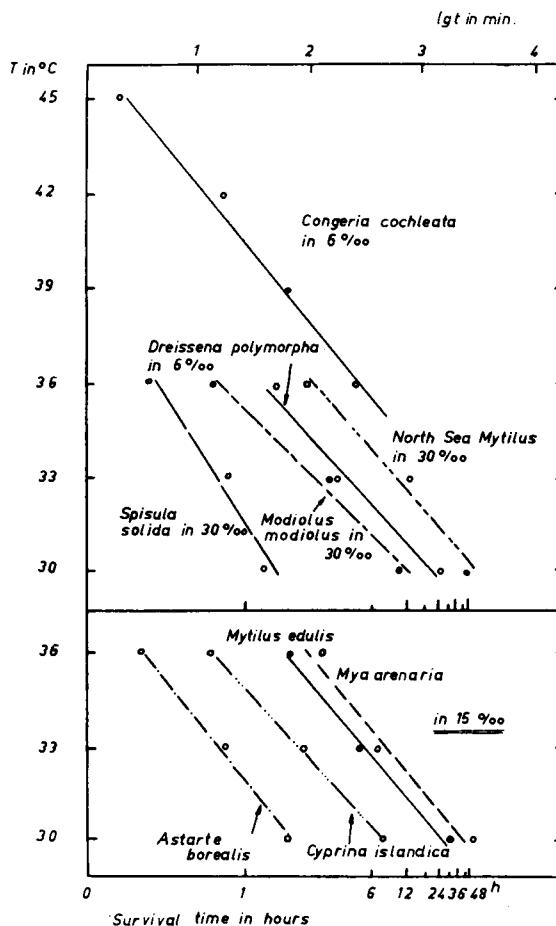


FIG. 1. Cellular thermal resistance time of isolated gill tissue from eight species of bivalves (After Reshöft, 1961).

TABLE 2. CELLULAR THERMAL RESISTANCE TIME (IN min) OF ISOLATED GILL TISSUE FROM 3 SPECIES OF BIVALVES
(After Vernberg, Schlieper and Schneider, 1963)

Species	Habitat	Experimental temp. (°C)	Resistance time (min)	
			Warm (25°C) acclimated	Cold (10°C) acclimated
<i>Crassostrea virginica</i>	Intertidal	44	118 ± 9	77 ± 3
<i>Modiolus demissus</i>	Intertidal	44	132 ± 8	68 ± 3
<i>Aequipecten irradians</i>	Sublittoral	37	92 ± 6	92 ± 4

Every factor which reduces the activity and metabolic rate of the cells (e.g. high total salinity, relatively increased calcium and magnesium concentration of the external medium) causes an increase of the cellular resistance

TABLE 3. CELLULAR THERMAL RESISTANCE OF *Modiolus demissus* GILL PIECES AS INFLUENCED BY DIFFERENT PERIODS OF THERMAL ACCLIMATION. EXPERIMENTAL DETERMINATIONS MADE AT 44°C (After Vernberg, Schlieper and Schneider, 1963)

Preacclimation time (days)	Resistance time (in min) (mean values)	
	Warm (25°C) acclimated	Cold (10°C) acclimated
3-5	134	69
14-20	155	123
23-30	98	124

against extreme non-optimum temperatures (Fig. 2). But the addition of a second stress factor (e.g. prolonged starvation, unusual low salinity, low oxygen concentration, etc.) reduces the normal cellular thermal resistance range.

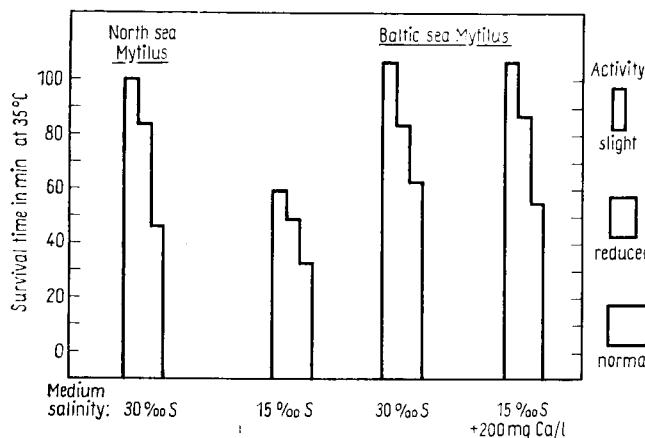


FIG. 2. Cellular thermal resistance time of isolated gill tissue from *Mytilus edulis* L. and the influence of salinity and composition of the external medium.
(After Schlieper and Kowalski, 1956.)

Finally, the photoperiod has also been shown to exert an influence on the temperature-resistance at the tissue level. This was reported in the case of rainbow trout where the tissue oxygen consumption rates tend to be highest in specimens maintained under short photoperiod (Evans *et al.*, 1962). Similarly dark adapted bivalves (*Modiolus demissus*) had a higher thermal cellular resistance than other specimens acclimated under a 14-hr light-10-hr dark period (Vernberg, Schlieper and Schneider, 1963).

(b) Salinity

The cellular osmotic resistance of bivalve species is genetically established like the cellular thermal resistance. There are similar relationships to the qualities of the habitat. We are able to differentiate between (1) stenohaline types with a small cellular osmotic resistance and adaptation range, and (2) euryhaline types with a broader cellular osmotic and adaptation range. Usually deep and open water forms are stenohaline, and shallow water estuarine forms are more euryhaline and show a greater osmotic plasticity at the cellular level (Fig. 3).

(0-30‰ S, 24 hours at 10°C)

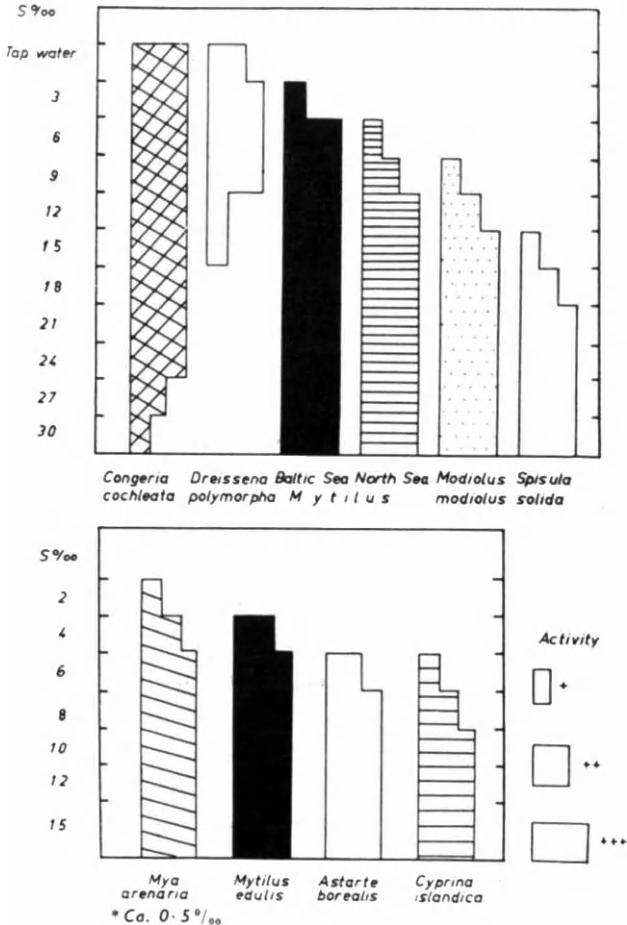


FIG. 3. Cellular osmotic resistance of surviving gill pieces after 14 hr at 10°C. (After Reshöft, 1961.) Upper part: *Congeria* and *Dreissena* from 6‰ S, Baltic Sea *Mytilus* from 15‰ S and three other species from 30‰ S. Lower part: 4 species from 15‰ S.
* Ca. 0-5‰

The cellular osmotic resistance range is largest at optimum temperatures; it is diminished at extreme, non-optimum temperatures. Therefore a cold- or deep-water species will show the greatest osmotic resistance (dilution tolerance) at low temperatures and a warm-water intertidal species will show the greatest osmotic resistance at higher temperatures (Table 4).

TABLE 4. CELLULAR OSMOTIC RESISTANCE OF SURVIVING GILL PIECES AFTER 48 hr
(After Vernberg, Schlieper and Schneider, 1963)

Salinity ‰ S	<i>Aequipecten irradians</i> (sublittoral)		<i>Crassostrea virginica</i> (intertidal)	
	Warm (25°C)	Cold (10°C)	Warm (25°C)	Cold (10°C)
33	+++	+++	+++	+++
30	+++	+++	+++	+++
27	+++	+++	+++	+++
24	+	++	+++	+++
21		++	+++	+++
18		+	+++	+++
15		+	+++	+++
12			+++	+++
9			+++	++
6			+++	+
3			++	

Activity: +++ = normal, ++ = reduced, + = slight.

The individual cellular osmotic resistance range may be extended by increasing the calcium concentration of the external medium

Salinity-correlated capacity differences are observed only with euryhalinic species. These types show their maximum cellular mechanical activity (e.g. highest ciliary frequency) at their optimum salinity range. The cellular metabolic rate (= the level of the cellular energy production) of euryhalinic species may be influenced by ionic and osmotic regulatory work.

(c) Hydrostatic Pressure

The cellular resistance of marine species to hydrostatic pressure is related to the extent of their vertical distribution or in some species also to the degree of their euryvalent abilities.

Stenobathic deep-sea species are barophilic; this means that their cells cannot exist without the high pressure of their natural habitat. Stenobathic shallow water species are barophobic, this means that their cells will soon die under increasing hydrostatic pressure. Only eurybathic species with a large vertical distribution area have an equivalently large cellular resistance range and can be studied at very different pressures without harm.

As one of the first steps in analysing the mechanism of cellular resistance to pressure we need to investigate the differences in the protoplasmic structure

and enzyme adaptation in stenobathic and eurybathic forms. The significance of phenotypic adaptation in the cellular resistance of eurybathic species to pressure can only be analysed by long-time experiments with pressure bombs which permit the maintenance of the investigated specimen in flowing sea water under constant pressure. At present we have no pressure equipment which permits such long-term experiments.

We have developed a simple method of measuring the specific cellular pressure-resistance of marine invertebrates. For this purpose surviving gill pieces of marine bivalves or other ciliated epithelia are submitted to various pressures at constant temperature for 24 hr. After the experiment the activity of ciliary beating is observed microscopically under normal pressure. In this way we can easily determine the specific values of the cellular pressure-resistance (Fig. 4). These results agree also with the zoogeographical observations of the distribution of the investigated species.

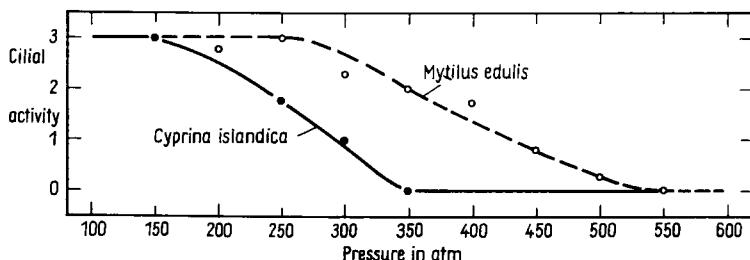


FIG. 4. Cellular pressure resistance of isolated gill tissue from *Mytilus edulis* and *Cyprina islandica* from the Baltic Sea at 5°C and 15‰ S (24 hr experiments with surviving gill pieces). (After Schlieper and Ponat, 1963b.)

Short-time experiments have shown that higher temperatures (within the optimum temperature range) cause an increase of the resistance of individual cells to pressure in relatively eurythermic species. Long-time experiments at different temperatures or prolonged preadaptation at different temperatures before raising the pressure will possibly show that the temperature influence on pressure-resistance may be negligible or may be at least of little importance.

(d) Oxygen and Carbon Dioxide Tensions

Species which are able to live or survive in water of low oxygen content have high cellular aerobic capacities. These types may also have an increased cellular resistance against exceptionally high carbon dioxide tensions.

Some of the species which survive anaerobic conditions well may also display an increased cellular resistance against hydrogen sulphide (H_2S), e.g. *Priapulus* spec.

The increased pumping and filtering rates of mussels after anoxibiotic periods are caused by stronger activity of the gill cilia. Similar cellular reaction can be demonstrated with anaerobic gill pieces after being returned to aerated sea water.

(e) Dissolved Organic Substances

The pumping rate of the filtering suspension feeders among the bivalves is regulated not only by nervous impulses but also by cellular reactions of the ciliated epithelium. Starving individuals in pure sea water and likewise isolated surviving gill pieces in pure sea water display a reduced rate of ciliary activity. But the cilial rate (ciliary frequency) of intact and of isolated surviving gill tissue—and therefore in intact animals also the pumping rate—increase immediately after adding dissolved organic food substances to the external medium. The same happens in intact animals after food intake.

The ciliary activity of isolated gill pieces (and also of intact animals) increases too by cellular reaction after the addition of stimulating products of protein breakdown (urea, amino acids, nitrates and ammonium compounds) to the external medium.

Similar cellular reactions—increased ciliary activity—can be observed after anaerobic periods in normal sea water, caused by the lactic acid produced during the previous anoxibiotic period.

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SOME PECULIARITIES IN TEMPERATURE ADAPTATIONS OF PROTOZOA AS COMPARED TO MULTICELLULAR POIKILOOTHERMS

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A NUMBER of experimental investigations on the temperature adaptation of protozoa have been carried out in the Laboratory of Cytology of Protozoa for the last 5-7 years (Poljansky, 1957, 1959, 1963 a, b; Sukhanova, 1959, 1961, 1962 a, b, 1963; Irlina, 1960, 1963 a, b, c). These studies have shown a wide adaptability of free-living and parasitic protozoa to various temperature conditions of the environment.

Among free-living protozoa the temperature adaptations of *Paramecium caudatum* have been studied most thoroughly (Poljansky and Orlova, 1948; Poljansky, 1957, 1959, 1963 a, b). The thermoresistance of *Paramecium* was measured by the death rate at high lethal temperatures (38, 40, 42°C), and this served as a criterion for the adaptation of the infusoria to various environmental temperatures. The experiments on thermoresistance were carried out on clone material cultivated at various temperatures from 0 to 28°C in sterile equilibrated Losina-Losinsky salt solution.

TABLE 1. THE DURATION OF *P. caudatum* SURVIVAL (THE AVERAGE TIME IN min) AT 40°C AFTER PRELIMINARY CULTIVATION AT VARIOUS TEMPERATURES (Poljansky, 1957)

Clone	12-13°C	18-20°C	24-28°C
A'	6.7 ± 0.16	13.9 ± 0.50	35.4 ± 0.86
	9.6 ± 0.20	17.6 ± 0.42	34.7 ± 0.30
B'	10.6 ± 0.30	19.1 ± 0.30	42.4 ± 0.77
	6.1 ± 0.05	19.3 ± 0.70	31.6 ± 0.69
D'	10.9 ± 0.10	Not measured	35.7 ± 0.30
	12.5 ± 0.10	23.3 ± 0.13	36.9 ± 0.20

Data on thermoresistance of *P. caudatum* cultivated at various temperatures are shown in Table 1. The table shows that the differences in thermoresistance among the infusoria of the same clone are very considerable. These differences depend on the temperature regime. The difference in the average survival

time shows also that the cultivation of *P. caudatum* at relatively high temperatures leads to an increase of their thermoresistance in comparison with the control kept at room temperature (18°C). By contrast, cultivation at a relatively low temperature considerably reduces the resistance to the effect of the high lethal temperature. Hence, the thermoresistance of *P. caudatum* is under the direct influence of the environmental temperature.

The same dependence of thermoresistance on the temperature conditions of the environment has been found in several other species of free-living infusoria: *Spirostomum minus* Roux, *S. ambiguum* Ehrbg., *Tetrahymena pyriformis* (Ehrbg.), *Oxytricha minor* Kahl (Irlina, 1960). Similar dependence of thermoresistance on the temperature regime was revealed in endoparasitic protozoa of amphibians: *Opalina ranarum* Ehrbg., *Nyctotherus cordiformis* (Ehrbg.), *Balantidium elongatum* Stein, *B. duodenii* Stein, *B. entozoon* Ehrbg., and others (Sukhanova 1959, 1962, 1963). The experiments on thermoresistance were performed on natural populations of endoparasitic protozoa taken from the intestine of amphibians and placed in Ringer's solution.

The study of thermoresistance of the intestinal infusoria and opalinids has demonstrated that one and the same species of the protozoa shows different resistance to high temperatures (lethal temperatures 36, 38, 40°C) in different

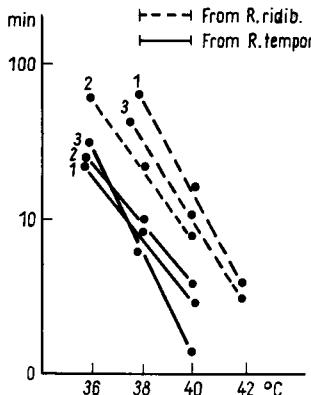


FIG. 1. The dependence of survival time (in min) of 3 *Balantidium* species on the lethal temperatures. 1—*B. elongatum*, 2—*B. entozoon*, 3—*B. duodenii* (Sukhanova, 1962).

species of its host. The difference in thermoresistance of the protozoa depends on the temperature conditions of their hosts. The most striking differences in thermoresistance were observed in protozoa from *Rana temporaria* L. and *Rana ridibunda* Pall. Figure 1 illustrates the thermoresistance of 3 *Balantidium* species. These infusoria are parasites of both *R. temporaria* and *R. ridibunda*. The curves show that all the species of *Balantidium* from *R. ridibunda* possess greater thermoresistance than those from *R. temporaria*. *R. ridibunda* is a sou-

thern species of amphibian and it has been adapted to comparatively high environmental temperatures. On the other hand, *R. temporaria* is a northern species and it has been adapted to comparatively low temperatures (Bannikov, 1943). In more thermophilic southern amphibians the same parasitic protozoa show greater thermoresistance than those from northern species. *R. ridibunda* and *R. temporaria* also exhibit different thermoresistance of their muscle and tissue proteins (Ushakov, 1956, 1958, 1959; Braun, Nesvetayeva, Fizhenko, 1959).

By contrast, the same species of endoparasitic protozoa show similar thermoresistance if they inhabit different species of amphibians which live under similar temperature conditions and show similar biological properties. For example, the same thermoresistance has been shown in the opalinids and the intestinal infusoria from *R. ridibunda* and *R. esculenta* L. as well as in *Balantidium elongatum* from *Triturus vulgaris* L. and *T. cristatus* (Laur.).

Under natural environmental conditions the thermoresistance of endoparasitic protozoa from amphibians exhibits a well-defined seasonal character and is in accord with seasonal changes of temperature. The data of these observations are shown in Table 2. As shown in the table, all the species of protozoa possess low thermoresistance in winter when the temperature in frog hibernation areas does not exceed +4°C. In summer the thermoresistance of the protozoa increases according to the environmental temperature. The same effect is obtained if both frogs and *in vitro* cultures of opalina are kept at low (+4°C) and at high (20–25°C) temperatures under laboratory conditions.

TABLE 2. THE AVERAGE SURVIVAL TIME OF OPALINIDS AND INFUSORIA (IN min) FROM *R. temporaria* AT 38°C DURING VARIOUS SEASONS (Sukhanova, 1962b)

Protozoa species	July	February
<i>Opalina ranarum</i>	17.74 ± 0.24 18.96 ± 0.17 22.00 ± 0.08	6.32 ± 0.08 7.56 ± 0.17 11.49 ± 0.08
<i>Nyctotherus cordiformis</i>	17.05 ± 0.58 22.08 ± 0.30 25.32 ± 0.13	6.79 ± 0.01 7.75 ± 0.03 9.80 ± 0.19
<i>Balantidium duodenii</i>	6.06 ± 0.03 6.35 ± 0.46 7.00 ± 0.03	Death in 1–3 min

When the environmental temperature is changed, the changes of thermoresistance of free-living and endoparasitic protozoa proceed very quickly, sometimes within several hours, and have a phasic character (Poljansky, 1957).

The problem of prolonged cultivation of protozoa at 0°C and the influence of such a low temperature on their thermoresistance was the subject of a

special investigation (Poljansky, 1959). The experiments were carried out on a clone of *P. caudatum*. The data obtained show that the cold-resistance of infusoria as well as their resistance to high lethal temperatures depends on the preliminary temperature regime. The temperature of 0°C appeared to be lethal for ciliates cultivated at 25–28°C, but, when the cultures of *P. caudatum* were kept at 5–6°C for about one and a half months and then transferred to 0°C, the infusoria did not demonstrate any pathological changes and were easily adapted to 0°C. It is possible to cultivate these ciliates at 0°C for several years if they are gradually adapted to the low temperatures. The infusoria cultivated at 0°C for a long time have a very low thermoresistance and 29°C is the lethal temperature for them. These experiments helped to account for the differences of opinions on this problem previously met in the literature.

In addition, these experiments which showed the possibility of adaptations of infusoria to 0°C led to another important and interesting question of the survival of protozoa under supercooling conditions below 0°C (Poljansky, 1963). The survival of infusoria under supercooling conditions had been little studied (Wolfson, 1935; Losina-Losinsky, 1948). The problem of the dependence of protozoan survival in supercooling on a preliminary temperature regime had not been studied at all.

TABLE 3. THE SURVIVAL OF THE "COLD" CLONE OF *P. caudatum* UNDER SUPERCOOLING TO -10 AND -15°C. THE FIGURES SHOW THE QUANTITY OF LIVING INFUSORIA IN PER CENT (Poljansky, 1963a)

	Duration of supercooling (min)						
	30	60	90	120	150-180	210-240	300
-10°	100	100	-	57·1	95·6	15·6	0
-15°	81·6	52·4	9·9	-	14·8	0	0

The object for these experiments was a clone of *P. caudatum* cultivated at 0, 4–5, 14–15, and 28–29°C. The supercooling of infusoria was carried out in glass capillary tubes. The data from the experiments show that the ciliates cultivated at 28–29°C (the "warm lines") show a very low cold-resistance. If they are supercooled to -3, -5°C very quickly, they immediately die (within 4–5 min). If they are supercooled to the same temperature slowly, they die within 15–20 min. At -10, -15°C the infusoria of the "warm lines" usually die in 1–2 min.

P. caudatum of the same clone cultivated at 4–5°C (the "cold lines") show much higher resistance in comparison with the "warm lines" (Table 3). The infusoria of the "cold lines" continue to live at -5°C for 7 hr, at -10°C for 4 hr, and at -15°C for 3 hr. The cold-resistance of the "cold lines" is more than 50–100 times as high as that of the "warm lines". Hence, the cold-resistance of *P. caudatum* depends on the preliminary temperature conditions and varies considerably as does the thermoresistance.

A comparative study of changes in thermostability of protozoa to the influence of lethal temperatures suggests that alterations of protoplasmic proteins might form the basis of cellular adaptations to high and low temperatures. The high value of the temperature coefficient or Q_{10} confirms this suggestion. The temperature coefficient of the lethal temperature effect on *P. caudatum* is very high and ranges from 60 to 100 (Poljansky, 1957). Q_{10} of *Opalina ranarum* is from 65 to 100 (Sukhanova, 1959). The protozoa with higher thermostability show higher value of Q_{10} as compared to the protozoa adapted to low temperatures.

The change of thermostability of protozoa is accompanied by a change in their resistance to the action of some other agents, for instance, ethanol (Alexandrov, 1963; Poljansky, 1957; Sopina, 1963; Irlina, 1963c). The action of ethanol on the living cells is closely connected with the processes of protein denaturation. Along with the rise of thermostability of protozoa, an increase in resistance to ethanol can be observed.

The alterations in the cell proteins are associated with considerable shifts in the cell metabolism. First of all, these alterations concern the enzymatic system of the cell as was shown in the experiments with *P. caudatum* (Irlina, 1963a). Metabolic changes can be detected by means of inhibitors of respiration and glycolysis. The adaptation of *P. caudatum* to 28–29°C results in predominance of respiration carried out by means of the cytochrome oxidase system. The ciliates cultivated at 14°C exhibited high sensitivity to monooiodoacetate which indicates a significant metabolic role played by glycolysis. A high sensitivity of this infusorian to malonate and high succinic dehydrogenase activity coexists with resistance to cyanide. The resistance of "cold" ciliates living at 4°C to respiratory inhibitors, as well as their weak succinic dehydrogenase activity indicates a relatively small role of respiration in the energy metabolism. Aerobic glycolysis appears to be the main source of energy in the "cold" ciliates. These experiments have demonstrated that enzymatic effects might be a physiological basis of temperature adaptations.

The protozoa cultivated at low and high temperatures show differing reactions to salt solutions (Irlina, 1963b). The measurements of the survival time for *P. caudatum* in different salines revealed the dependence of resistance of ciliates on cultivation temperature and salt concentration. The cultivation of *P. caudatum* for 1·5–2 months at 29°C results in a considerable decrease of their resistance to threshold concentration of calcium chloride and potassium chloride. The ciliates cultivated for the same period of time at 4°C survive in these solutions longer than the "warm" infusoria. The resistance in control *P. caudatum* living at 14°C is higher than in the "warm", but lower than in the "cold" infusoria.

The relationship are quite different in the case of concentrations of calcium chloride and potassium chloride well above lethal levels; the survival time of the "warm" infusoria in high concentration of calcium chloride and

potassium chloride is long, whereas the control and the "cold" ones survive for a shorter time.

Shifts in the metabolism which occur in the course of adaptations of infusoria and opalinids to different temperatures result in quantitative changes of the polysaccharide and neutral lipid stores in the cytoplasm (Kovaleva, 1962; Poljansky, 1963; Sukhanova, 1963). *P. caudatum* and *O. ranarum* cultivated at 4°C usually have a great amount of glycogen and neutral fat. The same species of protozoa living at room temperature possess a smaller quantity of glycogen and neutral fat. The protozoa cultivated at 28–29°C have a very small amount of glycogen and neutral fat.

The change of the temperature regime exerts an influence on the mitochondria of protozoa, as shown by the electron microscope investigation of *P. caudatum* (Wolfarth-Botterman, 1958; Mashansky, 1961). At a high temperature the mitochondria increase their size and lose their normal structure. When the infusoria are returned to room temperature, the mitochondria return to normal size and structure. Hence, the swelling of mitochondria is a reversible phenomenon. The swelling of mitochondria at a high temperature can also be seen in *Opalina ranarum* (Sukhanova, 1963).

All these data show the complexity of the process of adaptations of free-living and endoparasitic protozoa to environmental temperature. Protozoa, animals at the cellular level of organization, possess an adaptability to various temperature conditions. A vast majority of free-living and parasitic protozoa occur in the range of temperate temperatures. However, some species of sarcodina, flagellates and infusoria live in hot springs at 40–54°C. The endoparasitic protozoa of homeotherms are also adapted to comparatively high temperatures, the body temperature of their hosts.

A wide adaptability of protozoa to other environmental factors, such as salinity, hydrogen ion concentration, gas regime, etc. was shown by some authors. For example, the thermoresistance of *P. caudatum* depends on the concentration of calcium ions and other substances in solution. The wide individual adaptability of unicellular organisms to changing environmental conditions is due to cellular mechanisms responsible for changes in cytoplasmic proteins and the type of cell metabolism.

The role of cellular mechanisms in adaptations of most multicellular poikilotherms to various temperature conditions is different from that of protozoa. The experimental evidence obtained during the last few years (Alexandrov, 1952; Ushakov, 1956, 1958, 1959; Zhirmunsky, 1958, 1959; Dzamurova, 1960; Kusakina, 1960 and others) suggests that cellular thermoresistance may serve as a cytophysiological criterion for most metazoan species. In the process of their individual adaptation to different temperatures, multicellular animals, unlike protozoa, exhibit not so much the cellular changes but alterations at other levels of organization. Cellular thermoresistance of different species of multicellular poikilotherms is different and depends on ecological conditions of the species. Thus, the cellular adaptation is seldom realized

in the process of individual adaptation of the organism, but it plays an important part in the process of adaptive evolution.

Cellular thermoresistance of some multicellular poikilotherms may undergo regular changes connected with the phases of the reproductive cycle (Schlachter, 1961; Pashkova, 1962—for amphibians; Dregolskaya, 1962 and others—for molluscs and actinia). The dependence of thermoresistance on the phases of the hormonal cycle of amphibians was found in *Opalina ranarum* from the intestine of *Rana temporaria* (Sukhanova, 1963).

In some instances, it was found that the cellular level of organization also participates in the process of individual adaptations of multicellular poikilothermal animals, and that changes in the thermoresistance of cell proteins are brought about in the same way as those in protozoa (see Dregolskaya, 1963—for hydras; Ushakov, 1959; Gorodilov, 1961—for polychaetes). As a matter of fact, the same mechanisms may be involved in the process of individual adaptation of some multicellular animals to environmental temperature.

It may be suggested that certain changes in the mechanisms of temperature adaptations appear to have occurred in the process of evolution. Originally these mechanisms were intracellular and were associated with the changes in cell proteins. In the process of the formation of multicellular organization, thermoresistance of cells and that of their proteins became relatively stabilized and the function of individual adaptability was transferred to other levels of organization (the organ level, organismal and biocenotical levels). However, a physiologically more ancient mechanism of adaptation at the cellular level is partly kept and in some cases it may play a significant role in metazoan adaptation as well. It may be suggested, accordingly, that cellular mechanisms of adaptations must be realized in the cases when the habitat of species is variable, and when higher levels of organization are not enough to correlate functions of an organism with changing environmental conditions.

Further investigations in this field seem to be most promising.

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A COMPARATIVE STUDY OF CELLULAR THERMOSTABILITY OF MARINE INVERTEBRATES IN RELATION TO THEIR GEOGRAPHICAL DISTRIBUTION AND ECOLOGY

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THE investigations of cell thermostability of multicellular poikilothermic animals, which have been carried out by Ushakov and his collaborators on a vast amount of comparative experimental material, have shown that thermostability of homologous cells of the individuals of one species is rather conservative. As a rule, it does not depend on environmental temperature conditions of separate individual populations from which the investigated animals were taken. At the same time, cell thermostability of different taxonomically closely related species, is different and is in conformity with the environmental temperature conditions of these species. The differences in cell thermostability were found to be related to the peculiarities in the protoplasmic protein structure of these species. Hence, in poikilothermic animals there is a species adaptability of protoplasmic protein complexes to environmental temperature conditions. This enabled Ushakov (1955) to suggest cell thermostability as a cytophysiological criterion of species for multicellular poikilothermic animals.

In the present paper the experimental material on cell thermostability of marine invertebrates obtained by the workers of the Laboratory of Comparative Cytology is compared with data on geographical distribution and ecology of the investigated species. Cell thermostability of 122 species of invertebrates belonging to 7 phyla and 12 classes was studied by the method of Ushakov (Ushakov, 1959). Representatives of these species were collected in the Barents, Bering, Okhotsk, Japan, Yellow, South China and Black Seas.

Cell thermostability was evaluated from thermostability curves. To build these curves, semi-logarithmic graphs are used as a rule, the abscissa indicating the temperature (in °C), the ordinate showing the survival time of ciliated epithelium or the retention time of muscle excitability (in min, logarithmic scale). Each point on the graph represents the arithmetic mean of

5–7 experiments. The curve of cell thermostability thus built consists of two segments with different slopes.

By examination of the cell thermostability curve, Ushakov and Gasteva (1953) drew the conclusion that the right-hand part of the curve which is characterized by a high temperature coefficient, Q_{10} , represents a protoplasmic response to the injurious temperature effect and can serve as an index of cell thermostability. Therefore, in order to compare cell thermostability of different animal populations or species it is sufficient to examine data for the right-hand segment of the curve only.

On comparing cell thermostability in different populations or species it is necessary to exclude its seasonal fluctuations and to take into consideration its lability in some species of *Coelenterata* and possibly *Vermes* (see Ushakov, 1963). For sea animals the effect of salinity on cell thermostability has to be considered (Schlieper and Kowalsky, 1956; Dregolskaya, 1961; Ivleva, 1962; the author's experimental data). Therefore, the experiments, as a rule, were performed at the same season, and cell thermostability was determined separately in animals from seas of normal (30–34 per mille) and of lower salinity (17–18 per mille, the Black Sea).

Cell Thermostability in Animals of the same Species

The results of the experiments show that marine invertebrates, like terrestrial poikilotherms, are characterized by a certain species specificity of their

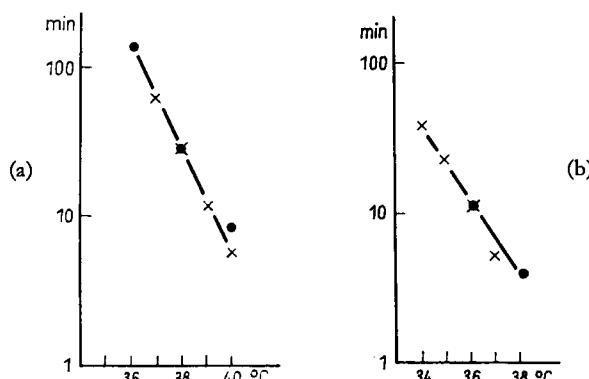


FIG. 1. Thermostability curves of ciliated epithelium cells of the actinians *Anthopleura xanthogramica* (Berkely) (a) and foot muscles of the bivalve mollusc *Prototaca staminea* (Conrad) (b) from different regions. In Fig. 1a: points—actinians from Putyatin Island (Sea of Japan), experiments of T. A. Schlachter; crosses—from Ptichy Island (Okhotsk Sea), experiments of A. V. Zhirmunsky. In Fig. 1b: points—molluscs from Putyatin Island, experiments of T. A. Dzhamusova; crosses—from Ptichy Island, experiments of A. V. Zhirmunsky. The abscissa gives the sea-water temperature (in °C), the ordinate, the survival time of cells (in min, logarithmic scale).

cell thermostability. This was confirmed first of all by the data indicating a considerable conservatism of the cell thermostability in animals of one species. Thus cell thermostability in different populations gathered either in different or in the same seas, but at various depths, proved to be identical. Such data were obtained for 14 species of echinoderms, molluscs and coelenterates (Ushakov, 1959; Schlachter, 1959; Dzhamusova, 1960; Dregolskaya and Altukhov, 1960; Zhirmunsky and Pisareva, 1960; the author's data).

Figure 1a represents the results of experiments performed on two populations of the littoral actinian *Anthopleura xanthogramica* from the Sea of Japan (latitude 43° N) and the Okhotsk Sea (latitude 57° N). As can be seen from the graph, despite considerable differences in the habitats of these actinians, the cell thermostability of their ciliated epithelium is the same. Similar results were obtained from experiments with foot muscles of two subspecies of the bivalve mollusc *Prototaca staminea* (Fig. 1b) collected in the intertidal zone of South Sakhalin and Komandorsky Islands.

Cell Thermostability of Taxonomically Close Species

A series of investigations demonstrated a relationship between cell thermostability of different but taxonomically close species and environmental temperature conditions where they occur. Relationships were found between species differing in their latitudinal area boundaries (latitudinal zonality), as well as species living at different depths (vertical zonality).

Species of a more southern area show as a rule a higher level of cell thermostability as compared with taxonomically close species living relatively more to the north. Similar relationships were detected for 57 species belonging to 18 genera (Ushakov, 1956, 1959; Schlachter, 1959; Dzhamusova, 1960; Zhirmunsky, 1960, recent data; Andronikov, 1963). Thus, there is a parallelism between cell thermostability of marine invertebrates and latitudinal distribution of the species.

Species living in the intertidal zone were found to show higher cell thermostability than taxonomically related sublittoral species. In addition, animals inhabiting the upper part of the intertidal zone exhibit higher cell thermostability compared with animals living in the same beach, but in the lower part of the intertidal zone, for example sympatric molluscan species belonging to the genera *Nerita* and *Donax* from the South-China Sea (Zhirmunsky and Chu Li-chun, 1960, 1963).

The same relationship was established for the animals inhabiting different depths of the sublittoral, namely for two sea mussel species—*Mytilis crassitesta* and *Mytilus grayanus* and also for two ascidian species—*Tethyum aurantium* and *Tethyum roretzji* (Zhirmunsky, 1963). Thus a parallel was found between the cell thermostability of marine invertebrates and their vertical distribution in the sea.

The results of experiments with two species of the gastropod *Acmaea* can be presented as an example showing the relationship between the cell thermostability of species and their latitudinal and vertical distribution in the sea. Figure 2 shows that the cell thermostability in *Acmaea cassis* (2) is considerably

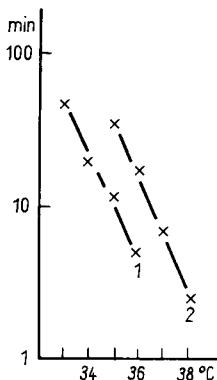


FIG. 2. Thermostability curves of ciliated epithelium cells of the bivalve molluscs *Acmeae scutum* Esch. (1) and *A. cassis* Esch. (2) collected on Ptichy Island (Okhotsk Sea). Designations on the axes the same as in Fig. 1.

higher than in *Acmaea scutum* (1). This is in conformity with the geographical distribution of the species. According to Golikov and Kusakin (1962) *A. cassis* is characterized by a wider geographic range and reaches more southern regions than *A. scutum*. In Japan, for example, *A. cassis* can be found as far as the north of the Chonsju Island, while *A. scutum* extends only up to Chokaido Island.

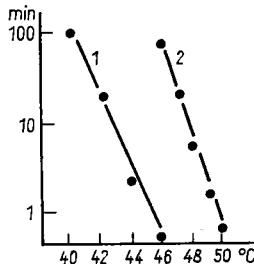


FIG. 3. Thermostability curves of ciliated epithelium cells of the bivalve molluscs *Modiolus philippinarum* Hanley (1) from the lower horizon of the intertidal zone (South China Sea) and *M. atra* (2) from the upper horizon (Yellow Sea) (Zhirmunsky, 1960). Designations on the axes the same as in Fig. 1.

As I observed on Ptichy Island in the Okhotsk Sea, *A. cassis* of the same habitat dwell in the higher part of the littoral as compared to *A. scutum*. Thus there is a parallel between the cell thermostability and the environmental temperature conditions of these species.

The data obtained shows that in some instance the vertical disposition of a species in the sea is more significant than its latitudinal distribution. Thus *Modiolus atrata* living in the littoral of a more northern area show higher cell thermostability than *Modiolus philippinarum*, a tropical species from the sub-littoral (Fig. 3; Zhirmunsky, 1960). Such discrepancies in the relationship between cell thermostability and latitudinal distribution of species are in accordance with zoogeographical evidence suggesting in some cases the prevalence of the vertical zonation law over the latitudinal zonation law (Shelford, 1911; Guryanova, 1959). As in the previous case, distinctions in cell thermostability of various species are related to the environmental temperature.

Cell Thermostability of Taxonomically Remote Species

The vast experimental material obtained on numerous marine invertebrates belonging to different taxonomic groups and inhabiting different latitudes—from 18 to 70° N—enabled us to make a broad comparison between the cell thermostability of the animals and their geographical distribution. This comparison was made separately for different tissues (ciliated epithelium, muscles, spermatozoa). Phylogenetic relations and vertical distribution of animals in the sea were taken into consideration.

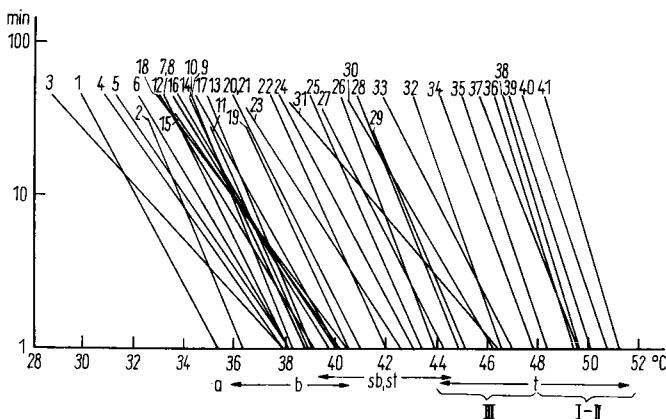


FIG. 4. Thermostability curves of ciliated epithelium cells of 41 animal species from the intertidal zone. List of species is given in Table 2. a—arctic species; b—boreal species; sb, st—south-boreal and subtropical species; t—tropical species; III—lower horizon; I-II—upper and middle horizons of the intertidal zone. Designations on the axes the same as in Fig. 1.

Thus the extreme values of cell thermostability differ by 16° for the ciliated epithelium of 41 species of intertidal animals (Fig. 4, Table 1). The temperature range causing thermonarcosis in 1 min (t_1) is 44–51°C for all the investigated tropical invertebrates regardless of their taxonomic position; for

TABLE 1. ZOOGEOGRAPHICAL POSITION OF INTERTIDAL AND FREQUENTLY OCCURRING
THEIR CILIATED
(a—arctic species, b—boreal species, sb—south-boreal)

Number of the curve in Fig. 4	Species	Class	Zoo- geographical position
1	2	3	4
1	<i>Saxicava arctica</i> (L.)	Bivalvia	a
2	<i>Prorotaca staminea</i> (Conrad)	Bivalvia	b
3	<i>Mya arenaria</i> L.	Bivalvia	b
4	<i>Leptasterias ochotensis</i> (Brandt)	Asteroidea	b
5	<i>Easterias retifera</i> f. <i>tabulata</i> Djakonov	Asteroidea	b
6	<i>Bunodactis stellata</i> Verill	Anthozoa	b
7	<i>Acmea scutum</i> Esch.	Gastropoda	b
8	<i>Modiolus modiolus</i> (L.)	Bivalvia	b
9	<i>Natica janthostoma</i> Deshayes	Gastropoda	b
10	<i>Cnidopus japonica</i> Verill	Anthozoa	b
11	<i>Metridium senile</i> (L.)	Anthozoa	b
12	<i>Lysastrosoma anthosticta</i> Fisher	Asteroidea	sb
13	<i>Acmea cassis</i> Esch.	Gastropoda	b
14	<i>Asterias rollestoni</i> Bell	Asteroidea	sb
15	<i>Asterias amurensis</i> Lütken	Asteroidea	sb
16	<i>Asterias rathbuni</i> ssp. <i>crassispinis</i> Djakonov	Asteroidea	b
17	<i>Nucella lima</i> (Martyn)	Gastropoda	b
18	<i>Buccinum percrassum</i> Dall	Gastropoda	b
19	<i>Mytilus edulis</i> L.	Bivalvia	b
20	<i>Patiria pectinifera</i> (Müller et Troschel)	Asteroidea	sb
21	<i>Nucella elongata</i> (Golikov et Kussakin)	Gastropoda	sb
22	<i>Anthopleura xanthogrammica</i> (Berkely)	Anthozoa	b
23	<i>Neptunea artritica</i> (Bernardt)	Gastropoda	sb
24	<i>Mya japonica</i> Jay	Bivalvia	st
25	<i>Venerupis variegatus</i> (Sowerby)	Bivalvia	st
26	<i>Venerupis japonica</i> Deshayes	Bivalvia	st
27	<i>Tridacna crocea</i> L.	Bivalvia	t
28	<i>Modiolus medcalfei</i> Hanley	Bivalvia	st
29	<i>Modiolus philippinarum</i> Hanley	Bivalvia	t
30	<i>Placenta sella</i> Gmelin	Bivalvia	t
31	<i>Lingula shantungensis</i> Hata	Brachiopoda	t
32	<i>Donax semigranosus</i> Dunker	Bivalvia	t
33	<i>Placenta placuna</i> L.	Bivalvia	t
34	<i>Donax dysoni</i> Deshayes	Bivalvia	t
35	<i>Donax cuneatus</i> L.	Bivalvia	t
36	<i>Modiolus atrata</i> (Lischke)	Bivalvia	st
37	<i>Donax faba</i> Gmelin	Bivalvia	t
38	<i>Nerita albicilla</i> L.	Gastropoda	t
39	<i>Nerita planospira</i> Anton	Gastropoda	t
40	<i>Nerita seabricosta</i> Lam.	Gastropoda	t
41	<i>Nerita plicata</i> L.	Gastropoda	t

IN THE INTERTIDAL ZONE SPECIES OF INVERTEBRATES AND THERMOSTABILITY OF EPITHELIUM CELLS

species, st—subtropical species, t—tropical species)

Place (sea) and time of collecting	t_1 (in °C)	t_{10} (in °C)	Names of the investigators referred to
	5	6	7
			8
Bering, 1962	35·3	32·0	
Bering, 1962	36·4	33·8	The author's experiments
Okhotsk, 1962	37·8	32·3	
Okhotsk, 1962	37·9	33·6	Experiments of Andronikov
Okhotsk, 1957	38·1	34·0	Schlachter (1959)
Barents, 1958	38·1	34·5	Zhirmunsky (1960)
Okhotsk, 1962	38·1	35·1	
Bering, 1962	38·2	35·1	The author's experiments
Okhotsk, 1963	38·2	35·8	
Japan, 1957	38·8	36·0	Zhirmunsky and Pisareva (1960)
Okhotsk, 1962	38·9	36·2	The author's experiments
Japan, 1957	39·1	35·4	Schlachter (1959)
Okhotsk, 1962	39·1	36·5	The author's experiments
Yellow, 1960	39·7	36·1	Experiments of Zhirmunsky, Sha Sue-shun and Sui Chen-dun
Japan, 1957	40·0	35·8	Schlachter (1959)
Okhotsk, 1962	40·0	36·0	
Okhotsk, 1962	40·0	36·6	Experiments of Andronikov
Okhotsk, 1962	40·4	35·9	
Okhotsk, 1957	40·5	37·7	Zhirmunsky and Pisareva (1960)
Japan, 1957	41·0	37·9	Schlachter (1959)
Japan, 1963	41·0	38·0	The author's experiments
Okhotsk, 1962	42·0	39·2	Dregolskaya (1963)
Japan, 1963	42·5	38·6	The author's experiments
Okhotsk, 1963	42·5	39·8	
Yellow, 1960	43·4	40·7	Zhirmunsky (1960)
Okhotsk, 1963	43·9	41·6	Experiments of Dregolskaya
South China, 1959	44·3	41·2	Zhirmunsky (1960)
Yellow, 1960	44·9	42·4	The author's experiments
South China, 1959	45·1	42·5	Zhirmunsky (1960)
South China, 1959	46·2	42·6	The author's experiments
Yellow, 1960	46·4	41·2	
South China, 1959	46·5	44·4	Zhirmunsky and Chu Li-chun (1963)
South China, 1959	47·0	43·9	Zhirmunsky (1960)
South China, 1959	47·9	45·6	Zhirmunsky and Chu Li-chun (1963)
South China, 1959	48·4	46·3	
Yellow, 1960	49·5	47·5	Zhirmunsky (1960)
South China, 1959	49·6	47·2	Zhirmunsky and Chu Li-chun (1963)
South China, 1959	49·7	47·8	
South China, 1959	50·1	48·1	Zhirmunsky and Chu Li-chun (1960)
South China, 1959	50·8	48·7	
South China, 1959	51·3	49·4	

subtropical and south boreal animals it is between 44 and 40°C, while for the boreal species it lies within 40–36°C. For the only arctic species of this group t_1 is 35·3°C. Similar data were obtained for the ciliated epithelium of sublittoral invertebrates, muscles and spermatozoa of intertidal and sublittoral invertebrates.

A more detailed examination of a series of thermostability curves for the ciliated epithelium of tropical intertidal invertebrates enabled us to divide the temperature zones mentioned above into subzones according to species distribution in the intertidal zone. In this case, for dwellers of the upper and middle horizons of the intertidal zone values of t_1 range within 51–47°C, while for low horizons and for the upper sublittoral t_1 lies between 47 and 44°C. For some species t_1 extends beyond the above-mentioned limits; this can be related to the ecological peculiarities of the animals (their living on different substrates, crawling under stones and in the holes, etc.).

Cell Thermostability of the Species as a Characteristic of Biocoenoses

Cell thermostability was also compared in different species typical of the same and neighbouring biocoenoses. The comparison was made on the basis of the material obtained from studies of communities from rocky soils of the Posyet Bay in the Sea of Japan. The results of this work are given in Table 2. The first column gives depths corresponding to a definite biocoenosis; the second one lists species typical of the biocoenosis; the fourth column gives the values of t_1 . Animals were grouped in biocoenoses on the basis of submarine observations made with the aid of an aqualung by the author in collaboration with research workers A. N. Golikov and O. A. Scarlato of the Zoological Institute of the Academy of Sciences of the U.S.S.R.

As can be seen from the table, cell thermostability of species of one and the same biocoenosis is similar. But it is different in species belonging to biocoenoses which inhabit different depths. Thus the t_1 for *M. crassitesta* and *Pterorystis burnetti* typical of the sublittoral with moderate degree of surf ranging from 0 to 2 m in both cases is 42·9°C. For the biocoenosis inhabiting the range between 2–4 m with *Arca boucardi* and *M. grayanus* as leading species and *Chlamys fareri* as a characteristic one, t_1 ranges between 40·1–40·5°C. For *Modiolus difficilis* and *T. roretzji* living at the depths of 3–7 m the t_1 is 39·2 and 39·4°C, respectively.

All these data show that taxonomically remote animals living under similar environmental temperature conditions exhibit similar levels of cell thermostability and that the biocoenoses can be characterized by the cell thermostability of common species.

Conclusion

Thus the above comparison enables us to conclude that cell thermostability of marine invertebrates is related to latitudinal distribution of species, depths

of their distribution in the sea and peculiarities of their ecology. It does not depend on the phylogenetic position of a species but is determined by its environmental temperature conditions.

The comparison of this conclusion with the data speaking for a considerable conservatism of cell thermostability give us reasons to suggest that cell thermostability is related not only to present environmental temperature conditions of a species, but that it also shows temperature conditions in which the species lived in past geological periods.

This is confirmed by the results obtained from comparison of cell thermostability of some living species with the data on their environmental tempera-

TABLE 2. CELL THERMOSTABILITY OF THE CILIATED EPITHELIUM IN DIFFERENT SPECIES OF INVERTEBRATES FROM THE POSYET BAY IN THE SEA OF JAPAN INHABITING VARIOUS DEPTHS

Depths (m)	Species	Class	$t_1 \dagger$ (°C)
0-2	<i>Mytilus crassitesta</i> Lischke	Bivalvia	42·9
	<i>Ceratostoma burnetti</i> (Adams et Reeve)	Gastropoda	42·9
2-4	<i>Arca boucardi</i> Jousseaume	Bivalvia	40·5
	<i>Chlamys fareri</i> Kuroda	Bivalvia	40·2
	<i>Mytilus grayanus</i> Dunker	Bivalvia	40·1
3-7	<i>Tethym roretzi</i> Drash	Ascidiae	39·4
	<i>Modiolus difficilis</i> (Kuroda et Habe)	Bivalvia	39·2

$\dagger t_1$ = temperature for narcosis in 1 min.

ture conditions at present and in past geological periods. For example, the oyster *Ostrea gigas* showing high cell thermostability (according to the author's data t_1 for its ciliated epithelium is 47°C) was found in the Miocene deposits in the North of Siberia where at the present time the climate is very severe. However, according to the paleontological evidence (Dall, 1893), during the Miocene the climate of this region was subtropical.

Hence, a study of cell thermostability in different specimens of the fauna inhabiting a given region combined with paleontological and zoogeographical investigations will help to analyse the temperature conditions under which the fauna were formed.

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THERMOSTABILITY OF MUSCLE TISSUE OF MOLLUSCS AS A CYTOPHYSIOLOGICAL CHARACTERISTIC OF A SPECIES

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ON STUDYING species specificity of cell thermostability of poikilothermal animals and its relation to intracellular proteins Ushakov (1958, 1959a, 1959b) suggested the use of cell thermostability as a cytophysiological species criterion. In the present investigation this index was used to characterize some molluscan species.

Cell thermostability can serve as a specific characteristic only in cases where it is identical in representatives of the same species and different in specimens of taxonomically close species. Therefore, we investigated muscle thermostability both in different populations of the same species and in taxonomically related species (species of the same genus).

Experiments were conducted on marine, fresh water and terrestrial molluscs; a total of 85 populations of 65 species have been investigated (Dzhamusova, 1960a, 1960b, 1960c, 1962, 1963; Dzhamusova and Shapiro, 1960). Muscle thermostability was measured by the time of the irreversible loss of muscle excitability to an induction current at high temperatures. For each given temperature a graph was made of the mean values of the retention time of muscle excitability. The abscissa gives the temperature in °C, the ordinate gives the logarithm of the retention time. The relation of the time of disappearance of muscle excitability to the temperature served as a characteristic of thermostability.

Figure 1a represents the data on muscle thermostability of different populations of the two marine species *Littorina squalida* Broderip et Sowerby and *Littorina mandchurica* Schrenck. Molluscs from two populations of each species were taken respectively from the Japan Sea which is warm in summer with a water temperature of 20–23°C and from the Sea of Okhotsk which is cold in summer with a water temperature of 10°C. In Fig. 1a the muscle thermostability of the two populations of *L. squalida* Broderip et Sowerby is characterized by the same straight line (line 1). This fact indicates that both populations did not differ in their muscle thermostability. Analogous data were obtained for two populations of *L. mandchurica* Schrenck (Fig. 1a

straight line 2). It should be noted that muscle thermostability of different *Littorina* species is characterized by different straight lines.

Figure 1b represents the data on muscle thermostability of different populations of the freshwater molluscs *Radix ovata* Draparnaud and *Radix auricularia* Linne. The molluscs *R. ovata* Draparnaud were taken from the cold Baikal (at the depth of 20 m), the warm spring (26–27°C) in the region of Baikal and from a pond in the Leningrad region (with a wide range of temperature fluctuations).

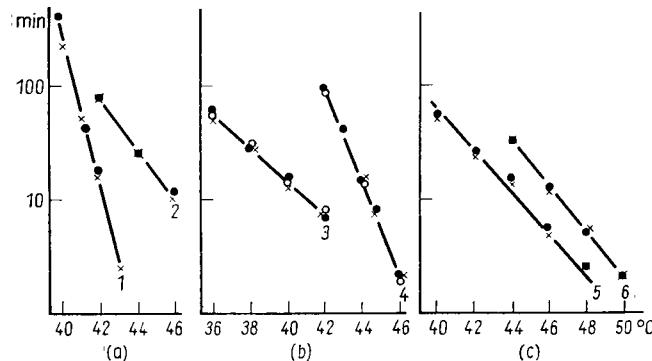


FIG. 1. Thermostability of the muscle tissue of molluscs of different populations within one species; a, 1—*Littorina squalida*; a, 2—*Littorina mandshurica* (dots—molluscs from the Sea of Japan, collected at the water temperature of 20–23°C; crosses—from the Sea of Okhotsk, collected at the water temperature of 10–12°C); b, 3—*Radix ovata* from the Lake Baikal (closed circles), from a warm spring of Gorjachinsky (open circles), from a pond of Leningrad district (crosses); b, 4—*Radix auricularia* from a pond of Leningrad district (crosses), from a continent pond near Vladivostok (open circles), from a lake of the Putyatin Island (solid circles); c, 5—*Circassina circassica* from Batumi (solid circles) and from Ordzhenikidze—Northern Osetia—(open circles); c, 6—*Fruticocampylaea narzanensis* from a woody ravine, (closed circles), from a semidesert region of Ordzhenikidze—northern Osetia (open circles). Abscissa—the temperature; ordinate—the time of excitability loss by muscle tissue at the thermal action (in minutes; the scale is logarithmic).

In Fig. 1b muscle thermostability of three different populations of *R. ovata* Draparnaud is represented by one and the same straight line (line 3). The same results were obtained for three different populations of *R. auricularia* Linne (straight line 4) collected in the Leningrad region, in Primorsk region of the Far East (near Vladivostok) on the mainland and on an island. Different straight lines are characteristic for muscle thermostability of different species of the genus *Radix*.

Figure 1c represents the data on muscle thermostability of different populations of land molluscs of species *Circassina circassica* (Mousson) and *Fruticocampylaea narzanensis* Krynicki. *C. circassica* (Mousson) were collected in the Caucasus, in the region of almost continental climate (Ordzhenikidze) and

in the subtropical region (Batumi). Muscle thermostability of these two populations is represented by the same straight line (line 5). The same straight line also represents the muscle thermostability of two different populations of *F. narzanensis* Krynicki (straight line 6). Populations of this species were taken from two different biotopes of the same Caucasian region near Ordzhenikidze: from the bottom of a woody ravine and a semidesert area.

TABLE 1. MUSCLE THERMOSTABILITY IN REPRESENTATIVES OF DIFFERENT POPULATIONS OF THE SAME SPECIES OF MOLLUSCS

No.	Species	Number of populations investigated	Number of pairs compared	
			Total	Number of pairs with the same muscle thermostability
	Marine			
1	<i>Littorina mandibularis</i> Schrenck	2	1	1
2	<i>Littorina squalida</i> Broderip et Sowerby	2	1	1
3	<i>Nucella beyseana</i> (Dunker)	2	1	1
	Freshwater			
4	<i>Unio tumidus</i> Philipsson	2	1	1
5	<i>Galba palustris</i> Müller	2	1	1
6	<i>Limnaea stagnalis</i> Linné	4	6	6
7	<i>Radix ovata</i> Draparnaud	3	3	3
8	<i>Radix auricularia</i> Linné	3	3	2
9	<i>Baicalia carinata</i> W. Dybowski	2	1	1
	Terrestrial			
10	<i>Helix vulgaris</i> Rossmassler	3	3	3
11	<i>H. lucorum</i> Linne var. <i>taurica</i> Krynicki	2	1	1
12	<i>Helicella derbentina</i> Krynicki	3	3	3
13	<i>Circassina circassica</i> Mousson	2	1	1
14	<i>Caucasolachea atrolabiata</i> Krynicki	2	1	1
15	<i>Fruiticocampylaea narzanensis</i> Krynicki	2	1	1
	Total (in %)	36	28 100 %	27 96 %

All the 15 species whose muscle thermostability was determined in representatives of different populations are given in Table 1. Of the total number of pairs of populations under comparison we calculated the percentage of pairs which exhibit a statistically reliable coincidence in thermostability of their muscle tissue. The investigation has shown for all molluscs investigated, aquatic and terrestrial that in 96 per cent of the cases muscle thermostability of different populations of one species was identical.

The results obtained show great conservatism of the thermostability of molluscan muscle tissue.

Figure 2a represents the data on muscle thermostability of different species of *Littorina*. All the 7 species of *Littorina* are characterized by different muscle thermostability straight lines. A coincidence was observed only between straight lines characterizing muscle thermostability of *L. mandchurica* Schrenck and *Littorina brevicula* Gould (lines 6 and 7). It appears that the differences in muscle thermostability of taxonomically close species of *Littorina* are related to differences in environmental temperature and conditions of species formation. North-Atlantic boreal species from the Barents Sea—*Littorina saxa-*

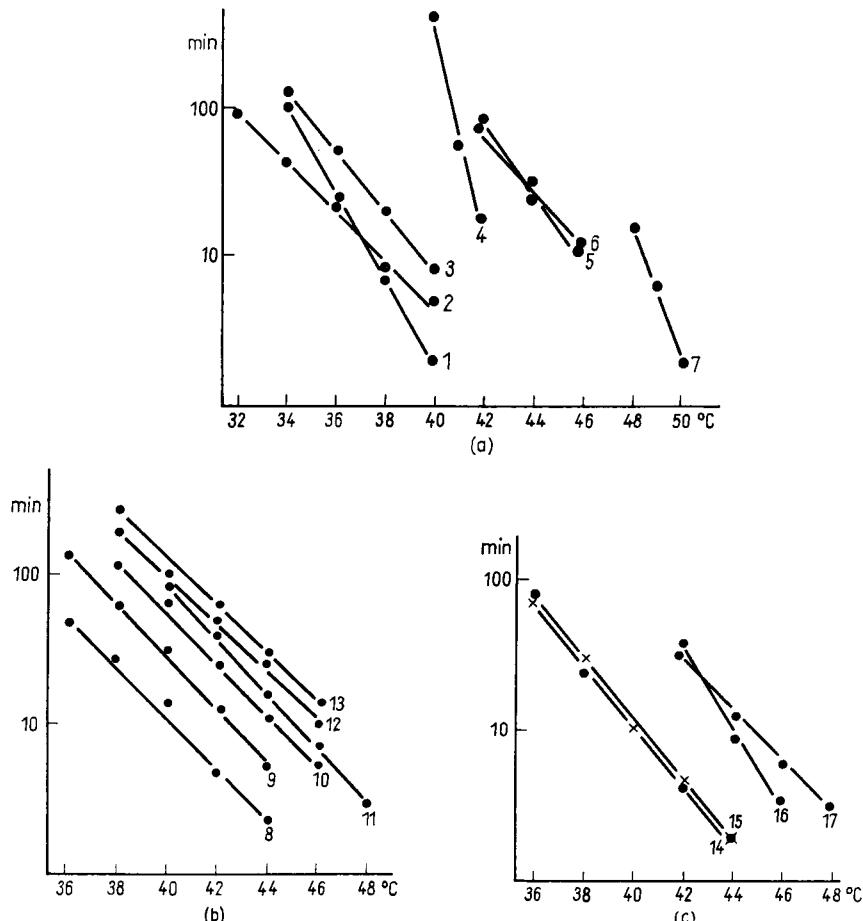


FIG. 2. Thermostability of the muscle tissue of different species of molluscs.
 a—genus *Littorina*: 1. *L. littorina*, 2. *L. obtusata*, 3. *L. saxatilis*, 4. *L. squalida*,
 5. *L. mandchurica*, 6. *L. brevicula*, 7. *L. granularis*; 1–6—own data of the author,
 7—data of A. V. Zhirmunsky (1960); b—genus *Anodonta*: 8. *A. complanata*,
 9. *A. anatina*, 10. *A. piscinalis*, 11. *A. cellensis*, 12. *A. beringiana*, 13. *A. woodiana*;
 c—genus *Eulota*: 14. *E. ravida*, 15. *E. middendorffii*, 16. *E. maacki*, 17. *E. fructicum*. Designations of the axes are the same as in Fig. 1.

tilis Olivi, *Littorina obtusata* (Linné) and *Littorina littorea* (Linné)—proved to be the least resistant to heat (straight lines 1, 2, 3). The level of thermostability was higher in the North-Pacific boreal species *L. squalida* Broderip et Sowerby (straight line 4) from the Sea of Japan and still higher in south-boreal species *L. mandshurica* Schrenck (straight line 5) and *L. brevicula* Gould (straight line 6) from the Sea of Japan. The highest thermostability was found in the tropical species *Littorina granularis* Grey (straight line 7). Muscle thermostability of this species was measured by Zhirmunsky (1960).

TABLE 2. MUSCLE THERMOSTABILITY OF REPRESENTATIVES OF DIFFERENT SPECIES OF MOLLUSCS

No.	Genus	Number of species investigated	Total	Number of pairs compared with the different muscle thermostability
	Marine			
1	<i>Littorina</i>	6	15	14
2	<i>Thais</i>	3	3	2
3	<i>Neptunea</i>	4	6	5
4	<i>Buccinum</i>	3	3	2
5	<i>Chlamys</i>	2	1	1
	Freshwater			
6	<i>Anodonta</i>	6	15	13
7	<i>Unio</i>	5	10	10
8	<i>Viviparus</i>	4	6	5
9	<i>Radix</i>	2	1	1
10	<i>Baicalia</i>	3	3	3
11	<i>Benedictia</i>	2	1	1
	Terrestrial			
12	<i>Sucinea</i>	2	1	1
13	<i>Eulota</i>	4	6	5
14	<i>Helix</i>	4	6	5
15	<i>Caucasotachea</i>	2	1	1
16	<i>Helicella</i>	4	6	4
	Total (in %)	56	84 100%	73 87%

In Fig. 2b one can see the data on muscle thermostability of the species belonging to the fresh water genus *Anodonta*. All six species are characterized by different muscle thermostabilities. Variations in muscle thermostability of different *Anodonta* species are also related to differences in environmental temperature and conditions of species formation. It has been found that representatives of the European species *Anodonta complanata* (Rossmassler)

(straight line 8) show the lowest muscle thermostability level while the South-Asiatic species *Anodonta woodiana* (Lea) the northern border of whose distribution lies to the south of the Primorsk region is the most resistant. Analogous data were obtained for fresh water molluscs (Fig. 2b).

All the closely related species of each genus which we have investigated are given in Table 2. On the basis of the total of the pairs of species compared we calculated the percentage of pairs whose difference in muscle thermostability is statistically reliable. It has been found that in 87 per cent of the cases investigated closely related species of molluscs can be distinguished according to this characteristic. As a rule these differences are related to the divergence in the environmental temperature and conditions of species formation.

The conservatism of muscle thermostability observed in representatives of the same species of molluscs and differences in muscle thermostability of closely related species give every reason to consider muscle heat-resistance as a cytophysiological species criterion.

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ANALYSIS OF SEASONAL CHANGES IN THERMOSTABILITY OF FROG MUSCLES

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SEASONAL fluctuations in thermostability of isolated tissues have been reported for muscles of frogs (N. Schlachter, 1961), fishes (Altukhov, 1963), reptiles (Ushakov, 1963) and for the ciliated epithelium of actinians (Dregolskaya, 1962) and molluscs (Dregolskaya, 1963).

By frequent measurements of the thermostability level (several times per month throughout a year) it was possible to detect seasonal changes (Fig. 1). Less frequent determinations (2–4 times a year) were insufficient to reveal any seasonal changes in heat-resistance of cells (Alexandrov, 1952; Arronet, 1959; T. Schlachter, 1959; Dzhamusova, 1960; Vernberg, Schlieper and Schneider, 1963).

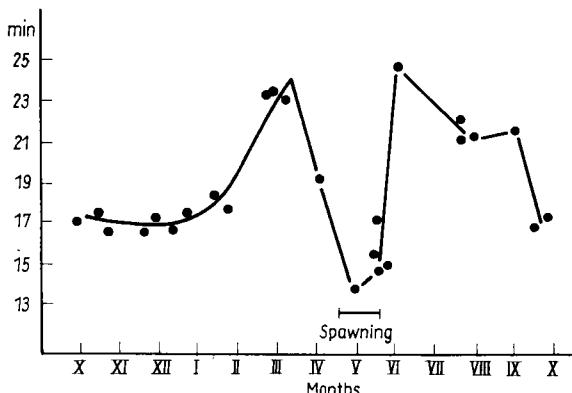


FIG. 1. Changes in muscle thermostability of frogs in different seasons of the year according to the data of N. Schlachter (1961). Abscissa—months of the year; ordinate—survival time of muscles at 38° .

We can observe seasonal changes in the tissue thermostability of poikilothermic animals. Thermostability of frog muscle tissue increases in the period preceding reproduction and decreases at the time of reproduction; in summer the resistance to heating increased again (Schlachter, 1959).

TABLE 1. CHANGES IN MUSCLE THERMOSTABILITY OF GRASS FROGS UNDER THE INFLUENCE OF ENDOCRINE FACTORS

Conditions of the experiment	Month	Development of non-excitability of muscles at 36°C			Development of non-excitability of muscles in 15% ethanol		
		Number of experiments	Changes in per cent of control samples	P-probability of differences	Number of experiments	Changes in per cent of control samples	P-probability of differences
I Natural spawning Artificially induced spawning (Immature frogs + hypophysis)	April	15	-59·7	0·02	10	-25·0	0·02
	February	25	-47·0	0·02	20	-23·0	0·02
	February	10	+8·0	0·12	7	+11·0	0·22
II Maintenance at 5°C and in the darkness The same + thyroidine feeding	July	24	-10·0	0·05	-	--	-
	July	17	+20·0	0·4	-	--	-

The fact that alterations of muscle thermostability in the spring were detected only in mature animals, and that they occurred at constant temperatures of the environment enabled N. Schlachter to suggest a possible role of endocrine glands in the changes of heat-resistance of muscle tissue in the spring.

To analyse seasonal changes in thermostability we investigated the effect of thyroid and hypophyseal hormones on the heat-resistance of frog muscle tissue. The experiments were performed on the m. sartorii of *Rana temporaria* L. The retention time of muscle excitability in Ringer's solution heated up to 36°C was used as a criterion of thermostability. Excitability was detected by muscle contraction in response to condensor discharge with impulses of 6·4 msec. The maximal strength of the test stimuli at the time of the loss of muscle excitability was 200 times higher than that of the normal threshold.

The changes in muscle tissue thermostability in spring were found to confirm the results of N. Schlachter. In the reproductive period the time for retention of muscle excitability in the heated Ringer's solution decreases on the average by 59·7 per cent (Table 1).

This decrease in muscle thermostability was relatively non-specific as was shown by a decrease in the resistance of fibres to the effect of 15 per cent ethanol prepared in Ringer's solution.

Similar changes in the properties of frog muscle tissue were revealed during artificially induced reproduction. The reproduction of frogs in winter was stimulated by injections of suspensions of hypophyses from the same species (2 hypophyses per injection, according to the technique of Kashchenko, 1963).

Examination of the frog muscle thermostability at the time of spawning in females and during the stable clasping reflex in males has shown that the time of retention of muscle excitability in Ringer's solution at 36°C decreased at that period (Table 1). This decrease in thermostability was also nonspecific and was accompanied by a decrease in muscle resistance to 15 per cent ethanol. The changes in the properties of muscle tissue observed in females during spawning were more pronounced than those in males at the time of the clasping reflex (Fig. 2). The injections of hypophyseal suspension into immature frogs in winter did not induce any reproduction. Measurements of their muscle thermostability 10 days after the injections showed no changes in the level of muscle thermostability as well as no changes in the resistance of the tissue to ethanol.

The similarity in the character of changes in the thermostability of muscle fibres in mature animals at the time of an artificially induced reproduction and also in the spring, as well as the absence of similar changes in immature frogs after hypophyseal injections, permit one to suggest that a decrease of muscle thermostability in mature animals might be related to the intensification of the gonadotrophic function of the hypophysis and the resulting endocrine function of sexual glands.

In order to analyse the increase in muscle thermostability at the time preceding reproduction and in the summer, we examined the muscle thermostability

during experimental increase of the amount of thyroid hormones in the organism. Experiments were performed in winter when, according to Sklower (1925), Meisenheimer (1936), Holzapfel (1937), Delsol (1955, 1957), and others, the thyroid gland of poikilotherms is not active and at that time the thyroid

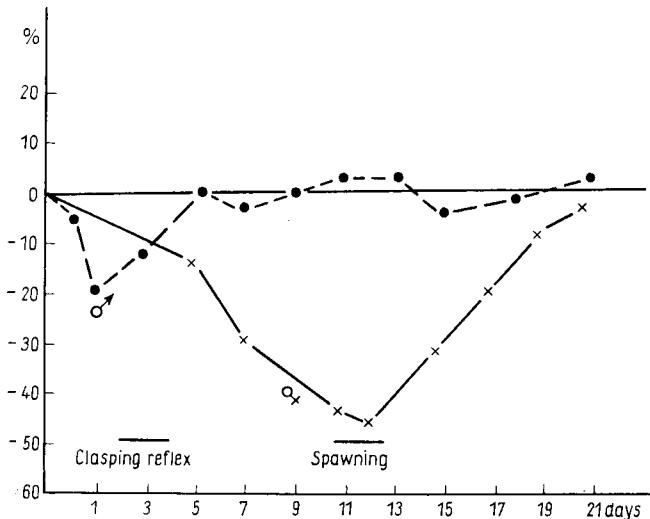


FIG. 2. Changes of muscle thermostability in males and females under the influence of the injections of hypophyseal suspension. Abscissa—number of days after injections; ordinate—changes in the retention time of muscle excitability at 36°C in per cent of the control samples. Continuous line—females; intermittent line—males.

gland practically does not respond to experimental changes in the environmental temperature (Eggert, 1936; Morgan and Mayer, 1936; Morgan and Stokes, 1936; Morgan and Fales, 1942; Evans and Hegre, 1938, 1940 and others). Three types of experiments were performed. The level of muscle thermostability was examined after the following treatments:

1. Thyroid feeding of frogs (50 mg daily, during 10–12 days).
2. Injections of pituitary thyrotropic hormones (15 and 75 ml units one or two times).
3. Thyroxine injections ($0.2 \text{ ml } 1 \times 10^{-2} \text{ mg/ml}$ daily, during 14 days).

In all the three series of experiments the level of muscle thermostability was found to increase (Fig. 3). This increase was non-specific and was followed also by an increase in the resistance to 15 per cent ethanol.

Thus, the increase in muscle thermostability as a result of experimental changes in the level of thyroid hormones in the organism suggests that an increase in the muscle thermostability in the period preceding reproduction

and in summer may be due to the rise in the activity of the thyroid gland. This conclusion is in accord with the data on the seasonal cyclic activity of the frog's thyroid gland.

Sklower (1925), Wolf (1934), Meisenheimer (1936) and Čehovic (1956) demonstrated a rise in the activity of the thyroid gland in the early spring and in the summer, i.e. when an increase in frog's muscle thermostability is

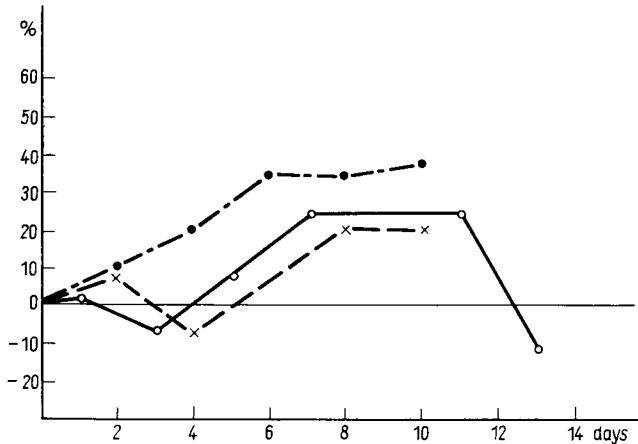


FIG. 3. Changes in muscle thermostability of frogs under the influence of thyroid hormones. Abscissa—time (in days) after the start of the experiments; ordinate—changes in the retention time of muscle excitability at 36°C in per cent of the control samples. Broken curve with points—changes under the influence of thyrotropic hormone. Broken curve with crosses—changes under the influence of thyroideine feeding; continuous line—changes in muscle thermostability under the influence of thyroxine.

observed. Moreover, the following experiment indicates the relationship of the level of muscle thermostability of frogs to the thyroid hormones in summer time. If frogs, in summer, are placed at low temperatures (4.5°C) and in darkness, their muscle thermostability decreases. The 20–25 days thyroideine treatment of such frogs in cold and darkness (50 mg every 2–3 days) resulted not only in the retention of the initial level of muscle thermostability, but even in slight increase of resistance (Table 1). It enables us to suggest that in summer there exists a certain relationship between the level of muscle thermostability and the activity of the thyroid gland.

The above results make it possible to conclude that changes in muscle thermostability observed in frogs in different seasons are related to the changes in the activity of endocrine glands. In the period preceding reproduction of animals in summer an increase of muscle heat-resistance is connected with the increase in the activity of the thyroid gland, while a decrease in muscle resistance, observed during reproduction, is due to the increase in the gonadotrophic function of the hypophysis and endocrine function of sexual glands.

According to Schlachter (1961), Ushakov (1963), Chernokozheva and Schlachter (1963) seasonal changes in cell thermostability are of cyclic character and are repeated annually.

The control level of thermostability of muscles for the first group of animals is 45.1 ± 1.9 min (mean from 15 experiments), for the 15 per cent ethanol 20.0 ± 0.6 (mean from 15 experiments); for the second group the level of thermostability is 51.0 ± 1.2 (mean from 54 experiments).

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THE RELATION OF TISSUE HEAT- RESISTANCE OF POLYCHAETES TO OSMOTIC AND TEMPERATURE CONDITIONS OF THE ENVIRONMENT

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THE role which cellular reactions play in the adaptation of animals to environmental conditions is far from clear. Numerous investigations of recent years (Alexandrov, 1952; Ushakov, 1959, 1961; Dzamusova, 1960; Zhirmunsky, 1960; Vinogradova, 1961; Ushakov and Kusakina, 1960 and others) have shown that in the majority of poikilothermal animals individual resistance adaptations to the temperature factor are not conditioned by changes in the heat-resistance of the cells and cell proteins. However, it was found that in some aquatic eurythermal and euryhaline invertebrates the changes in environmental conditions influence the cell reactivity. Hence, there is every reason to suggest the presence of intracellular mechanisms which provide for a normal vital activity of organisms under new conditions (Lobashev, 1949; Savvatayev, 1952; Gorodilov, 1961; Dregolskaya, 1961, 1963; Ivleva, 1962; Schlieper and Kowalski, 1956; Reshöft, 1961).

In the present investigation an attempt was made to determine the degree to which osmotic and temperature factors alter the heat-resistance of the muscle tissue of Polychaeta in the process of short-term and long-term acclimation of the animals to changed conditions.

Since the relation between the tissue survival time and the temperature represents a straight line in semilogarithmic system of coordinates, attention was paid to the changes of both coefficients characterizing thermoresistance: " τ "—the temperature at which the survival time of the tissue is 1 min (equal to t_1 in Zhirmunsky's paper in this volume) and " k "—the tangent of the angle of slope of the straight line to the abscissa. The coefficients are calculated by the method of the least squares. The statistical significance is estimated according to t -values by Student.

The Influence of Salinity

The effect of the total salt content of the environment upon the heat-resistance of the muscle tissue of *Nereis diversicolor* Müller and *Nereis succinea* Luckart has been studied. Animals which usually live in the Black Sea in the

region of Sevastopol at 18 per mille were acclimated to sea water of different salinities for five days. At the end of the acclimation period, measurements of tissue resistance to the action of lethal temperatures were made with each group of Polychaeta. The method of measurement was described earlier (Ushakov, 1959; Ivleva, 1962).

The character of changes in tissue resistance of *Nereis* under the influence of osmotic conditions is shown in Fig. 1. Along with the increase in the salt concentration of the media the heat-resistance of the cells increased. This increase however was not proportional to the change in the salt content and proved to be different for the two species. Doubling of the salt concentration

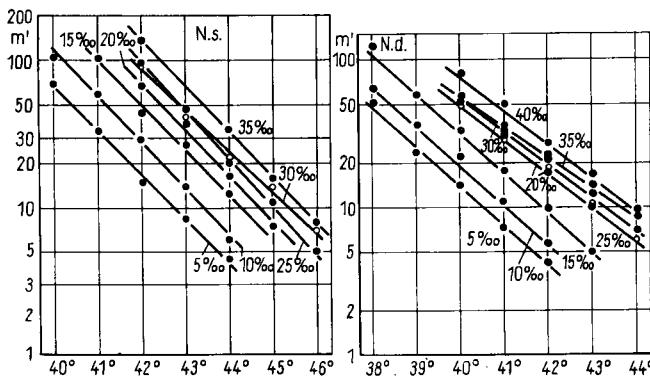


FIG. 1. Muscle tissue heat-resistance of *Nereis succinea* (*N. s.*) and *N. diversicolor* (*N. d.*) after acclimation for 5 days in solutions of different salinity. Abscissa— $T^{\circ}\text{C}$, ordinate—retention time of tissue excitability in min (logarithmic scale).

of the medium (5–10, 10–20, 15–30, 20–40 per mille) increased respectively the cell resistance by 1·4, 2·8, 2·2, 1·6 times in *N. diversicolor* and by 1·5, 2·4, 1·7 times in *N. succinea*.

Within the high salinity range there was a tendency to stabilization of the response and this stabilization was more pronounced in *N. diversicolor* than in *N. succinea*.

The degree of shifting of the indices of heat resistance is determined by the duration of existence of Polychaeta in new salt conditions. The resistance of the muscle tissue of *Nereis* acclimated to the medium the salinity of which was half of the normal concentration was minimal during the first few days of acclimation. Then the resistance slightly increased but did not attain the normal level even after 250 days of acclimation. When the medium was two times more concentrated than normal, the coefficient " k " changed considerably during the first 24 hr. On the 5–7th days the coefficient " k " returned to the initial level. Within this period the heat-resistance attained a new level and this remained unchanged during the whole time of acclimation (more than for two months).

To obtain data revealing the role of intracellular mechanisms of the cell thermostability changes during acclimation to media of different salinity, the following experiments were carried out. The thermostability of the muscle tissue of *N. diversicolor* acclimated at 35 per mille was measured in solutions with the salinity of 35, and also 18 per mille (the last value was taken as a normal salinity). The data are given in Fig. 2. The same character of changes of thermostability indices was found in both cases, the normal reactions being established at a certain different stable level some time after the acclimation.

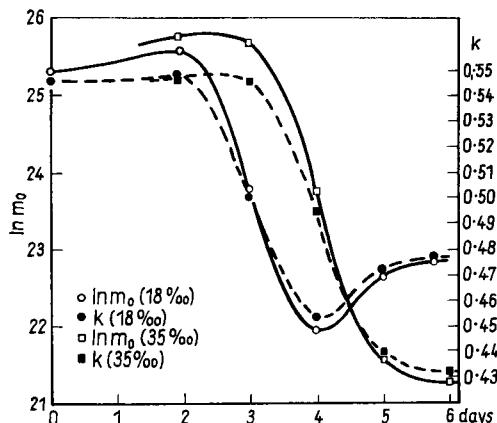


FIG. 2. Changes in the indices of muscle tissue heat-resistance of *N. diversicolor* taken from the sea water of 18 per mille and acclimated to the salinity 35 per mille and then tested for thermostability at 35 per mille, (squares) and at 18 per mille (circles). Abscissa — days of acclimation to 35 per mille; left ordinate — logarithm of retention time of tissue excitability ($\ln m_0 = k$); right ordinate — “ k ”.

These data are in good agreement with the observations previously made on tissue resistance of Polychaeta from different bodies of water. *N. diversicolor* and *Nephthys bombergii* from the Black Sea and Mediterranean populations differed in their tissue heat-resistance even in cases of readings taken in media of equal salinity. These differences cannot be related to the effect of salinity of solutions in which measurements were made (Pora, 1960) or to the influence of some cations (Schlieper and Kowalski, 1956). Rather, they appear as a result of the adaptation of animals and cells of muscle tissue to salinity conditions of the habitat.

The Influence of the Temperature

The heat-resistance characteristic of *N. diversicolor* tissue was determined under seasonal changes of temperatures in natural conditions and during a long-term acclimation of animals to low temperatures.

The tissue heat-resistance of the Black Sea population of *N. diversicolor* changed during the course of the year. It considerably increased during hot

months (July, August) and dropped in March and April. Changes in heat-resistance do not necessarily follow directly the alterations of the water temperatures. As can be seen from Fig. 3 a decrease in the thermostability was observed in March, only after the animals had lived at low temperatures (8–9°C) in January and February. Yet, the summer increase of resistance coincided with the rise of external temperature. The level of heat-resistance was greatest in the second half of August, i.e. in the period of the highest possible heating of the mud where the worms occurred (in the day time up to 20–32°C). A drop in temperature in September and October was followed by a gradual decrease of the tissue resistance which in November coincided with the June readings. This heat-resistance level was retained till February despite a further decrease of the external temperature.

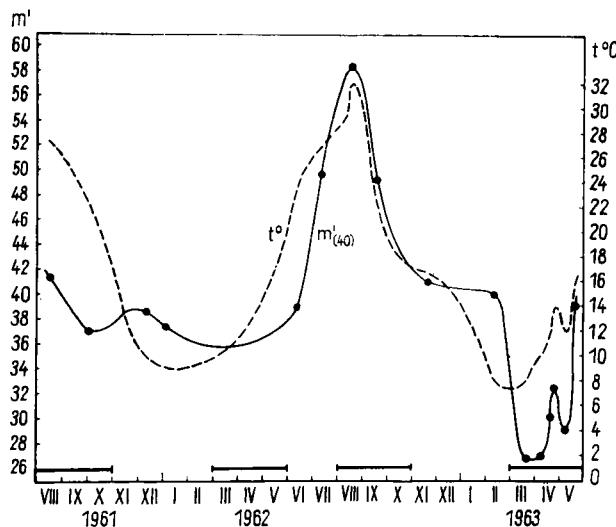


FIG. 3. Changes in muscle tissue heat-resistance of *Nereis diversicolor* within a year. Dotted line indicates temperature changes of the mud, solid line — muscle tissue resistance to heating at 40°C. Periods of reproduction are shown by the lines above the abscissa.

The character of the shifting of the heat-resistance throughout a year might turn out to be more complicated, were the measurement made more often than once or twice a month. However, we could not have missed temporal rises and falls in the heat-resistance during the period of reproduction as recorded for frogs by Schlachter (1961) and Pashkova (1962), for actinians and molluscs by Dregolskaya (1962).

Polychaetes unlike frogs, actinians and molluscs may show a change in the heat-resistance related directly with the environmental temperature. This suggestion is confirmed by the following fact: during the spring spawning (March–May) the heat-resistance is low, while in the period of summer—

autumn reproduction it is high. However, we have not enough experimental evidence to draw final conclusion concerning the effect of hormones on the heat-resistance of polychaetes.

The dependence of tissue heat-resistance on temperature was revealed clearly in experiments on acclimation of Polychaeta to low temperatures ($1.8-5.2^{\circ}\text{C}$). Compared to worms taken from the natural condition which increased their heat-resistance during July and August, the experimental *Nereis* transferred while they were in the condition of low temperature, in June, changed their tissue heat-resistance in the following way (Fig. 4): by end of the first month of acclimation (the end of June-July) the resistance was re-

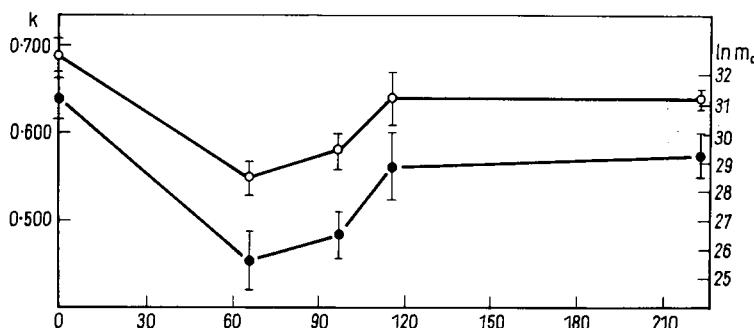


FIG. 4. Changes in indices of muscle tissue heat-resistance of *N. diversicolor* during acclimation to low temperatures. Abscissa—days of acclimation; left ordinate—the index “*k*” (white circles); right ordinate—logarithm of retention time of excitability $\ln m_0 = k$ (black circles).

duced but the change observed was statistically unreliable. During the second and third months the heat-resistance differed from the initial value and the decrease observed was statistically reliable. Then the resistance increased and on the 116th day of acclimation it attained the initial level. No change in heat-resistance was observed during the following 100 days of acclimation.

The above data show that the heat-resistance level of muscle tissue of Polychaeta can change considerably under the influence of osmotic and temperature factors. The degree of alterations depends on the intensity and duration of the environmental influence and is also determined by physiological peculiarities of the species.

Temperature changes are followed by the shifting in the muscle tissue heat-resistance (and of the coefficient “*k*” first of all) which, however, returns to the initial position in the process of a further acclimation. Changes in the index “*k*” apparently indicate serious disturbances in some tissue co-ordinating mechanisms the analysis of which needs additional research.

The acclimation to changed salinity conditions, unlike temperature acclimation, can be completed at room temperature within 5–7 days. During this

period the heat-resistance attains a new level and does not return to the initial position upon a further acclimation.

Under the influence of temperature, changes in the heat-resistance of Poly-chaeta cells occurred only in extreme conditions: in nature, during summer heating of water above 28°C or during winter cooling below 8°C; under experimental conditions at the cooling of worms below 5°C. In the thermal range between 10–25°C the heat-resistance indices remained at essentially the same level.

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A STUDY OF CELL THERMOSTABILITY OF SOME COELENTERATA

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IT HAS been demonstrated that within a species the cell thermostability of relatively high poikilothermal animals, such as molluscs, arthropods, echinoderms, fishes, amphibians and reptiles, is to a considerable extent independent of environmental temperature changes (Ushakov, 1955-1963; Arronet, 1959; Ushakov and Kusakina, 1960; Amosova, 1963; Andronikov, Dzhamusova and Kusakina, 1963; Zhirmunsky and Schlachter, 1963). On the other hand, Protozoa kept in various temperatures are able to immediately change their resistance to high temperatures (Poljansky, 1957; Irlina, 1960; Losina-Losinsky, 1961). In this connexion it would be interesting to study cell thermostability of relatively low Metazoa, such as sponges and coelenterates.

The present communication gives the results of the investigation on thermostability of some coelenterates, actinians and hydras.

Cell thermostability of actinians was studied: (1) in animals of one species (a) depending on temperature changes under natural and experimental conditions, and (b) in animals of different populations within one species; (2) in representatives of different species. The experiments were performed on the ciliated epithelium of mesenterial filaments. The time between immersion of the ciliated epithelium preparation in heated sea water and cessation of ciliary movement served as a measure of thermostability.

Thermostability of the Black Sea *Actinia equina* showed considerable fluctuations within a year. Survival time of cells at 39°C is 33 min in April and 114 min in August. The comparison of these fluctuations with thermal changes of sea water shows that thermostability tends to follow the temperature changes in the sea water. However, in the period of reproduction(spring-beginning of summer) thermostability decreases. The decrease in the thermostability level is also typical for such animals as molluscs (Dregolskaya, 1963 a), fishes (Altukhov, 1963), frogs (Schlachter, 1961; Pashkova, 1962) and lizards (Ushakov, 1963).

Attempts to obtain shifts in actinian thermostability by changing the rearing temperature failed (Zhirmunsky, 1959; Dregolskaya, 1962). But we found out that under laboratory conditions, independently of the rearing temperature, the rhythm of seasonal fluctuations of actinian cell thermostability coincides

with that of the animals taken directly from nature (Table 1) (Dregolskaya, 1962).

In actinian populations from different points of the area of distribution of each of the three species (*Epiactis prolifera*, *Anthopleura xanthogrammica* and *A. equina*) cell thermostability was the same within either species despite considerable differences which sometimes occur in the environmental temperature of some populations (Zhirmunsky and Pisareva, 1960; Dregolskaya, 1962; Zhirmunsky, 1963).

TABLE 1. SURVIVAL TIME (min) OF THE CELLS OF ACTINIANS KEPT UNDER DIFFERENT TEMPERATURE CONDITIONS

Time and conditions of experiments	Water-temp. (°C)	39°C	40°C	41°C
August, on the day when animals were collected	25	114.5 ± 4.9	32.0 ± 1.6	12.0 ± 0.4
October, on the day when animals were collected	17	105.6 ± 7.0	29.0 ± 1.8	9.8 ± 0.9
November, on the day when animals were collected	15	68.7 ± 1.5	24.6 ± 0.8	9.3 ± 0.0
November, after 35-day cultivation in the heat	25	78.6 ± 4.0	25.6 ± 1.4	9.8 ± 0.5

Cell thermostability of marine animals is influenced by the salinity of the water (Schlieper and Kowalski, 1956a, 1956b; Dregolskaya, 1961; Reshöft, 1961; Ivleva, 1962 and others). That is why actinians of different species (*Bunodactis stella* from the Barents Sea, *E. prolifera* and *A. xanthogrammica* from the Sea of Okhotsk (Zhirmunsky, 1963) were taken from the waters of the same salinity (30–34 per mille). These species differ in their cell thermostability (Fig. 1). The differences are of an adaptive character since they are related to the environmental temperature conditions of species: the more thermophilic the species, the higher is its cell thermostability.

Thus cell thermostability of actinians is as conservative as that of other poikilothermal animals. This can be seen from the fact that within a species it does not depend on the environmental temperature of separate populations and the rearing temperature. Moreover, the rhythm of seasonal fluctuations in the thermostability of animals in nature is retained under laboratory conditions at different rearing temperatures. Taxonomically close species of actinians exhibit different cell thermostability which enables us to use it as a cytophysiological species criterion.

We studied thermostability of hydras in relation to the temperature regime of an environment, under natural and experimental conditions. Temperature reactions in a few species of hydras were compared (Dregolskaya, 1963 b).

The experiments were done on isolated digestive and interstitial cells. The survival time of cells in special salt solution† heated to a specified temperature level was used as an index of thermostability. The cell death was determined by irreversible shrinkage of the protoplasm.

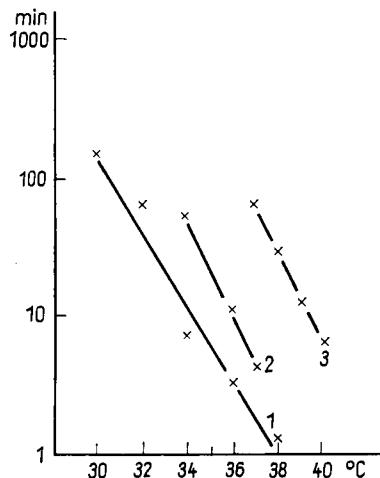


FIG. 1. Relation of survival time of ciliated epithelial cells in different species of actinians to temperature. Abscissa—temperature (in °C), ordinate—survival time (in min, logarithmic scale). 1—*Bunodactis stella*, 2—*Epiactis prolifera*, 3—*Anthopleura xanthogrammica* (Zhirmunsky, 1963).

The level of cell thermostability of *Hydra oligactis* kept at 10°C for a month proved to be half that of hydras kept at 28°C. As can be seen from Fig. 2 after hydras had been transferred from heat to cold, changes in their cell thermostability were observed in 2 hr. From the very beginning these alterations of the cell thermostability level in hydras are of fluctuating (phasic) character (Fig. 2). However, on the 8th day cell thermostability attains a new stable level which is lower than that of the hydra cells kept in the heat.

In *H. oligactis*, cell thermostability depends on the environmental temperature of different populations of this species. But these differences are not stable: after the maintenance of hydras at the same temperature for a week, the thermostability of their cells becomes identical.

Then an attempt was made to reveal differences in cell thermostability between several species of *Hydra* (*H. oligactis*, *Hydra vulgaris*, *Chlorohydra viridis*). The animals were preliminarily kept for a month at similar temperatures. This method for determining in species the specific levels of thermostability was applied by Schlieper (1960) for molluscs, and by Irlina (1960), and Losina-Losinsky (1961) for Protozoa. As a result of our experiments,

† The content of salt solution: 0.75 g of NaCl, 0.03 g of KCl, 0.02 g of CaCl₂, 1000 cm³ of distilled water.

statistically significant differences in cell thermostability of hydra species were obtained. Thus, the survival time at 37°C was 44 min, 48 and 71 min for *H. oligactis*, *H. vulgaris* and *C. viridissima* respectively. The differences in the cell thermostability are in accord with the differences between the temperature of the environmental conditions of the species.

Thus, within the phylum Coelenterata, temperature reactions of cells differ as follows: in actinians, in the same way as in higher poikilothermic animals, the level of cell thermostability is conservative; in hydras, as in Protozoa, the

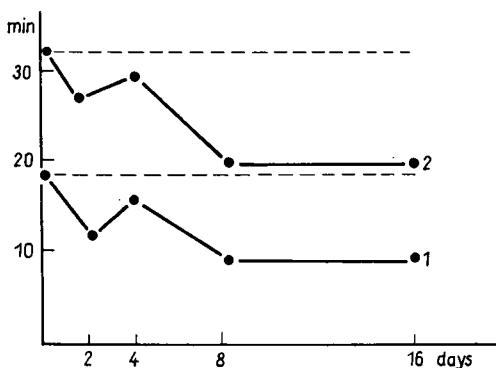


FIG. 2. Survival time of digestive cell in *Hydra oligactis* (1) and *Hydra vulgaris* (2) at 37°C at different times after the transfer of hydras from heat (23°C) to cold (10°C). The initial level of cell thermostability for hydras from 23°C is represented in hatched lines. Abscissa—intervals for determination of thermostability (in days); ordinate—survival time of cells (in min) (Dregolskaya, 1963 b).

level of thermostability exhibits considerable lability. Two suggestions were made to explain differences between actinians and hydras. These differences can be due either to the level of phylogenetic development of the group or to differences in the amplitude of temperature fluctuations of their habitat. Thus in the Black Sea where actinians live, daily fluctuations in temperature are insignificant (2–3°C), while in shallow ponds populated by hydras the amplitude of daily thermal fluctuations proved to be rather large (up to 10°C).

In order to support the first of the above suggestions a series of experiments were done on the Black Sea hydroid *Cordylophora caspia*. This animal lives in the same conditions as the actinians, but phylogenetically it is closer to *Hydra*. After exposure for 10 hr of the animals to heat (23.5°C) and cold (5°C), the level of their cell thermostability proved to be the same, and also equal to that of the control samples taken from the temperature of 14°C. Under similar experimental conditions shift in the cell thermostability of hydras occur in 2 hr. Thus the experiments confirm the suggestion that in Coelenterates dif-

ferences in the species ecology influence considerably the character of cell thermostability. But additional investigations are needed to check this suggestion.

Actinians and hydras show a species specificity of cell thermostability. But whereas the environmental temperature in actinians does not play the main role for determination of this specificity, in hydras it can be found only when the animals are kept under similar temperature. The cell thermostability of Coelenterata can be used as a cytophysiological species criterion.

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HEAT-RESISTANCE OF CELLS OF ANIMALS FROM THERMAL SPRINGS

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ANIMALS living in thermal springs are most suitable subjects for the investigation of temperature adaptations. A comparison of the cellular properties of specimens from populations inhabiting thermal springs with populations of the same species from water basins of normal temperature gives information concerning the part which cells play in adaptation of an organism to different temperatures. Such comparisons are of interest, particularly when we study the degree of conservatism of cellular thermostability and the possibilities of changing this property under high environmental temperature.

Heat-resistance of muscle cells was determined in frogs (*Rana ridibunda* Pall.) from Caucasian and Bulgarian thermal springs, molluscs (*Radix ovata* [Draparnaud]), fishes (*Carassius auratus* Gibelo and *Cobitis sibirica* Gladkov) from thermal springs of the Baikal shore (Dzhamusova and Shapiro, 1960; Kusakina, 1962; Svinkin, 1962).

In every case studied, the cellular heat-resistance in specimens of the same species from thermal springs and from water of normal temperature was found to be identical. In fish (*C. auratus*) the heat-resistance of the cholinesterase of muscle homogenates was also identical (Kusakina, 1962).

However, the coincidence in the heat-resistance of the cells and the protoplasmic proteins in dwellers of thermal springs and normal temperature water basins does not indicate the absence of cellular adaptation in these animals. Ushakov and Zander (1961) described adaptive changes in the functional state of muscles of frogs living in thermal springs. Ushakov and Glushankova (1963) discovered certain differences in lipids of frogs dwelling in thermal springs and in normal temperature regions. Attention must be called to the fact that the heat-resistance of muscles in such frogs *in situ* is higher than that of isolated muscles (Alexandrov, Galkovskaya and Losina-Losinsky, 1960).

The absence of difference in cellular heat-resistance of fishes, frogs and molluscs inhabiting thermal springs and normal temperature regions indicates the marked conservatism of this feature in higher poikilotherms.

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THE EFFECT OF MAINTENANCE TEMPERATURE OF MULTICELLULAR POIKILOTHERMS ON THE THERMOSTABILITY OF INTACT ORGANISMS AND THEIR CELLS

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1. Prolonged maintenance of poikilothermic animals at various temperatures (referred to as "acclimation" by a number of American authors) is a successful method for studying temperature adaptation. It enables us to determine the influence of temperature conditions of animals on the resistance of intact organisms and their cells to high temperature, which is significant for the use of thermostability in taxonomy.

2. Numerous investigations performed on representatives of some systematic groups of multicellular poikilotherms have shown that thermostability of intact organisms is related to the environmental temperature in which animals were kept during the experiment. Changes in the environmental temperature involve adequate shifts in the thermostability of the organisms. Species specificity of thermostability in systematically related species can be displayed only in the case when animals belonging to different species are kept at the same temperature. Hence, the conclusion may be drawn that thermostability of intact organisms shows a considerable lability (Ushakov, 1956, 1963; Fry, 1957; Arronet, 1959; Zhirmunsky, 1959; Ushakov and Kusakina, 1960; Zhirmunsky and Schlachter, 1963, and others).

3. On determining the dependence of cellular thermostability on the environmental temperature from which animals were taken before the experiment, some investigators found this criterion to be fairly stable (Mikhailchenko, 1958; Arronet, 1959; Ushakov and Kusakina, 1960; Dregolskaya, 1962, and others). In some instances, however, it was detected that cellular thermostability of animals alters with a change in the maintenance temperature (Lobashov and Korenevich, 1947; Reshöft, 1961; Dregolskaya, 1963; Suzdalskaya and Kiro, 1963, and others).

4. An analysis of these phenomena was made on frog *Rana temporaria* L. (Zhirmunsky and Schlachter, 1963; Pashkova, 1963). Zhirmunsky and T. Schlachter (1963) demonstrated that in winter, cellular thermostability is

the same for ciliated epithelium and *m. sartorii* previously maintained in heat or cold. In summer the level of cellular thermostability increased; maintenance of frogs in the cold led to a decrease in muscular thermostability down to the winter level.

5. Pashkova (1963) exposed frogs to temperatures exceeding by 10–15°C the winter and summer temperatures of the habitat. The maintenance of animals under such a temperature for 10–15 days affected the muscular thermostability differently depending on the month of the year. In October–December thermostability of *m. sartorii* in frogs under investigation did not differ from that in frogs taken directly from nature and used as controls. In February muscular thermostability of frogs increased; in April it fell, to rise again in July.

6. A comparison of the obtained data with experimental findings of N. Schlachter (1961) and Pashkova (1962) on seasonal changes in the muscular thermostability explains these seemingly contradictory changes. According to these authors muscular thermostability in autumn and winter seems rather stable; in spring thermostability falls, to rise again afterwards, and in summer it decreases again. Special experiments showed that in every case studied seasonal changes in cellular thermostability resulted from alterations in endocrine system activity (Pashkova, 1963).

When animals are transferred to conditions of increased temperature, seasonal shifts of cellular thermostability occur earlier than in the natural conditions. It follows that the maintenance of frogs in a warm environment accelerates the process of alteration.

7. As may be concluded from the comparison of cellular thermostability of control frogs and those kept in the heat, spring fluctuation in the cellular thermostability is not related to changes in the environmental temperature. In summer these shifts occur simultaneously with temperature changes. Variation in the environmental temperature influences thermostability indirectly, via changes in the thyroid gland activity. It is not clear whether these changes have adaptive significance.

8. Unlike frogs, thermostability of actinian cells in summer do not change simultaneously with alterations in the environmental temperature. Cellular thermostability of actinians kept under increased temperature did not differ from that of actinians in their natural habitat at this season of the year (Dregolskaya, 1962).

The case is different with hydras (Dregolskaya, 1963), whose thermostability (as in protozoans and intact organisms) responds by adequate alterations to thermal changes of the environment. It is not improbable that such variability of temperature responses in hydra cells is related to the position of these animals in the phylogenetic system. One should not exclude the hypothesis that this variability reveals special adaptations in the Hydra to the conditions of the wide temperature range.

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SPECIFIC AND NON-SPECIFIC CHANGES IN RESISTANCE OF *PARAMECIUM* *CAUDATUM* ADAPTED TO DIFFERENT TEMPERATURES

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IN THE process of adaptation to low and high temperatures, protozoans change their resistance to such injurious agents as ethanol, potassium cyanide, glucose and increased concentrations of certain salts (Poljansky, 1957; Irlina, 1963 a, b, c).

It might be expected that such alterations are accompanied by changes in the protein substrate and the rearrangement of metabolic processes. Therefore, a study of the reactions of ciliates (adapted to various temperatures) to the factors differently affecting the metabolism may lead to an understanding of physiological alterations which occur in the process of metabolism.

Measurements of the survival time for *P. caudatum* in different salt solutions have shown that the resistance of ciliates depends equally on cultivation temperature and salt concentration. For example, the cultivation of ciliates for 1.5–2 months at 29°C ("warm" infusorians) results in a considerable decrease of their resistance to threshold concentration of calcium chloride and potassium chloride. The ciliates cultivated for the same period of time at 4°C ("cold" infusorians) survive in the solutions for longer periods of time. The resistance in control ciliates (living at 14°C) is greater than in the "warm", but less than in the "cold" infusorians.

The relation is different in the case of concentrations of calcium chloride and potassium chloride above the lethal threshold. Survival time of the "warm" ciliates increases in these solutions, whereas that of the control and the "cold" ones decreases.

A question arises whether the resistance of *P. caudatum* to potassium cyanide changes similarly? In the threshold concentration range of potassium cyanide for inhibiting the cytochrome respiratory system, the "cold" ciliates appear to be the most and the "warm" ciliates the least resistant. But at higher concentrations, when potassium cyanide acts as a poison on the entire protoplasm, the "warm" infusorians exhibit higher resistance than the "cold" ones. Such differences in the effect of high and low concentrations were shown also by Troshina (1951) in her experiments with frog muscles.

A non-specific increase in resistance of "warm" ciliates at the suprathreshold concentration might be due to the fact that the adaptation to high temperature leads to changes in the proteins of the protoplasm which render them more resistant to denaturant influences. The same rise of resistance to denaturant action of various agents was observed in plant cells, the heat-resistance of which has been experimentally increased (Alexandrov, 1963).

A non-specific increase in resistance of frog muscle cells after their exposure to super-optimal temperatures was also observed by Schlachter (1959).

Possible biochemical alterations of the thermostable proteins (when bacteria are kept at high temperatures) and the existence of non-specific resistance to certain agents were considered by Köffler *et al.* (1957).

It might be expected that in the process of the adaptation of ciliates to different temperatures there occur not only substantial changes in the physico-chemical properties of the protoplasm but also alterations in metabolism. Metabolic changes can be detected by means of inhibitors of respiration and glycolysis. The adaptation of *P. caudatum* to high temperatures (29°C) results in the prevalence of respiration related to the cytochrome oxidase system. The fact that "warm" infusorians show a high sensitivity to 2×10^{-3} M KCN (pH 5.4) (Poljansky, 1957; Losina-Losinsky, 1961; Irlina, 1963b) confirms this hypothesis. It seems that glycolytic processes do not play a significant part in the metabolism of "warm" infusorians for, as our experiments have revealed, they exhibit a considerable resistance to monooiodoacetate (2×10^{-5} pH 5.4). Cytochemical reactions confirm these experimental results (Kovaleva, 1962; Poljansky, 1963). The "warm" infusorians survive in malonate solution (2×10^{-4}) for longer periods than the "cold" and the control ones. The cytochemical reactions show that succinic dehydrogenase activity is insignificantly active in the "warm" ciliates and succinic acid probably cannot serve as a respiratory substrate of "warm" *P. caudatum*. According to the survival time of "cold" ciliates in solutions of respiratory inhibitors and glycolytic inhibitors it can be suggested that glycolysis is the main source of energy. This supposition is confirmed by biochemical evidence as well (Kovaleva, 1962; Poljansky, 1963). The resistance of "cold" ciliates to respiratory inhibitors, as well as their weak succinic dehydrogenase activity indicate that the role of respiration in energy change of "cold" infusorians is relatively small.

Control ciliates cultivated at 14°C exhibited the sensitivity to monooiodoacetate, which indicates that glycolysis plays a significant role in their metabolism. This was also confirmed by cytochemical data (Poljansky, 1963). A high sensitivity of the control ciliates to malonate and a high level of succinic dehydrogenase activity coexist with the resistance to cyanide.

Thus changes in cultivation temperature result in metabolic changes, predominance of a certain functional enzymic system and in disturbances of the correlation between respiration and glycolysis. It appears that enzymic systems are the physiological basis of temperature adaptations. Hoffman (1952) provided evidence showing the metabolic changes of *Streptococcus cremoris* as a

result of cultivation at various temperatures. The fact that an organism has no capacity to change its enzymic activity according to new thermal conditions precludes its adaptation to these new temperature conditions (Hagen and Rose, 1962). It appears that metabolic changes are responsible for the specific character just as substantional alterations determine the non-specific character of reactions in protozoans adapted to a certain temperature.

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PHENOMENA OF CONSERVATISM AND CHANGES IN THE LEVEL OF CELLULAR HEAT-RESISTANCE IN CULTURES OF THE MYOCARDIA AND THEIR CYTOPHYSIOLOGICAL INTERPRETATION

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1. Explants of the myocardia of frogs (*Rana temporaria* L. and *Rana ridibunda* Pall.) were cultivated at different temperatures in Carrel flasks in various media (plasma, embryonic extract, serum) and in Tyrode solution. Taking into account greater lability of cells of young animals (Rumyantsev, 1960a) we first explanted fragments from hearts (atrium cordis, ventricle) of immature first-year frogs.

2. Thermoresistance of the explants was measured by retention time of excitability at 40–48°C. Heat-resistance of the myocardial fibres is 1·7° higher in *R. ridibunda* than in *R. temporaria* which is in good agreement with the evidence of Alexandrov and Ushakov obtained from experiments with other tissues. Cultivation at 16–19°C for 38–45 days does not affect this divergence, despite the fact that this temperature is sub-optimal for tissues of *R. ridibunda*.

3. Species divergence in the myocardial heat-resistance does not undergo any significant change even after a 1–1·5-month cultivation at 26°C (*R. temporaria*) and 16–19°C (*R. ridibunda*), i.e. in conditions opposite to ecological peculiarities of these species.

4. Such "inversion" of the thermal optimum also reveals the conservatism of the heat-resistance of the fibroblast-like cells of the growth zone, although the majority of them run their mitotic cycle at temperatures abnormal for the species. Formation of neutral red granules was repressed (paranecrosis according to Nassonov) in the cells of *R. ridibunda* cultivated at 16–19°C at temperatures 2–2·5° higher than in cells of *R. temporaria* cultivated at 26°C.

5. Along with an apparent conservatism of the thermostability of cells in tissue cultures, an insignificant but quite evident increase in the heat-resistance (by 0·3–0·5°) was observed as the result of a prolonged cultivation of *R. temporaria* myocardia at 16–19 and 26°C. At that time the nature of this phenomenon was not clear.

6. Contrary to our suggestion, an analogous phenomenon was found to be more pronounced in cells of the adult organisms. Heat-resistance of fibres of the heart ventricle apex of adult frogs increases by 1·5–2·0° on the 17–20th day of cultivation at 16–19°C. At a higher temperature of cultivation (25°C) the same effect appears more rapidly; at 10–12°C (i.e. under temperatures low enough to suggest "heat adjustment" in cells of *R. temporaria*) resistance to heating rises 1·1–1·4°C but more slowly (Rumyantsev, 1961).

7. The following evidence also testifies against the possibility that an increase in heat-resistance in this case is due, to a significant degree, to "temperature adjustment". Unlike analogous experiments of Poljansky with infusorians, the heat-resistance of the myocardial cultures which had been preliminarily raised by 1·5–2·0° (after a 20-day cultivation at 25°C) retained its unusually high level during 20 days of subsequent incubation at 1–4°C. Incubation performed at 1–4°C in control cultures with a standard level of heat-resistance also does not cause a well-expressed "cold adjustment". A rise in thermotolerance of the myocardia cultivated for a long time under temperatures not lower than 10–12°C does not coincide with an increase in the resistance to the action of alcohol that is characteristic for the effects of heat "hardening" of "adjustment" type.

8. Hence, we have discovered a phenomenon *sui generis*. The nature of this phenomenon was revealed by a comparative analysis of the heat-resistance in muscle fibres with different structures and specialization, as well as by the consideration of structural and functional changes accompanying the heat-resistance increase of the myocardia explants.

9. Heat-resistance differs in various types of striated muscle fibres as follows: non-tonic fibres < tonic fibres < fibres of the lymph heart < fibres of the ventricle < fibres of atrium cordis without automatism < spontaneously contracting fibres of the atrium cordis. The reverse sequence of this series exactly reflects the gradual morphophysiological differentiation of the striated muscle fibre as Orbeli (1945), Chlopin (1950), Ginetsinsky (1947) and Itina (1959) use the term.

10. On the basis of these data it may be suggested that an increase in the myocardial heat-resistance is determined by its morphophysiological dedifferentiation outside the organism, which is in good agreement with a well-known rule saying that tissues *in vitro* exhibit primitive features. This suggestion is confirmed by a histological analysis which revealed a great deal of dedifferentiation (embryonalization) of the myocardial fibres. As soon as a dedifferentiation maximum is attained and the level of heat-resistance increased (by the end of the third week of cultivation), muscle fibres show a primitive (according to Orbeli) function—myogenous automatism which they lacked before dedifferentiation.

11. The proposed explanation is also confirmed by the fact that in frog ventricle muscles *in situ* it is possible to produce regions with increased heat-

resistance by damaging the heart 20–30 days before heat-resistance is determined. As has been demonstrated (Rumyantsev, 1960b), the myocard injured *in situ* dedifferentiates at the stumps of surviving muscle fibres.

12. Changes in contractile proteins may provide a basis for an increase of cellular heat-resistance. This supposition becomes more probable due to accumulation of ribonucleoproteins in the course of this process (Rumyantsev, 1960b).

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TEMPERATURE ADAPTATIONS IN ENDOPARASITIC PROTOZOA OF AMPHIBIANS IN CONNEXION WITH PECULIARITIES OF THEIR ECOLOGY AND LIFE CYCLE

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1. Experimental studies of temperature adaptations were performed on natural populations and cultures *in vitro* of Opalinida and other infusoria parasitizing the intestine of amphibians. The adaptation to environmental temperature was estimated by the degree of thermostability determined by the death rate of protozoans after exposure to high temperatures. The heat-resistance of endoparasitic ciliates and opalinids as well as that of free-living species (Poljanskij, 1957; Irlina, 1960) depends on the thermal environmental conditions of the host. In more thermophilic southern amphibians the same parasitic protozoans show a greater heat-resistance than those from northern species (Sukhanova, 1959, 1962). This is in agreement with the heat-resistance of amphibian tissues themselves (Alexandrov, 1952; Ushakov, 1956, 1959 and others). The infusorian *Nyctherus cordiformis* (Ehrbg.), for example, possesses the highest heat-resistance in *Bufo viridis* Laur.; it is lower in *Rana ridibunda* Pall. and the lowest in *Rana temporaria* L. It has been found that in cultures *in vitro*, under similar temperature conditions, *N. cordiformis* of the three hosts show differences in heat-resistance during 2 or 3 weeks.

2. Under natural environmental conditions the heat-resistance of endoparasitic protozoans of amphibians has a well-expressed seasonal character and is related to seasonal changes in temperature (Sukhanova, 1961, 1962), the same as the heat-resistance of amphibians (Arronet, 1959).

3. The environmental temperature affects considerably not only the heat-resistance of endoparasitic protozoa but also the course of their life cycles. When mature *R. temporaria* are transferred into room temperature conditions (18–25°C) in winter, in several days infusoria parasitizing in their intestine begin to divide and cysts appear in *N. cordiformis*. The heat-resistance of infusoria also increases in about 24 hr. Considerable shifts are observed in the life cycle and heat-resistance of *Opalina ranarum* Eherb. Cyst formation under

natural conditions occurs only in spring, during the breeding season of the frogs. In other seasons of the year no cysts can be formed. If mature frogs are transferred to room temperature in winter, cyst formation begins in Opalina in about 7–10 days. In other experiments, cyst formation in Opalina was found in 40–70 per cent of mature frogs. No cyst formation was observed in populations of Opalina taken from one- or two-year frogs removed from hibernation sites into room temperature conditions. Analogous results were obtained from winter experiments with cultures *in vitro*. Opalina cultures were transferred from low temperatures of 4°C into conditions of room temperature. In a few days an intensive division begins in cultures of Opalina taken from mature frogs: in some cases it is accompanied by the formation of precyst individuals and cysts.

Heat-resistance of Opalina (from winter frogs) increases rather quickly when amphibians and cultures *in vitro* are exposed to room temperature. An increase in the heat-resistance of Opalina in cultures occurs more rapidly than in the host organism.

4. To show the dependence of the life cycle of Opalina and their thermostability on hormonal influences of the host, a series of experiments were made with injections of hypophyseal suspension into *R. temporaria*. After hypophyseal injections performed in January and February mature frogs were placed in conditions of room temperature and they spawned on the 7–9th day. An intensive formation of cysts was noted in 60–70 per cent of these frogs in the Opalina populations.

Injections of hypophyseal suspension into 1–2-year old frogs in early spring did not produce any effect on Opalina: not a single population showed cyst formation.

Experimental results obtained from hypophyseal injections and removal of *R. temporaria* into room temperature conditions in winter suggests that the process of cyst formation in Opalina is influenced by hormones of amphibian sexual glands. A transfer to high environmental temperatures involves an alteration in the condition of sexual glands which produces a considerable influence of sexual gland hormones of a host on cyst formation in Opalina (Bieniarz, 1950; Čehovic, 1956; McConnachie, 1960; Mofty and Smyth, 1960).

The injection of hypophyseal suspension into *R. temporaria* causes a significant change in the heat-resistance of Opalina. Heat-resistance of Opalina taken from females of *R. temporaria* increases with a rise of environmental temperature only during the first 24 hr. Then heat-resistance regularly decreases and by the end of the second day it is 2–3 times lower than that of the control. It remains at this low level up to the moment of spawning. In male populations of Opalina heat-resistance decreases below the level of the control only at the very beginning of hypophyseal treatment. Then it begins to rise, attains the control level and remains there until spawning. Hence, heat-resistance of Opalina from females is 2–3 times lower than that from *R. temporaria*.

males who have been stimulated by hypophyseal suspensions injected during the period of maturation of sexual glands.

The same relationship is observed in early spring in nature during the whole preparatory period before spawning. Regular changes also occur in the heat-resistance of the tissues of *R. temporaria* depending on phases of their sexual cycle (Schlachter, 1961; Pashkova, 1962).

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DISCUSSION

Discussion of G. I. Poljansky and K. M. Sukhanova's Paper

C. L. PROSSER: I would like to ask Prof. Poljansky whether adaptive changes in the thermostability of Protozoa appear as a result of selection during a prolonged cultivation, or not?

G. I. POLJANSKY: No selection was possible in our experiments because we cultured isolated lines. Moreover, changes in the thermostability appeared very quickly. A cytological control was always carried out. The investigation was performed on *Paramecium caudatum*, which is known to have no periodical autogamy. We have found that genetic differences do exist between individual populations, and genetic mechanisms determining the thermostability are now under a special study.

The data presented here dealt, however, with individual adaptations of a number of lines from single paramecia, which change their thermostability very quickly.

Long-term modifications (Dauermodifikationen) can arise during a prolonged cultivation and they may become superposed with the individual changes of thermal resistance. We are studying this phenomenon now, but I did not dwell upon it specifically.

N. L. FELDMAN: I wonder what is your opinion towards the cause of the change in the metabolic pattern of *Paramecium* during development of temperature adaptation: is it a change in the temperature optimum of enzymatic activity or an exchange of one enzymatic system for another?

G. I. POLJANSKY: Investigations of such kind have only been initiated and I hope I shall give you a more precise answer some time later. However, I suspect the former possibility to be more probable.

V. Y. ALEXANDROV: Did you study the thermostability of parasitic Protozoa from two different species of frogs living in the same water basin?

G. I. POLJANSKY: Such experiments were performed. Species differences in the thermostability of Protozoa proved to persist in spite of the fact that the host species inhabited the same water basin. However the presence of two or more host species in one and the same basin does not mean that they exist in identical temperature conditions. Microclimatic temperatures could be different as a result of different behavior.

C. L. PROSSER: We have to distinguish between adaptation processes in the zone of normal and of extreme temperatures. What were the temperature limits used in your experiments?

G. I. POLJANSKY: Most experiments were carried out within the limits of natural temperatures. For ciliates, these temperatures range from 0 to 25°C. In experiments on supercooling at -15°C, we did not study enzymatic systems. Our supposition on metabolic changes which occur in the course of adaptation is based on our experiments on the action of some metabolic inhibitors on the ciliates. We made no direct determinations of enzymatic activity.

Z. I. BARBASHEVA: I do not understand how ciliates of the same species can have different thermostability, if thermostability is determined by the properties of proteins, and the species properties of proteins are conservative.

G. I. POLJANSKY: The thermostability of Protozoa, as well as that of metazoan cells, is thought to be determined by properties of the proteins. Judging by the high Q_{10} values, we are dealing, during measurement of the thermostability, with protein alterations of the irreversible denaturation type. But upon changing the cultivation temperature, the thermostability becomes modified very strongly, by several times, far exceeding the range which needs statistical evaluation. This fact is beyond any doubt. Shifts of the thermostability of Protozoa are apparently connected with reversible changes of the properties of proteins. It is difficult to say now, of what kind these changes might be exactly. But the fact that changes of the environmental temperature produce entirely different effects on the thermostability of Protozoa on the one hand, and on that of metazoan cells, on the other, remains certain.

Z. I. BARBASHEVA: Let me not agree with the opinion of G. I. Poljansky, that system adaptations acquire greater significance, the higher is the complexity of organization. System adaptations are better developed, the higher the level of organization. However, they are used by the animal only when a rapid adaptation to changed environmental conditions is necessary. Upon a prolonged action of altered conditions, and a true adaptation or acclimatization, cellular adaptation is the most important part of the adaptation mechanism.

The action of various factors, unlike as they might be, gives rise to a non-specific resistance. Thus, action of low temperatures and hypoxia produces a simultaneous increase in resistance of mammals to radial acceleration, cramp poisons, viruses and bacteria, to anaemia, overheating, etc. This non-specific rise of resistance is based upon changes of the cellular metabolism (increase of the activity of respiratory enzymes, of glycolytic processes, etc.). Such a non-specific rise of stability is caused by factors inhibiting the metabolism (hypoxia, low temperature, inhibition of the activity of endocrine glands). Factors intensifying metabolism produce quite an opposite effect: the thermostability of cells was found to decrease following an injection of thyroxine (I. M. Pashkova) or a prolonged action of a high temperature (K. Schlieper). Perhaps, this is the reason why the adaptation to cold is accompanied by an

increase in resistance to overheating, while training to a high temperature does not increase the resistance to cold.

P. P. RUMYANTSEV: I would like to speak about the most interesting observations made by G. I. Poljansky and K. M. Sukhanova in respect to the significance of the level of organization for the determination of the character of temperature adaptation of the organism. The authors compared organisms of different steps of organization. It is possible, however, to investigate some other aspects of this problem as well. It is known that cultivation of cells in tissue culture results both in removing the "organismal" integrating mechanisms and in revealing the phylogenetically ancient properties of cells.

In our experiments, cultivation of the frog myocardial pieces proved to be insufficient to reveal the ability of cells to change easily their thermoresistance in response to the changes of the cultivation temperature. At the same time, as it is mentioned in my abstract, the dedifferentiation of cells leads to a change of their thermoresistance character. I suppose, that in my experiments not a good method of investigation was used: tissue integration appeared still preserved in the piece of the myocard. Moreover, one must keep in mind that heart tissues are functionally specific ones. A method of cultivation of cell suspensions by means of a constant shaking, which Dr. De Druyn showed me in Amsterdam, would perhaps have been better. It might be possible to compare the cells in such a culture with Protozoa. Experiments of this type might give an answer to the question under discussion.

B. P. USHAKOV: The difference in the character of the reactions of Protozoa and of metazoan cells was revealed very clearly in the experiments carried out simultaneously in our laboratory and in that of G. I. Poljansky. In winter frogs, kept at various temperatures, the thermostability of the whole organism has been studied as well that of its parasitic Protozoa, of ciliated epithelium cells, and of spermatozoa. During a very short interval (5 days) the thermostability of the frog organism and that of intestinal Protozoa changed, while the thermostability of the ciliated epithelium and that of the spermatozoa persisted at the initial level. (These results appeared to be opposite to our *a priori* supposition that the thermostability of spermatozoa would follow the temperature of the environment as it does in the case of Protozoa). A necessary reservation: these experiments were carried out in winter; now we have reasons to believe that we could have obtained quite a different result in summer.

G. I. POLJANSKY: I share completely the views of P. P. Rumyantsev; it would be really very interesting to carry out extensive investigations of the cellular resistance in conditions of the disturbed integrating system. Concerning the critical remarks by Z. I. Barbasheva, I would like to point out the necessity to distinguish facts from hypotheses. Our hypothesis may be somewhat not very good and questionable, but the facts prove definitely,

that the character of temperature adaptations is quite different in Protozoa and multicellular organisms. Naturally, this does not mean that adaptations on the cellular level are lacked by Metazoa.

Discussion of A. V. Zhirmunsky's Paper

H. PRECHT: I address my question not only to Dr. Zhirmunsky but also to his collaborators.

You have found no change in thermostability of muscles on adaptation to heat in the majority of higher animals examined in winter. In actinians in contrast to Protozoa and hydras' tissue thermostability turned out to be rather conservative. On the other hand, certain observations during the spawning time show that hormonal influences lead to changes in the thermostability of tissues (thyroxine decreases for instance heat-resistance). Therefore, all changes of the activity of the thyroid gland should cause changes in the heat-resistance of the muscle tissue. The fact that you never found such changes in winter time, although the animals lived in different adaptation temperatures, would logically point out that thyroid gland in its activity is not influenced by adaptation temperature. This is also valid for the other hormonal glands which, according to your experiments, change the thermostability of tissues. The hormones of all these glands can have no importance for capacity and resistance adaptation. Prof. Rao would not agree with this conclusion. In our laboratory we found (1) cases of changes of the resistance of tissues and isolated organ functions at extreme temperatures with adaptation temperature, though sometimes these changes are of paradoxical character. We did not examine so many species as you did, which allows you to speak of a rule, while our findings may be exceptions. But Prof. Schlieper and other authors made observations like ours, examining also few species. (2) We found facts which indicate the importance of the thyroid gland for capacity adaptation (for instance in the experiments of Jankowsky with frogs). Jankowsky did not consider the influence of light. But also the observations of Suhrmann and others seem to prove that hormones may play a role in temperature adaptation. (3) Measurements of the activity of the thyroid gland of fishes and amphibians held at different temperatures have often shown an influence of adaptation temperature (see Precht, *Helgoländer wiss. Meeresforschg.*, in print).

You may object that my experiments with eels in regard to capacity and resistance adaptation prove a direct influence of adaptation temperature on the tissue and exclude a co-operation of higher systems like hormones, because the adaptation of the muscle tissue of eels with differently adapted anterior and posterior ends depended on the adaptation temperature of each part. But I think, these observations should not be generalized. I would propose that you examine also the capacity adaptation in the normal range of temperature, because your findings exclude logically also the participation of the mentioned hormones on capacity adaptation.

B. P. USHAKOV: I shall try to answer this question, because it is addressed also to me. The data of hormonal influence on cell thermostability, seem very contradictory. However, they can be made a bit clearer, thanks to studies performed by I. M. Pashkova. The acclimation of animals in laboratory conditions was not found to result in changes of thermostability in autumn and winter, the periods of no general or reproductive activity. At this same time the cell thermostability in nature is also kept at a constant level. In this period, according to some authors, temperature fluctuations do not influence the activity of endocrinal glands. Different results are obtained when animals are examined in the active period of their life. The rise in environmental temperature accelerates the course of natural seasonal hormonal changes. Under these conditions the changes of cell stability are observed. Till recently we performed our experiments on acclimation only in winter time. Now we consider it important to carry out our work in summer time as well. In this case it is likely we shall obtain results similar to those reported by Prof. Schlieper, Prof. Precht and some other investigators. I hope we shall make our experimental contribution for the solution of the question touched upon by Prof. Precht.

C. L. PROSSER: What do you think about the differences in the response of an intact organism and of its cells?

A. V. ZHIRMUNSKY: The response of the whole organism to the temperature changes is quite opposite to that of cells. We kept actinians from the Barents Sea in cold and heat. In these conditions the thermostability of the organisms changed very quickly, while the thermoresistance of cells appeared to be unchanged. Thus, you see, that the temperature reactions of the whole organisms are very labile. As to the cell reactions, they possess a considerable conservatism.

H. PRECHT: What are, in your opinion, the causes of the change in the thermostability of actinians, as an example of whole organism acclimation?

A. V. ZHIRMUNSKY: I am sorry to say but we have just only established this fact and have not had enough time to study it. I believe, however, that these changes can be connected with metabolic changes, as it was shown in Fox's experiments on medusae, which had different intensity of respiration depending upon the rearing temperature.

B. P. USHAKOV: In connection with Prof. Prosser's question, I would like to note that while speaking of the conservatism of cell thermoresistance it is absolutely necessary to distinguish between "capacity adaptation" and "resistance adaptation". When we speak of the conservatism we mean by this only the "resistance adaptation". It should be taken into consideration, that in our experiments we usually deal with irreversible thermal injuries of cells and protoplasmic proteins. "Capacity adaptation" is revealed very often on acclimation.

We also have some papers in which functional changes as well as variations in lipid contents and enzymatic activity on acclimation are described.

M. A. ROSIN: I should like to call your attention to the experiments carried out by myself and my colleagues and consider them from the viewpoint of the problem of the resistance of the whole organism and its cells. We studied the influence of some benzimidazole derivatives upon the cell resistance. Some of these combinations were shown to increase the resistance of the frog sartorius muscle to the action of a high temperature (36°C), the resistance of frog's cardiac nerve cells to hypotonic solution, the resistance of frog's sciatic nerve to a local mechanical pressure, and finally, the resistance of cornea epithelium of frogs and mice to the action of ethanol etc. Among these combinations 2-benzyl-benzimidazole (dibazole) appears to be most effective. A positive effect of dibazole upon resistance was usually revealed both after the injections of dibazole solution into the animals and after its direct action upon isolated cells. Dibazole displays its action within a very wide range of concentrations: from 1×10^{-5} to 1×10^{-11} .

The effectiveness of benzimidazole derivatives depends upon the hormonal conditions in the organism. For example, the effect of dibazole upon cell resistance appeared very insignificant, if any, while spring frogs were examined. However, it appeared very clearly in winter. The derivatives of benzimidazole investigated did not increase the resistance of hypophysectomized frogs. Only after hypophyseal injection was made to the hypophysectomized frogs, the cell resistance of some tissue of the latter increased as in cases of both injection of dibazole solution and its direct influence upon isolated cells.

Discussion of C. Schlieper's Paper

V. Y. ALEXANDROV: You obtained changes in thermostability upon a prolonged keeping of the molluscs at 10 and 25°C . The temperature of 25°C is superoptimal. Have you observed any changes in the cell thermostability when the animals were kept under optimal temperatures?

C. SCHLIEPER: The experiments on *Modiolus modiolus* and *M. demissus* were carried out only at 10 and 25°C in summer and at the very beginning of the autumn. These littoral species are very easily acclimatized to the high temperature. However, I did not carry out any prolonged experiments with stenothermal animals.

B. P. USHAKOV: What is your explanation of the complicated process of development of an animal's resistance on acclimation (increase, decrease, the second increase and decrease again)?

C. SCHLIEPER: I think such a complex process can be explained by the changes of physiological state of the molluscs. The thermostability decreased at the end of acclimation period because of starvation. A similar phenomenon

can be observed in the process of acclimation to the reduced partial pressure of oxygen.

A. V. ZHIRMUNSKY: I am glad to have the opportunity to say a few words concerning the question touched upon by Prof. K. Schlieper and Dr. I. V. Ivleva. Prof. Schlieper and Dr. Kovalskaya showed that the changes in cell thermostability may occur due to changes in the salinity. These data were

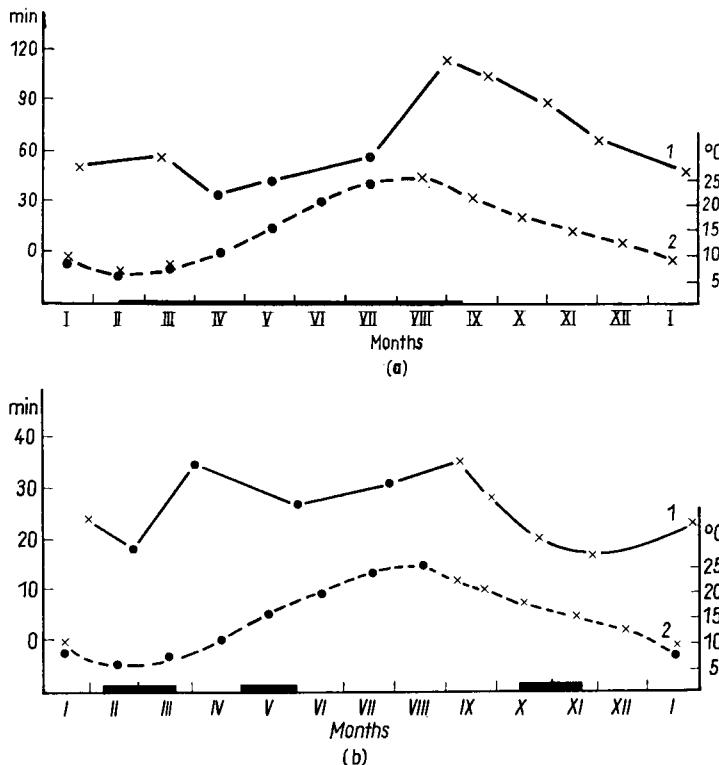


FIG. 1. The survival time of glimmering epithelium cells of *Actinia* (a) (from Dregolskaya, 1962) and *Mytilus* (b) (from Dregolskaya, 1963) from the Black Sea under the effect of high temperatures (a—39°C, b—38°C)—1; the temperature of sea water in different months—2. Abscissa—months, ordinate: to the left—the survival time of cells in min, to the right—the temperature in °C. Solid circles—measurements made in 1959; crosses—measurements made in 1961. The period of reproduction is marked by the thickened line on the abscissa.

confirmed by a number of other findings and they must be taken into account in experiments to be performed on all sea animals. However, I. N. Dregolskaya obtained different data concerning the change of cell thermostability of hydras. Now it is known for sure that hydras constitute the only group of

animals which change their cell thermostability very quickly and according to the environmental temperature.

Dr. Gorodiliv's experiments showed that the cell thermostability of Arenicola changed according to the increase of the sea water temperature. However, there is no evidence that the environmental temperature *per se* influences these changes.

Very interesting data were obtained by Prof. K. Schlieper which concern the changes of cell thermostability in the littoral molluscs and of the absence of similar changes in the sublittoral molluscs. In connection with these experiments are the data reported by Dr. Dregolskaya and Dr. Zhirmunsky on acclimation of the littoral actinians *Bunodactis stella* and *Actinia equina*. A prolonged keeping of the actinians in "cold" and "warm" conditions revealed no change in the thermostability of their glimmering epithelium cells. Thus, cell thermostability of the littoral actinians unlike that of the littoral molluscs, is conservative. It is possible that the change in reactions of the littoral molluscs is closely connected with the seasonal rhythm of their cell thermostability.

Dr. Dregolskaya has shown that in some sea invertebrates, in *Actinia* and *Mytilus* in particular, the cell thermoresistance undergoes some seasonal changes connected with the reproductive cycle of the animals. In Fig. 1 is pictured the heat-resistance of *Mytilus galloprovincialis* and 3 reproductive periods marked by thick lines on the abscissa coincide with 3 falls of the cell thermostability of the mollusc.

Dr. Pashkova kept grass frogs at different temperatures and showed that the altered temperature can accelerate or retard seasonal changes in cell thermoresistance. That is why the changes in the thermostability of the molluscs examined by Prof. Schlieper may be connected with seasonal changes. The other possibility can be also admitted; I mean the changes of thermostability as a result of the organismal stress-reaction in response to a sharp alteration in the environment. However, in this case the thermostability must return to the normal level after some time, as it was demonstrated by Dr. Pashkova, myself and Dr. Schlachter.

I would like to make another brief remark concerning Dr. Feldman's paper. As it was previously reported, with special reference to sea animals, the thermostability of the whole organism is connected with the vertical distribution of these animals in the sea. However, some opposite data are also available. We believe that this contradiction depends on the fact, that the whole organism can easily change its thermostability according to the environmental temperature. Therefore, some investigators would observe different values for the thermostability in one and the same species inhabiting different littoral horizons. When one deals with investigation of cell thermostability, more precise results are usually obtained because the thermostability of cells, unlike that of the whole organism, is much more conservative.

N. L. FELDMAN: I would like to say some words in respect to papers read

by Dr. Zhirmunsky and Prof. Schlieper. The fact is that cell thermostability not only of the animals but also of plants inhabiting the littoral zone is in conformity with the environmental temperature. Cell thermostability of sea grasses and algae is different in various littoral horizons. The cells of *Zostera nana* from low-tide zone usually die after 5-min heating at 42.6°C. By contrast, the cells of *Zostera marina* from a intertidal zone die following 5-min heating at 36.3°C. Similar examples were given by Dr. Lutova concerning 4 *Fucus* species. The plants from the upper littoral horizons undergo greater fluctuations of the temperature than the plants from the lower ones, and this accounts for their possession of higher thermostability in comparison with the plants from sub-littoral and from lower horizons of littoral.

THE ROLE OF SH AND SS IN THE RESISTANCE OF CELLS TO HIGH AND LOW TEMPERATURES

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THE physical and chemical environment of a plant may lead to injury in many different ways. The ability of the plant to survive such potentially injurious environments may be called environment resistance. Plant resistance against several such injurious factors is known. In all cases, there are only two basic methods of resistance available to the plant: (1) it can exclude the injurious factor from its tissues and (2) it can survive the penetration of its tissues by the injurious factor. The first method is called avoidance, the second tolerance or hardiness. These are the theoretical possibilities. But each kind of injury must be examined individually in order to find out whether or not the plant has succeeded in developing the two kinds of resistance. Table 1 shows the possibilities in the case of several factors.

TABLE 1. TWOFOLD NATURE OF RESISTANCE TOWARD AN INJURIOUS FACTOR

Environmental factor	Condition of resistant plant cells possessing	
	Avoidance	Tolerance
1. Low temperature of chilling	Warm	Cold
2. Freezing	Unfrozen	Frozen
3. High temperature	Cool	Hot
4. Drought	High vapour pressure	Low vapour pressure
5. Radiation	Low absorption	High absorption
6. Salt (high conc.)	Low salt conc.	High salt conc.
7. Flooding (O_2 def.)	High O_2 content	Low O_2 content

Since plants are poikilotherms, they are unable to develop low temperature avoidance. Freezing avoidance, of course, occurs in seeds and other equally dry plant parts. Even some normally moist plant parts have been found to avoid freezing at subfreezing temperatures. But in the vast majority of cases the plant does not possess enough frost avoidance to remain unfrozen throughout a winter in cold climates. Therefore, low temperature resistance is nearly always frost-tolerance.

Heat-resistance is due to avoidance in the case of "under-temperature plants" (Lange, 1959). But these plants are rare, and again, just as in the case of low temperature resistance, high temperature resistance is nearly always tolerance. Consequently, although the resistances dealt with here are really frost-tolerance and heat-tolerance, they should include nearly all low temperature and high temperature resistance.

The relation of SH to frost resistance has been shown in many ways (Levitt *et al.*, 1961, 1962; Levitt, 1962; Schmutz *et al.*, 1961; Waisel *et al.*, 1962). When cabbage or saxifrages are frozen, and their SH content determined before and after the freeze, no appreciable change occurs if the freeze is non-injurious, but there is a marked decrease in SH if the freeze kills the plant (Fig. 1). In the case of vernalizing wheat, it has been possible to show the same effect, though it is quantitatively smaller, and the decrease in SH content

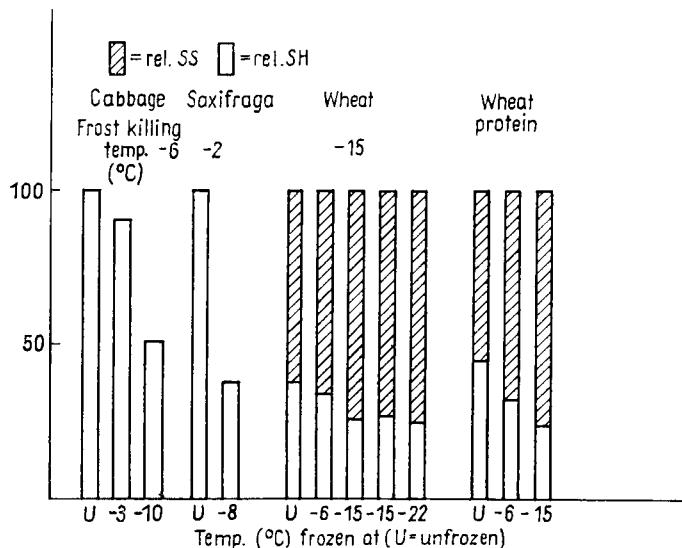


FIG. 1.

is accounted for by an equivalent increase in SS (Fig. 1). Similar results have been obtained in the case of heat injury (Table 2). It is, of course, difficult to prove whether this conversion of SH to SS is the cause or the result of the injury. When the proteins are isolated, however, a similar conversion of SH to SS has been found in them (Fig. 1). But in this case, a small conversion of SH to SS occurred even at a freezing temperature above the killing point. This result favours the concept that the SH → SS conversion precedes the injury and is, therefore, more likely to cause it than to result from it.

Recent results have shown a similar conversion of SH → SS as a result of chemical injury (Mazzolani, unpublished), though it is again difficult to prove whether the conversion is cause or effect.

The above results indicate a relation between conversion of SH → SS and injury due to freezing, heat, and other factors. But what is the relation, if any, to resistance? As mentioned above, we are concerned here with tolerance, e.g. the ability of the plant to survive freezing of its tissues. Since many plants readily increase in frost-tolerance (or hardiness) on exposure to low temperatures, it is easy to determine the effect, if any, of such a hardening process on the

TABLE 2. EFFECT OF HEAT KILLING (15 min AT 58°C) ON SH CONTENT OF SUPERNATE FROM KHARKOV WHEAT AFTER ABSORBING 60 g H₂O PER 100 g GRAIN

	SH	SH + 2SS	% SH (of SH + 2SS)
<i>A. Unvernalized (3 days at 20°C)</i>			
Control	0.25	0.85	30
Heat-killed (1)	0.16	0.80	20
(2)	0.12	0.90	13
<i>B. Vernalized (40 days at +3°C)</i>			
Control	0.52	1.36	38
Heat-killed	0.20	0.96	21

plant's SH content. Repeated analyses have shown an increase in the SH content during at least the early stages of the hardening process and, in fact, a series of wheat varieties differing in hardiness showed an excellent correlation between hardiness and the SH content, though the sugar content was not correlated (Table 3). But these results are highly unexpected, for it has long been known that rapid growth is associated with high SH content and low frost-

TABLE 3. FROST-HARDINESS AND SH CONTENT IN WHEAT VARIETIES
(From Schmutz, Sullivan and Levitt, 1961)

Variety	Hardiness from field experience	Frost killing temp. (°C)	SH content of supernatant (μ mol/gfw leaves)
Anna Migliori	Very hardy	-15	0.84
Carsten VIII		-14	0.93
Eroica II		-12.5	0.84
Criewener 192	Hardy	-12.5	0.81
Derenburger Silber		-12.5	0.77
Austro Bankut		-12.5	0.73
General v. Stocken	Moderately hardy	-12.5	0.65
Pfeuffers Schernauer		-12.5	0.52
Heine VII		-11	0.58
Panter		-11	0.49
Etoile de Choisy	Slightly hardy	-12.5	0.46

resistance. In agreement with this relationship and in spite of the increase in the SH content found during hardening, tender plants that were growing rapidly and unable to harden actually did have the highest SH content. Furthermore, Ivanov (1939) had earlier found an inverse correlation between the GSH content and hardness in citrus.

We are, therefore, faced with the paradoxical situation in which both hardness and lack of hardness are associated with high SH. This was resolved when it was found that the increase in SH during hardening was more apparent than real. It was actually an artifact that arose due to the more rapid oxidation of the SH in the non-hardy material. In other words, hardness involved a high resistance of the SH-groups to oxidation, and the SH content of the hardy plant might actually be lower than that of a non-hardy, rapidly growing plant, if it could be determined under conditions that completely eliminate such oxidation.

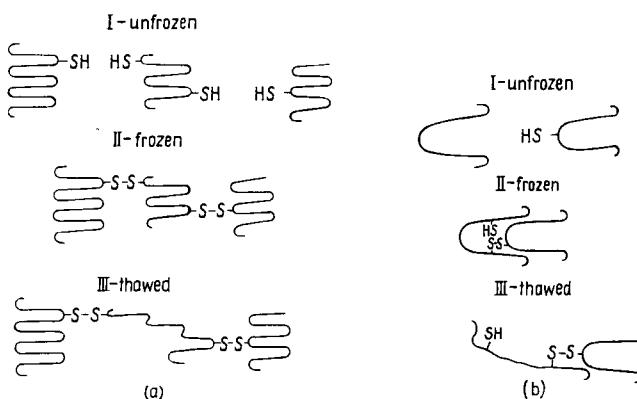


FIG. 2.

But the apparent increase in SH on hardening is just as important as if it were real, even though it arises during the preparation of the sample. It can readily explain the results obtained when the plant is frozen. If the plant is frost-resistant, it can be frozen without injury because its SH-groups resist oxidation to SS. If the plant is not resistant, freezing leads to an oxidation of SH to SS. The role that freezing plays in the process is to dehydrate the protoplasm and to bring the SH-groups of adjacent proteins close enough together to permit them to react with each other. The way in which this may conceivably occur is illustrated in Fig. 2. Due to the formation of inter-molecular SS bonds, adjacent protein molecules may become joined together on freezing, and this may lead to unfolding of the molecules on rehydration during thawing.

It should be emphasized that the links may occur due to $\text{SH} \rightleftharpoons \text{SS}$ interchange and therefore do not require oxidation of the SH. This type of junc-

ture between protein molecules has been found to occur in artificially thiolated proteins (e.g. thiogel). The interchange is due to a chain reaction which may be initiated by a small-moleculed SH substance. This fact may explain why actively growing tissues have high SH contents and cannot survive freezing. The high SH may be partly due to glutathione (GSH) which can act as the primer for such chain reactions between proteins. Conversely, during the hardening process, the GSH oxidizing activity of the tissues increases and all the GSH is oxidized to GSSG. In this form, it can no longer act as a primer, and the tissues can be frozen without initiation of this chain reaction.

In this way we can explain both the $\text{SH} \rightleftharpoons \text{SS}$ conversion during freezing that is severe enough to injure, and the apparent SH increase during hardening that is really a resistance to SH oxidation. This hypothesis can lead to an understanding of many other aspects of frost-hardiness that have not been satisfactorily explained until now.

The role of sugars and other protective substances can readily be explained if they accumulate in the protoplasm. Here they would hold water osmotically against the dehydrating effect of freezing and would therefore help keep the SH-groups of adjacent proteins far enough apart to prevent or slow up the reaction. It would, therefore, require a lower freezing temperature in order to dehydrate sufficiently to bring the protein molecules close enough for reaction. There is, in fact, no aspect of frost-hardiness that cannot fit into this hypothesis. Much indirect evidence in favour of it is available in the literature. Hartley *et al.* (1962) have revealed the presence of dimers in bovine plasma albumin due to the tying up of the SH groups in SS linkages between two protein molecules. They believe that these are preparation artifacts that can be formed by ethanol. Since ethanol dehydrates the proteins in the same way as does freezing, this result supports the above hypothesis. Spragg *et al.* (1962) decreased the SH: SS ratio in proteins of pea seeds and found that germination was decreased. They interpreted their results to mean that chemical reactions take place *in vivo* between GSH and protein SH.

But it should be emphasized that the above is still a hypothesis based on indirect evidence. It is still necessary to obtain direct evidence before it can be established as a theory. It is necessary to prove: (1) that proteins do unite as a result of intermolecular SS formation during injurious freezing, (2) that this does lead to unfolding and therefore denaturation of the proteins. If these two points can be proved, the hypothesis will open up a whole new field of research and may conceivably lead to a new method of artificially inducing frost-resistance.

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BIOCHEMICAL MECHANISMS OF THE DEATH OF PLANTS AND THEIR TOLERANCE AND ADAPTATION TO HIGH TEMPERATURE IN NATURAL CONDITIONS

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PHYSIOLOGICAL processes occurring in plants under the influence of high temperatures attracted the attention of scientists after the discovery of irreversible damage of metabolism caused by a prolonged, intermittent effect of super-optimal environmental temperatures. Lethal temperatures are not typical even for the southern regions of the U.S.S.R., but super-optimal temperatures occur very often in middle and northern latitudes of the vast agricultural territory. Moreover, the data of our laboratory shows that the temperature of the plant is often higher than that of the ambient atmosphere because of the impairing of some physiological functions. We know perfectly well the visual signs of temperature injuries, but we know much less about the effect of temperature on the growth and productivity of plants. This means: depression of mitosis, retardation of growth, anomalous development of the flower, prolonged fading (non-infectious), functional diseases of hot-house plants and others. All this shows the importance of a high temperature effect on plants in natural conditions.

In the early thirties Professor Sukhorukov (1936) studied the effect of high temperatures of the soil and the atmosphere on the plant under natural conditions (prolonged effect and gradual rise and fall of temperature; different effect on different organs). Sudden exposure to sublethal and lethal temperatures made mechanisms active other than those which existed when super-optimal maximal temperatures were gradually applied.

That gave rise to a new trend where the emphasis was laid on studying changes of metabolism which we think to be the principal link in the chain of phenomena from the initial temperature effect up to the final response of the organism. With the increase of temperature effect (temperature vs. time), if there is enough moisture available, we have first a decrease and then an increase of cytoplasmic viscosity, a decrease of biocolloid dispersion, an increase in permeability, exosmosis of electrolytes and non-electrolytes, vacuolization and granulation of cytoplasm, lipophanerosis and in some cases

lysis of the organoids of the cell (Fig. 1). A gradual increase of temperature for many days ($38\text{--}52^{\circ}\text{C}$) causes changes of the nuclei in the cells of the periblem tissues of the root tip of the wheat seedlings, which means great changes

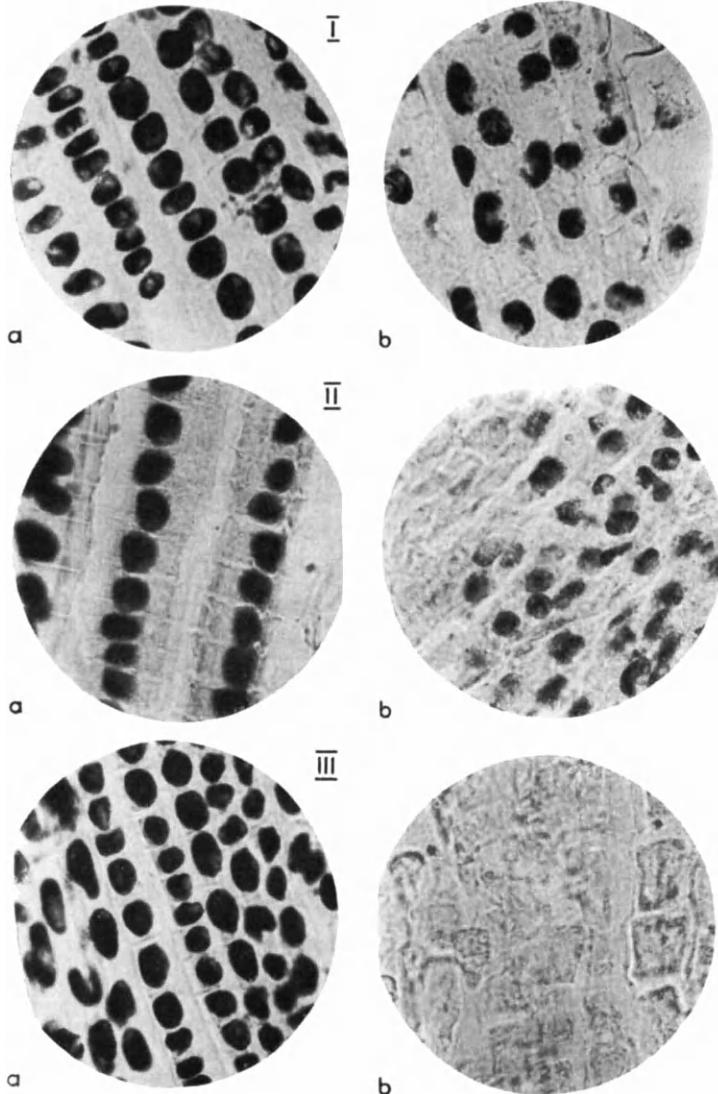


FIG. 1. Change in the cell nuclei of the periblem tissue of a Milturum 321 wheat root (3–5 mm from top) under the influence of increasing temperatures. I: 4 days; a (control): $22^{\circ}\text{C} \times 48\text{ hr}$; b (experiment): $38^{\circ}\text{C} \times 24\text{ hr} + 42^{\circ}\text{C} \times 24\text{ hr}$. II: 5 days; a: $22^{\circ}\text{C} \times 72\text{ hr}$; b: $38^{\circ}\text{C} \times 24\text{ hr} + 42^{\circ}\text{C} \times 24\text{ hr} + 48^{\circ}\text{C} \times 24\text{ hr}$. III: 6 days; a: $22^{\circ}\text{C} \times 96\text{ hr}$; b: $38^{\circ}\text{C} \times 24\text{ hr} + 42^{\circ}\text{C} \times 24\text{ hr} + 48^{\circ}\text{C} \times 24\text{ hr} + 52^{\circ}\text{C} \times 24\text{ hr}$. Staining by Feulgen. Magnification $\times 1000$.

Experiments of K. P. Volgina and M. P. Andronova.

in the molecular structure and final disintegration of the nucleic acids. Successively we saw a depression of mitotic division, swelling, loosening, partial or complete dissolving, diffusion of the nuclear substance into cytoplasm (karyolysis). This sequence is analogous to the lysis of the nucleus of the hyphae of *Fusarium* when the growth of the mycelium is checked under the influence of increasing temperature or high concentration of growth substances, phenomena which were discovered in our previous investigations (Altergott, Lavygina and Kuvshinova, 1941). Karyolysis shows that as a result of the increase of temperature (or with the prolongation of the time of temperature action) the processes of depolymerization and disintegration become more pronounced (Altergott, 1936, 1937, 1960). This result becomes the cause of the development of the protective qualities of the organism, because the appearance of new, more tolerant structures and processes is possible if the original ones are partly impaired, and the phenomenon is reversible. The activity of the enzymes of respiration, phosphorylation, exchange of nucleic acid and protein are of primary importance when heating is very long. High thermal optima and maxima of peroxidase, polyphenoloxidase, cytochromoxidase account for the specific oxidation processes in the cell. The effect of respiration will be changed, if the synthesis in the cell is depressed. (Sukhorukov, 1957; Altergott, 1960, 1963). At a certain stage the increase of the oxidation potential in the cell can be regarded as a protection reaction against poisonous products of metabolism, which are made harmless by oxidation.

Together with the oxidation of sugars, which, in addition, block sensitive groups of protein, many compounds are intensely oxidized—ascorbic acid, glutathione, tannin, anthocyanins and sometimes proteins (Sukhorukov and Novoselova, 1952; Altergott, 1963). Intensive disintegration of P-organic

TABLE 1. PHOSPHORUS COMPOUNDS IN TWO-DAY ALBIDUM WHEAT SEEDLINGS
UNDER INFLUENCE OF RISING TEMPERATURES (P μ g PER ABSOLUTE DRY WEIGHT
OF ONE PLANT)

Conditions. I: $37^\circ \times 24$ hr; II: $37^\circ \times 24$ hr + $42^\circ \times 24$ hr; III: $37^\circ \times 24$ hr + $42^\circ \times 24$ hr + $48^\circ \times 24$ hr; IV: $37^\circ \times 24$ hr + $42^\circ \times 24$ hr + $48^\circ \times 24$ hr × $52^\circ \times 24$ hr.
Control at 24°C .

P-compounds	Heating. Final temp. (°C)							
	I (37)	Control II (24)	Control III (42)	Control IV (24)	Control V (48)	Control VI (24)	Control VII (52)	Control VIII (24)
Lipoid	6.84	4.41	4.80	5.68	3.69	13.83	0.41	11.61
ATP	5.50	4.95	4.12	5.22	1.18	9.21	0.54	9.72
P-organic								
P-mineral	1.33	1.23	1.00	0.98	0.74	0.96	0.40	1.07

TABLE 2. NITROGEN COMPOUNDS, CHLOROPHYLL AND BIOS IN THE LEAVES OF TOMATO "LUCHSHI IZ VSEKH" (THE BEST) UNDER A PROLONGED EFFECT OF HIGH TEMPERATURE AND MOISTURE IN A HOOTHOUSE (32-37°C; 75-85%)

Leaves	Nitrogen, mg/g dry weight			Chlorophyll, percentage of dry weight	Free	Bios: the amount of yeast cells per unit area of tomato chamber		
	Total	Protein	Non-protein			Percentages of control (blank)	Bound	Percentage of control (blank)
Lower tiers, old, heavily damaged	30·13	24·06	6·07	0·76	0·50	400	0·25	200
Second growth slightly damaged	29·42	29·10	0·32	1·31	0·75	600	0·38	300
Young growth shoots	51·10	47·35	3·75	2·34	1·63	1300	1·25	1000

compounds and storage of mineral phosphorus are taking place. Some of the data worked out by K. P. Volgina and M. P. Andronova are given in Table 1.

In a complex of processes taking place in the plant at high temperature, depression of protein synthesis and check of nitrogen exchange accompanied by an accumulation of ammonia play the principal part. The last stage—pathological respiration causing the oxidative self-destruction of the plant is all important as a result of the thermal injury of the plant (Sukhorukov, 1936, 1957; Sukhorukov and Malysheva, 1955; Altergott, 1963). The impossibility of the use by injured cells of the energy of oxidative processes leads to increased heat emission (Sukhorukov, 1957; Altergott, 1963). In the above-ground parts of the plant injured by high temperature, easily resynthesized products of depolymerization and slight disintegration are stored, as well as biologically active compounds (auxin, biotin, vitamins, toxins, amides, organic acids and amino acids) which move from injured organs to physiologically active centres, and can be used by newly appearing organs. Such migration of the products of disintegration from the tomato leaves injured by a prolonged heat treatment in the hothouse, into newly appearing seedlings has been shown by G. A. Kotlyarova (Table 2).

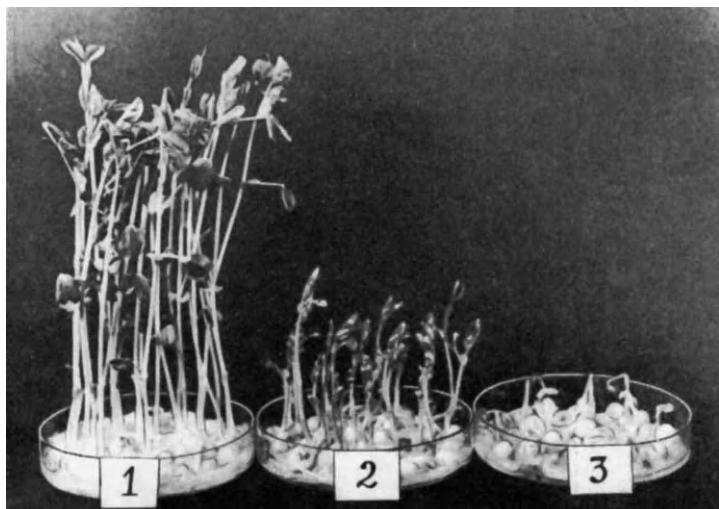


FIG. 2. Effects of different temperatures on 4-day-old pea seedlings. 1: 22°C (control). 2: Gradually increasing temperature (till 48°C): 12 hr at 38°C + 12 hr at 40°C + 12 hr at 42°C + 12 hr at 22°C (rest) + 2 hr at 48°C. 3: Quick rise of temperature till 48°C; 48°C—2 hr.

The protective capacity of the plant is determined by its inherited characters, and by the effect of high temperatures. With continuous depression of protein synthesis and growth, tolerance is determined by the presence of "protective" readily oxidized compounds, by the presence of oxygen in the

cell, by the formation of organic acids, by the concentration and the content of amino acids. Therefore, supplying the cell with sugars, amino acids, purine and pyrimidine bases, growth and protein synthesis stimulators renders toxins and ammonia harmless, facilitates the synthesis of constitutional compounds and increases the factors of protection capacity and tolerance (Altergott and Sevrova, 1960). When the plant is subjected to high temperatures intermittently and there are periods of rest in between, the plant acquires its principal protective function against different autotoxins—its growth capacity (Sukhorukov, 1957; Altergott, 1961). In our opinion, secondary growth of woody plants, appearance of shoots, inflorescences, bulbs and tubers when the functions are impaired, secondary flowering and fruit-bearing—all these are protective reactions of the plant as the aftermath of the effect of high temperatures (Sukhorukov and Novoselova, 1952; Altergott, 1961). Protective reactions of the plant against superheating are given diagrammatically in Fig. 2.



FIG. 3. Wheat *Albidum* 3700 in the phase of the beginning of ripening. Two-day seedlings were heated under conditions: $38^{\circ}\text{C} \times 12\text{ hr} + 40^{\circ}\text{C} \times 12\text{ hr}$ $+ 42^{\circ}\text{C} \times 12\text{ hr} + (22^{\circ}\text{C} \times 12\text{ hr}) + 48^{\circ}\text{C} \times 12\text{ hr} + (22^{\circ}\text{C} \times 48\text{ hr}) + 50^{\circ}\text{C} \times 2\text{ hr} + (22^{\circ}\text{C} \times 48\text{ hr}) + 52^{\circ}\text{C} \times 2\text{ hr}$.

Alternation of periods of reversible thermal injury with those of reparation of the injury is the basis of plant adaptation to high ambient temperatures. This two-step regime, which makes possible adaptation and induced heat-tolerance of the plant was adopted for induced breeding of seedlings. The increase of the stability of protein synthesis in these plants has been studied as well as the raising of the cardinal thermal points of the action of enzymes. Viable fruit-bearing seedlings of wheat have been grown with heating for 1–2 hr at 52–53°C (Fig. 3).

Practical methods of increasing heat-tolerance of plants are as follows:

1. Growing of a strong plant with high synthetic capacity using necessary fertilizers (chiefly phosphorus, potassium) and moisture (Altergott, Shatilova and Khuntsaridze, 1939a).
2. Frequent application, in regions of irrigation, of a repeated artificial rain at the time of subjecting the plant to high temperatures, which results in lowering the temperature of plant organs, in maintaining the necessary water supply, in washing out the ammonia and reducing the effect of microclimate (Altergott, 1937). Combining watering with the introduction of macro- and microelements, growth stimulators and compounds which stabilize protein synthesis is advisable (Genkel and Tsvetkova, 1950; Petinov, and Molotkovsky, 1956; Shkolnik and Makarova, 1958).

The above brief account contains the results of many years research of heat-tolerance of plants. It is a development, with the help of experiments, of the concepts of the author's teacher, Professor K. T. Sukhorukov, who has sponsored the work.

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THE EFFECT OF TEMPERATURE ON RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF PEA SEEDLINGS

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IN STUDIES on the adaptation of plants to environmental temperature conditions, much attention has been given to the respiratory process. Changes in respiration rates are generally made use of as a criterion of the functional resistance of the plant tissues. Lately, interest in respiration has increased due to findings which imply the possibility of alterations by unfavourable temperature conditions of the efficiency of respiration as an energy supplying process (Zholkevich, 1955; Petinov and Molotkovsky, 1960; Altergott, 1963 and others).

This report deals with that part of our investigations of plant respiration vs. temperature relations, in which measurements are made with mitochondria. There is, as yet, little evidence of the effect of temperature on plant mitochondria (Biale *et al.*, 1957; Lieberman *et al.*, 1958; Hall and Arnon, 1962). In our experiments the mitochondria were isolated from 7–20-day pea seedlings (“Green early” variety) grown in soil or on nutrient solutions. The method of mitochondrial isolation was described earlier (Mosolova and Sisakjan, 1961; Semichatova and Bushuyeva, 1963).

At first, the direct influence of temperature on the mitochondrial activity was studied, i.e. the oxygen uptake and the decrease of inorganic phosphate content in the media were estimated after the exposure of isolated mitochondria to different temperatures from +2 to 43°C. From each mitochondrial preparation several samples were examined at 20°C (control) and the rest—simultaneously—at higher (or lower) temperatures. The results were calculated as a percentage of the control.

It can be seen from Fig. 1 that the effect of temperature on oxygen uptake and binding of inorganic phosphate (P_i) by mitochondria is very different. The oxygen uptake increases with the increase of temperature up to 38–40°C while the binding of P_i at 25°C is already markedly depressed. At 35°C there is no decrease of P_i in the medium and in some experiments its content was even somewhat higher. The ratio P/O is greatest at +2°C and sharply de-

creases with the increase of temperature. At 35°C a complete uncoupling of respiration and phosphorylation takes place.

In order to evaluate the relation of these findings to the processes occurring in the intact cells, another series of experiments was performed. The intact seedlings were subjected to different temperature conditions and only after

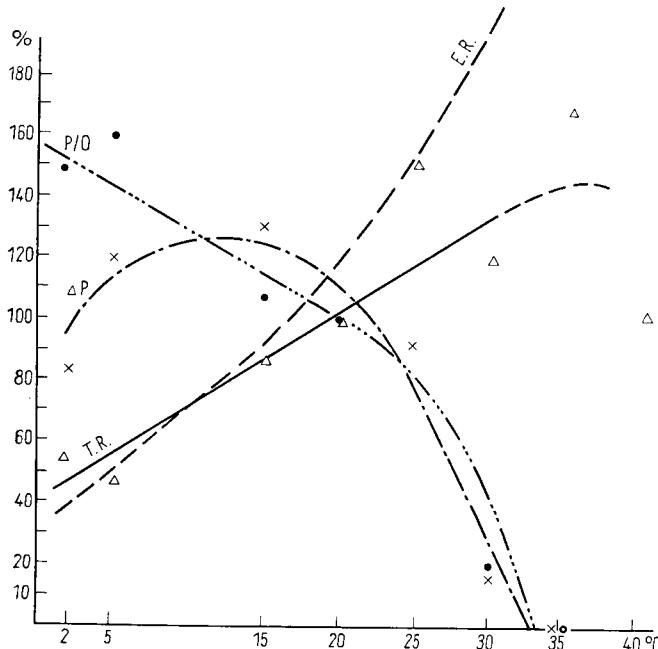


FIG. 1. The respiration, phosphorylation and P/O changes of isolated mitochondria at different temperatures (exposure of 1 hr) E.R.—endogenous respiration; T.R.—total respiration in the presence of succinate. ΔP —decrease in P_i content in the medium (bound P_i). The points represent mean values (for 2–3 experiments, 3 determinations in each) calculated in per cents of the control (20°C).

the temperature treatment were their mitochondria isolated. One sample of seedlings was kept for 10 min in water at the required temperature. The control sample was at the same time immersed in water at room temperature. The data expressed as a percentage of the control values are represented in Fig. 2. They are very different from those obtained on isolated mitochondria which had been treated by high temperature.

Significant alteration in the uptake of oxygen and P_i took place only after the exposure of seedlings to a temperature of 42°C . A higher temperature at first caused a similar depression of respiration and phosphorylation. Above 46°C phosphorylation was slightly more affected than was the uptake of oxygen. Hence, the change of P/O does not take place up to 46°C and in spite

of a gradual decrease in respiration rate its efficiency does not change until the depression by temperature attains nearly 70 per cent.

Now let us consider the possible explanations of the different effects of the temperature on mitochondria in the isolated state and in the intact tissue.

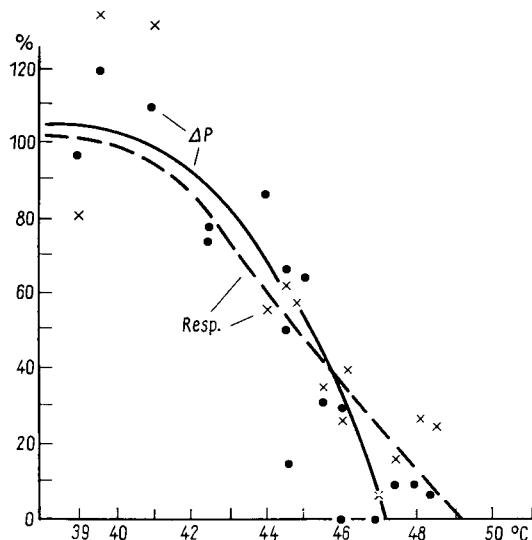


FIG. 2. Changes of the respiration in the presence of succinate (Resp.) and of phosphorylation (ΔP) by mitochondria isolated after a 10-min exposure of the seedlings to high temperature. Resp. = Total respiration—endogenous respiration. The points—as in Fig. 1. Measurements were made at 20°C.

First of all, attention must be drawn to the fact that in the first series of experiments (Fig. 1) the duration of the temperature treatment was 1 hr and in the second (Fig. 2) 10 min. This raised the possibility that isolated mitochondria might have been able to bind P_i even at higher temperatures than 35°C but only at the beginning of the exposure. However, by the method we used phosphorylation of even a short duration could be detected since in the presence of hexokinase the inorganic phosphate is fixed in a relatively stable compound with glucose. Thus this difference in exposure time could not be of much significance.

Differences between the effect and the after-effect of temperature could arise if the direct influence of temperature is highly reversible. Special experiments were undertaken to evaluate the possibility of such an explanation of the results. The isolated mitochondria were subjected to a temperature of 40°C for 10 min and then transferred to 20°C. Phosphorylation remained depressed. Evidently the temperature damage to isolated mitochondria is not reversible.

Rather, the reality of the cessation of phosphorylation in isolated mitochondria can be questioned because the process was measured by the decrease in the content of P_i in the medium. Figure 1 shows that the endogenous respiration of mitochondria is greatly increased with the increase in temperature. Apparently some substances in the mitochondria are liberated and oxidized. It would be desirable to verify whether a process similar to endogenous phosphorylation can take place, i.e. a process of phosphorylation of the inorganic phosphate that originates inside the mitochondria under the influence of high temperature. Such a process could at least partly account for the differences obtained. However, the irreversibility of the temperature damage to mitochondria does not support this possibility.

The fact that tests of isolated mitochondria cannot duplicate their behaviour under intracellular conditions was already stated in connexion with other experiments (Lehninger, 1956). Without going into a detailed analysis of the factors concerned in the greater temperature resistance of mitochondria when in the cells, we will only suggest that it is not a matter of protective sugars or ATP availability, since these substances were present in the media used for mitochondria.

TABLE 1. RESPIRATION AND OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA FROM PEA SEEDLINGS

Mitochondria isolated at 14,000 g with sucrose (0.3 M) and tris-HCl (pH 7.3). The reaction mixture included a final volume of 3 ml: sucrose 600 μM , tris-HCl 200 μM , glucose 150 μM , MgSO_4 3 μM , succinate 60 μM , K_2HPO_4 20 μM , AMP 3 μM , ATP 1 μM , hexokinase 0.2 mg, NaF 30 μM and 0.5–0.8 mg of protein N of mitochondria. All the values are in microatoms per mg of protein N. Averages from 3 determinations.

No. exp.	Temp.	Total respir.		Endo- genous respir.		Exo- genous respir.		P_i bound		P/O	
		Exp.	Contr.	Exp.	Contr.	Exp.	Contr.	Exp.	Contr.	Exp.	Contr.
1	39	4.0	4.1	1.5	1.0	2.5	3.1	3.0	3.1	1.2 ± 0.22	1.0 ± 0.10
2	40	4.8	3.4	1.2	1.2	3.4	2.2	3.2	2.0	0.9 ± 0.10	0.9 ± 0.17
3	41	3.3	3.9	0.7	0.8	2.6	3.1	1.9	2.0	0.7 ± 0.10	0.6 ± 0.10
4	44–45	5.8	8.1	1.3	1.0	4.5	7.1	5.8	11.6	1.3 ± 0.10	1.6 ± 0.10
5	44.5–45	3.5	4.8	2.0	2.1	1.5	2.7	0.3	2.1	0.2 ± 0.10	0.8 ± 0.10
6	44	6.7	8.6	2.7	1.5	4.0	7.1	5.8	6.7	1.4 ± 0.15	0.9 ± 0.22
7	45–46	4.1	7.5	2.1	2.6	2.0	5.6	1.4	4.5	0.7 ± 0.15	0.8 ± 0.13
8	46	2.2	4.2	1.4	1.2	0.8	3.0	0	4.0	0	1.3 ± 0.23
9	46	2.5	4.0	1.4	0.6	1.3	3.4	0.7	2.5	0.6 ± 0.10	0.7 ± 0.10
10	47	1.3	8.2	0.9	1.0	0.4	7.2	0	8.2	0	1.1 ± 0.10
11	47	1.9	3.3	1.0	1.0	0.9	2.3	0.4	1.8	0.4 ± 0.10	0.8 ± 0.10
12 ^a	48	0.7	2.0	0.4	0.2	0.3	1.8	0.1	1.0	0	0.6 ± 0.12
13	49	1.5	8.4	1.3	0.8	0.2	7.6	0.5	12.3	0	1.6 ± 0.10

^a Without the correction for protein N.

Recently, an opinion was presented that the uncoupling of oxidation and phosphorylation and the disturbance of energy supply due to it is the cause of the temperature depression of respiration and the death of the cells (Molotkovsky, 1961). But as was shown above, the decrease in the phosphorylation rate does not precede the suppression of respiration but starts with and proceeds simultaneously with it. This applies not only to the respiration of mitochondria but to the respiration of intact seedlings as well. Other experiments have shown that a significant depression of their respiration (more than 20 per cent) after a 10 min exposure to a high temperature occurs at the same temperature of 42°C which calls forth the decrease of respiration and phosphorylation in isolated mitochondria.

When disturbances of different processes take place at the same intensity of the damaging factor, the depression of one of these processes can be the cause of the decrease of the other (or others) only if they are very closely linked with one another.

But it is well known that an uncoupled oxidation is possible. Considerable variations in the P/O value in different experiments (see Table 1) provide the proof that in pea seedlings also the coupling of respiration and phosphorylation is rather loose. Thus a conclusion can be drawn that the decrease in respiration rate at high temperature under the conditions of our experiments cannot be attributed to the depression of phosphorylation. It is more likely that the disturbance of both respiration and phosphorylation is connected with the direct effect of temperature on the structural components of the cells.

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PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF PLANT CELL RESPONSES TO CONTINUOUS ACTION OF HIGH TEMPERATURE

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DURING eight years we carried out the following series of experiments: we transferred barley seedlings or separated 10 mm pieces of coleoptiles from optimal (17–18°C) to extreme (44°C) temperatures. The course of the most important physiological processes was followed in time, from the beginning of the heat treatment up to complete death of the cells. Thus we obtained, in a manner of speaking “history of the heat disease of the cell”. First, let us consider the results obtained from investigations of one property only, namely, the capacity of protoplasm to excrete or lose substances into the solution.

Figure 1 shows that at the beginning of the temperature exposure and at its termination, just before the death of the cell, the protoplasm tends to lose weakly water-soluble substances, including monosaccharides, amino acids and —one which is specially important—potassium (Belikov and Kirillova, 1962). It should be noted that an initial increase in the loss of substances is partially reversible, this fact being responsible for the wave-like character of the time course of the excretion. In other words, a thermal irritant causes the appearance of one of the paraneurotic signs (Nassonov and Alexandrov, 1940) just after its application to a plant cell.

Depending on the intensity of treatment the index under investigation changes only quantitatively: alterations affect the number and height of the waves and the time when the maximum occurs. Consequently, the injurious effect can be studied over a broad temperature range. However, the temperature of 44°C seemed to be preferable. Under lower temperatures the cells remain alive for a long period of time, whereas higher temperatures considerably increase the rate of disease.

Thus, by means of a comparatively simple test revealing changes in the permeability of protoplasm, one can see significant alterations in the functional state of a plant cell. Common features in responses of plant and animal cells are quite evident. It is well known for instance, that muscle excitability of

different animals (tested directly or through nerves) results in the liberation of potassium, inorganic phosphorus and other water-soluble substances (Troshin, 1956).

It should also be noted that the excretion rate of substances from excited tissues can be used to determine heat-hardening of plants (Belikov and Kirillova, 1959; Oleinikova, 1963).

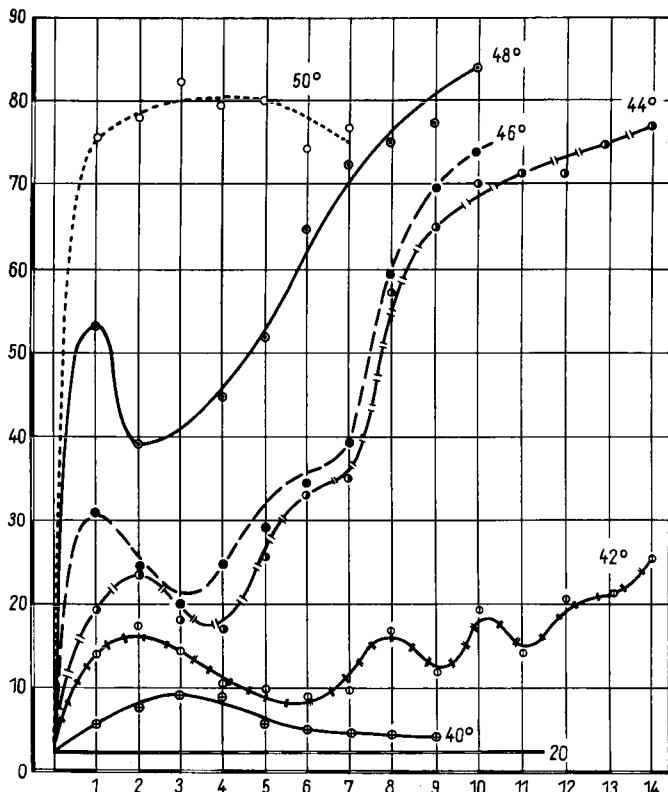


FIG. 1. Relation of the excretion of substances by barley coleoptiles to temperature. Abscissa shows hr. Ordinate shows units of interferometer scale.

However, let us follow the heat disease. Protoplasmic streaming increases (Fig. 2) and simultaneously one can observe the expenditure of high energy phosphates (Belikov and Dmitrieva, 1963), as well as free monosaccharides, first fructose and then glucose (Belikov and Dmitrieva, 1962). Respiration increases sharply, only to fall soon afterwards. However, for rather a long period of time (2 hr) it retains a level which is higher than that of the control. Hence, a stimulated cell performs work and expends energy and substances. It must be noted that not only free monosaccharides and reserve polysaccharides are spent, but also protoplasmic proteins and phospholipids (Belikov

and Dmitrieva, 1962, 1963) and this naturally results in a decrease in the protoplasmic viscosity. Thus, here we can see processes of destruction, damage of protoplsam and the relationship of the latter with stimulation. Under thermal influence one can observe simultaneously an appearance and a quantitative increase of properties characterizing stimulation and damage. It should be

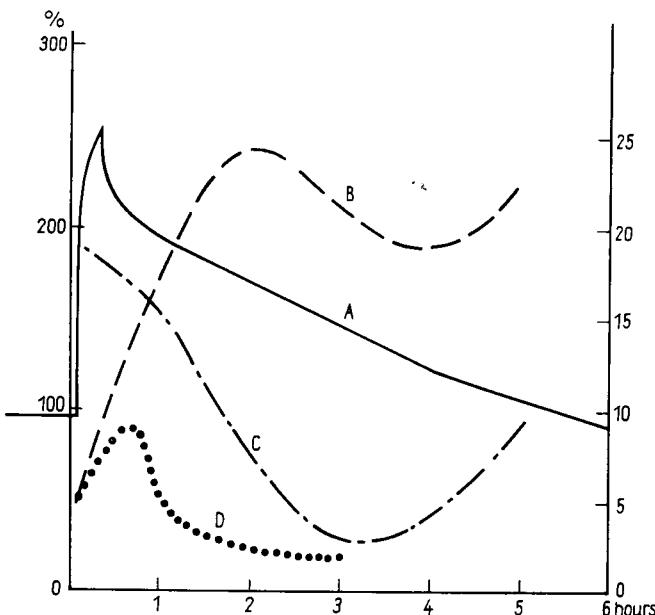


FIG. 2. Influence of high temperature (44°C) on different cell functions. Abscissa shows hr. Left ordinate shows the rate of respiration; right ordinate shows: B—units of interferometer scale; C—time of convex plasmolysis (in min); D—rate of spherosome streaming (μ/sec).

specially noted that the phase of "thermal disease" described here is typical for early signs of a destruction that is not yet complete. It must be mentioned that at this period neutral red becomes wholly localized in vacuoles and all the cells show an ability to plasmolyse (Belikov and Kirillova, 1959). It is important that the damage itself provides conditions for reparatory processes and hardening. Actually a breakdown of polypeptides leads to the accumulation of amino acids and amides, including such active substances as proline and glutamine. The breakdown of polysaccharides is accompanied by sucrose synthesis ("sucrose reaction") (Belikov and Dmitrieva, 1962). A protective role of sucrose in hardening is well known and need not be specially explained. Moreover, phosphatide destruction results in the accumulation of mineral phosphates which provides favourable conditions for a new activation of respiration, and naturally for the restoration of the spent energy reserves (ATP). The products of nucleic acid breakdown seem to act similarly. This

can be seen from an increase of the heat-resistance caused by adenine (Galston, 1959) and kinetine (Mothes, 1960) treatment of plants.

Then a stoppage of the protein breakdown is observed; viscosity of the protoplasm increases, and its streaming slackens and stops completely; respiration becomes stabilized, and excretion of water-soluble substances temporarily decreases. A decline of the protoplasmic sensitivity to repeated thermal treatment is the integral characteristic of all the alterations above described (Belikov and Dmitrieva, 1963).

Thus, two phases and hence a wave-like time course of changes in the principal properties characterizing a functional state of the cell are observed in the excited protoplasm (Figs. 1 and 2). We cannot neglect the fact that the bends in the curves do not occur quite simultaneously, and we are inclined tentatively to explain this phenomenon as stimulation of various cellular organelles at different times.

The complete death of cells as a result of heating is followed by irreversible changes, in particular by a linear increase in the protoplasmic viscosity, and loss of substances.

This gives a brief account of the changes occurring in the plant cell under the influence of continuous heating. Our studies are far from complete. However, at the present stage of investigation they permit us to suggest that in an analysis of the regulatory activity of the plant cell one can use concepts obtained from studies of the nervous-muscle system. At any rate it was shown that in response to thermal irritation there appear phasic changes, excitation and relative non-excitability.

In conclusion it must be noted that the idea of Vedensky (1901), Ukhtomsky (1951) and Nessonov (1959) concerning the integrity in responses of plant and animal cells to the action of various factors is a fruitful initial stage in the solution of new problems.

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REPARATION OF HEAT INJURY IN PLANT CELLS OF DIFFERENT AGES

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THE result of any unfavourable effect, heating included, depends not only on the degree of damage produced directly by the agent, but also on the capacity of the cell to repair the damage (Alexandrov, 1952, 1956). Therefore, when studying changes in heat-resistance related to the development of plant cells, we investigated their "primary injurability" determined by the cessation of protoplasmic streaming immediately after a 5-min heating, as well as their capacity to restore their streaming in a certain period after a 5-min heating.

The studies of age in the "primary injurability" of cells previously made on the lower epidermal cells of the leaves of a number plants showed that these changes are connected with growth: young growing cells are less resistant to a short period of intensive heating (5–40 min) than are adult cells. The cessation of growth in leaves upon transition from upper to lower tiers, growth inhibition of young leaves in winter or in the period of flowering and artificial growth repression by maleic hydrazide cause an increase in heat-resistance. Figure 1 shows the experimental results of a change in heat-resistance due to growth cessation of the leaves of upper tiers in *Kalanchoë*. The cessation of growth was induced by a treatment with 0·25 per cent maleic hydrazide. In control plants upper growing leaves possess lower heat-resistance than adult leaves. After cessation of growth these differences disappear as a result of an increase in heat-resistance of the leaves of upper tiers (Gorban, 1961, 1962).

The purpose of this work was to study age changes in the reparatory capacity of cells. In this case lower epidermal cells of *Zebrina pendula* and *Tradescantia fluminensis* were also used as objects of investigation. The cells of the leaves of the first upper tiers are defined as young, the same cells of the third tier as adult cells and cells of the leaves of the lowest tier are referred to as old cells.

The reparatory capacity was determined by the extent of the maximum temperature zone of reversibility or reparation and by reparation rate. The width of the maximum temperature zone of reversibility or reparation is obtained from the difference between the maximal and minimal high temperature

maintained for 5 min that reversibly represses protoplasmic streaming. For example, if the protoplasmic streaming stops at 45°C, and if the recovery of the protoplasmic streaming may still be obtained after 52°C, the zone of reparation equals 7°C.

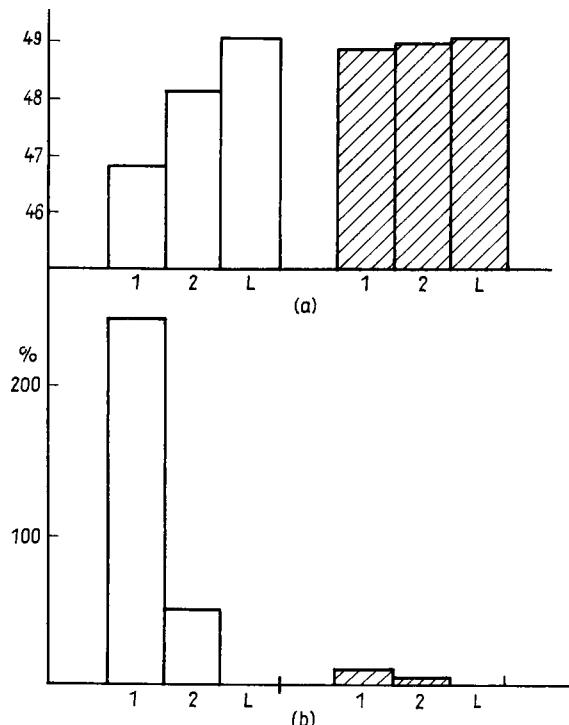


FIG. 1. The effect of maleic hydrazide on the growth and heat-resistance of leaf cells of *Kalanchoë blossfeldiana*. Abscissa—tiers; ordinate—(a) temperature stopping the protoplasmic streaming after 5-min heating; (b) the relative increase of the leaf surface within 10 days; the hatched column—treated plants; non-hatched columns—non-treated plants; *L*—the last tier of leaves.

It has been found that in young and adult cells of *Z. pendula* the maximum temperature zones of reversibility or reparation are the same and equal 5.7°C. Young cells attain the maximum reversibility zone by the third day and adult ones by the seventh day from the beginning of the experiment. Hence, the reparatory process in young cells is completed earlier and the reparation rate in young cells is higher than in adult ones.

Further, we studied reparation rate during the first half of the day after heating and characterized it by the extent of the reparation zone attained for a certain period of time. Observations have shown that 12 hr after heating, the reversibility zone in young Zebrina cells is 2.4°C, while in adult cells it is

0.7°C, i.e. considerably less. Therefore, if heat-resistance is determined immediately after 5-min heating at the minimum temperature stopping protoplasmic streaming, for young cells it will be 1.9°C lower than for adult ones (44.8 and 46.7°C respectively). But if heat-resistance is determined 12 hr after heating in both kinds of cells it will be identical due to a higher reparation rate of young cells (47.2 and 47.4°C respectively). The results show the role of reparatory capacity for establishing the heat-resistance level of cells.

Figure 2a shows the results of determinations of the minimum temperatures for reversibly repressing protoplasmic streaming and the maximum temperatures after the action of which protoplasmic streaming is restored during 3, 6 and 12 hr of reparation in young, adult and old *Tradescantia* leaves.

As is seen from Fig. 2a, in summer the minimum temperatures stopping protoplasmic streaming are lower in young cells than in adult cells. They are the same in adult and old cells. In 3, 6 and 12 hr the difference in heat-resistance between young and old cells decreases due to a more rapid reparation of young cells. Figure 2b presents reparation zones corresponding to the above data. Figure 2b indicates that in summer in 3 hr the reparation zone in young cells is greater than in adult cells, and in adult cells it is greater than in old ones. These differences are statistically reliable. After 6 and 12 hr reparations there is also some difference between the reversibility zones of young and adult cells, but it is not reliable, while the reversibility zone of old cells is less than that of adult and young ones. It is seen from Fig. 2 that in order to attain a reparation zone corresponding approximately to 2°, young cells need 3 hr, adult cells 6 hr and old cells 12 hr of reparation. Hence, the reparation rate, after a heating that equally inhibits protoplasmic streaming, is twice as high in young cells as in adult cells and four times as high as in old cells.

Hence, the reparation rate decreases with age. In winter when the leaves of the upper tiers cease their growth, simultaneously with the disappearance of the difference in the "primary injurability" of cells of different ages we observe the disappearance of the differences in maximum heating temperature for 5 min after the effect of which protoplasmic streaming is restored in 3, 6 and 12 hr. The reparation zones thus became identical (Fig. 2b). In winter, reparation zones in cells of all ages are considerably less than in summer.

Thus this investigation has shown that the maximum temperature reparation zone does not change with age but the reparation rate decreases. A decrease in reparation rate takes place not only upon transition from growing to adult cells but also on further ageing of adult cells. Upon transition from summer to winter the reparation rate falls in cells of all ages irrespective of cessation of growth. Hence, changes in the reparation rate depend on the age of cells or the season and are not so closely related to the process of growth as in the case of "primary injurability" which falls when growing cells are converted into adult ones and does not change with further ageing.

It can be suggested that a greater reparation rate of young cells depends on a greater intensity of their metabolic processes. The results obtained are in

agreement with the concept of Child. From these experiments, performed chiefly on animals, the conclusion can be drawn that the regions with an increased

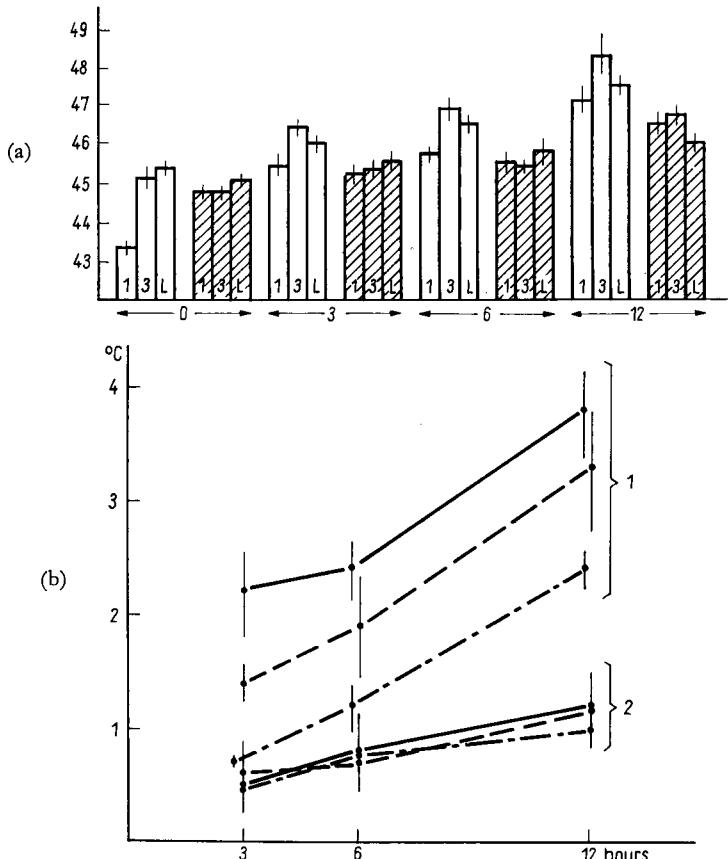


FIG. 2. The changes in the rate of reparation with the changes in age of the cells. (a) Minimal temperature of 5-min heating (0) and maximal temperatures after a 5-min exposure to which it becomes possible to repair the protoplasmic streaming within various periods of time in the leaves of the first (1), the third (3) and lowest (L) tiers of *Tradescantia fluminensis* in different seasons of the year. Abscissa—periods of protoplasmic streaming reparation (in hr); ordinate—temperature of a 5-min heating which stopped protoplasmic streaming; hatched columns—the results obtained in February; non-hatched—in July. (b) Zone of protoplasmic streaming reparation corresponding to the data shown in Fig. 2a. Abscissa—the period of protoplasmic streaming reparation; ordinate—the width of the reparation zone, i.e. the difference between the maximal temperature of a 5-min heating after which the reparation of the stopped protoplasmic streaming is possible and the minimum temperature stopping the protoplasmic streaming. The continuous line—leaf cells of the 1st tier; hatched line—those of the 3rd tier; hatched-dotted line—the cells of the lower tier. 1—winter; 2—summer.

intensity of metabolism which is characteristic of young cells exhibit a greater "primary injurability" and better reparatory capacity (Child, 1929).

All the above facts enable us to understand partially the contradictory data in the literature on changes in heat-resistance at different stages of cellular development. When cells were observed immediately after a short period of intensive heating, Reuter (1937), Alexandrov, Lyutova and Feldman (1959), Feldman (1960), Feldman and Kamentzeva (1963) noticed a greater stability of old cells as compared with young ones. The intensity of the agent and the short term exposure did not permit the processes of reparation to develop and a greater "primary injurability" of young cells was responsible for the result. According to Sachs (1864) old leaves observed immediately after heating were more resistant but a few days after the experiment in some instances young leaves proved to be more stable. Probably in these cases the result was due to a better reparatory capacity of young cells.

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INFLUENCE OF HEATING ON THE RATE OF PROTOPLASMIC STREAMING AND CELLULAR RESISTANCE OF PLANTS

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ALEXANDROV and his colleagues have shown that the effect of moderately high temperature causes an increase of heat-resistance together with a decrease of photosynthesis and a slowing down of growth (Alexandrov, 1963).

The aim of the present work was to investigate the influence of the heating on the ratio between the rate of protoplasmic streaming and that of the heat-resistance of the cell.

The experiments were conducted on *Campanula persicifolia* L. The investigations were carried out on the epidermal cells of a leaf midrib. The rate of protoplasmic streaming was determined by the time of spherosome movement between two points of the ocular micrometer. Thermostability was measured by the temperature which stopped protoplasmic streaming after a 5-min heating. Heat-resistance and the rate of streaming were measured at different intervals (from 30 min to 21 hr) while keeping the pieces of leaves in a moist chamber heated to 25·2, 31·1, 33·0, 35·0, 37·0, 39·0 and 41·0°C. Heat-resistance and the rate of streaming were also determined at different intervals after heating for 21 hr at the above-mentioned temperatures. The pieces of leaves were immediately transferred from ordinary conditions into the thermostat with the given temperature. At the end of the treatment, the material was immediately transferred to room temperature.

The rate of the spherosome movement was always measured at room temperature. Before determining the rate of movement and of thermostability the pieces of leaves were infiltrated in a syringe using Alexandrov's method (1954).

The rate of spherosome movement was expressed as a percentage of the controls. The pieces of leaves which were kept for the same period of time at room temperature in a moist chamber were taken as the controls. The thermostability was expressed as the increase over the controls (in degrees). Determinations of the rate of protoplasmic streaming of the controls showed insignificant fluctuations in time (see Table 1). The thermostability of the

controls gradually considerably increased (Table 1)—an increase on the 8–10th day constituted 1·5–1·8°C.

The heating at 25·2, 31·0, and 33·0°C caused a reversible slowing down of the rate of spherosome movement which became obvious after an hour of heating (see Fig. 1). The change in time and the extent of slowing down of the spherosome movement directly depend on the temperature of heating.

TABLE 1. CHANGES IN THE RATE OF PROTOPLASMIC STREAMING
AND OF THERMOSTABILITY OF THE CONTROL

Days		1	2	3	4	5	8	10
Series I	Rate in μ/sec	2·81	3·0	2·92	2·79	3·0	2·89	
	Thermostability in °C	43·2	43·5	43·7	44·1	44·7	45·0	
Series II	Rate in μ/sec	2·52	2·52	2·69	2·62	2·71	—	—
	Thermostability in °C	42·9	43·2	44·2	43·4	44·0	—	44·4

After heating at 25·2°C the maximum decrease in the rate of movement was marked in 10 hr after the beginning of heating. However, after this time, in spite of continued heating, an acceleration of movement occurred and at the end of the heating (21 hr) the rate of protoplasmic streaming attained the rate of the controls.

At the higher temperatures (31·0 and 33·0°C), the rate of spherosome movement, after slowing down by 25–26 per cent, gradually increased without attaining the rate of the controls. This was restored only 3–4 hr after the cessation of heating which lasted 21 hr.

Thermostability in all these experiments did not differ from that of the controls.

When heated at 35°C, a slowing of the spherosome movement was also observed. The maximum slowing was 29 per cent. This rate of movement was maintained during the whole period of heating (21 hr) and was restored only 6 hr after the cessation of heating.

The thermostability under the influence of this temperature gradually increased and attained its maximum within 4 hr from the beginning of heating (an increase of 1·1°). The same level was preserved for the whole period of heating and for 24 hr after its cessation.

On heating at 37·3°C the rate of spherosome movement decreased with fluctuations by 31–27 per cent and remained at this level during the whole period of heating (21 hr). When the material after the cessation of heating was transferred into a moist chamber at room temperature it was discovered that the protoplasmic streaming slowed down even more (by 43·5 per cent) within 2 hr. In 4 hr the acceleration of movement almost attained the rate of the controls. Then again the rate slowed down and in 7 hr it was 30 per cent slower than the controls and a slow recovery to the level of the controls took place within 4–7 and sometimes within 10 days. The question arises whether these peculiar changes in the rate of protoplasmic streaming, when the pieces

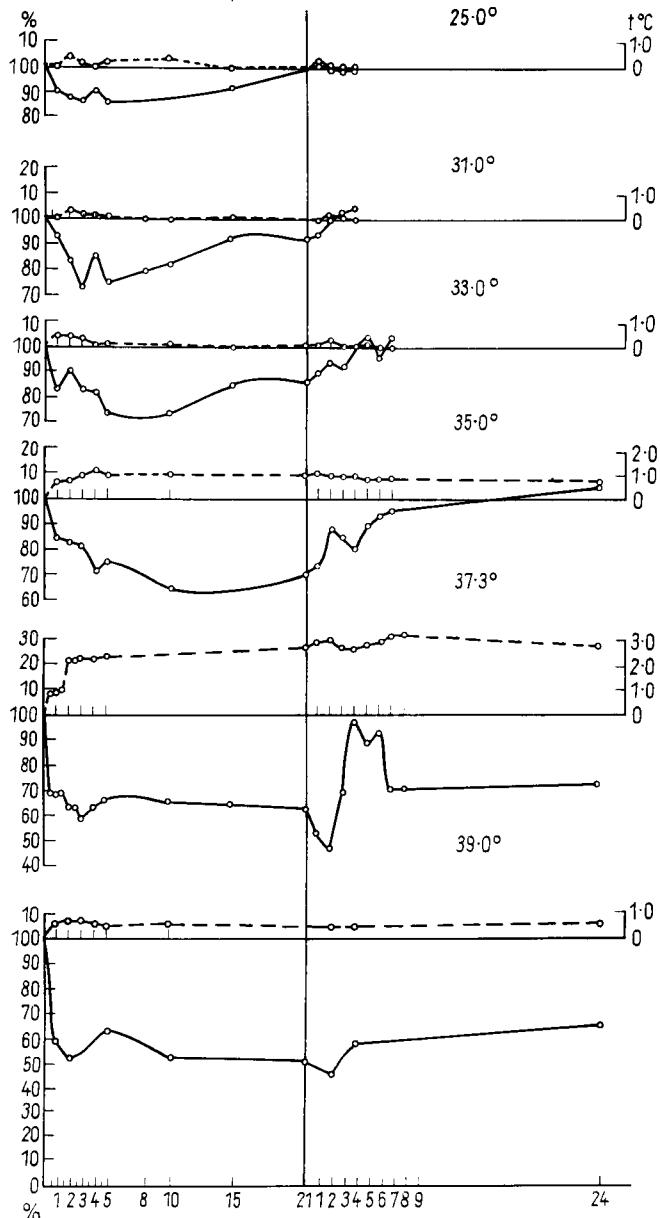
Campanula persicifolia L.

FIG. 1. Changes in the rate of protoplasmic streaming and of heat-resistance of the epidermal cells of the leaf of *Campanula persicifolia* under the influence of heating. Abscissa shows: on the left—time of heating; on the right—time after the cessation of heating (in hr). Ordinate shows: on the left—the rate of protoplasmic streaming (per cent of the control values); on the right—the increase of heat-resistance (per cent in comparison with the controls). Continued line—relative rate of spherosome movement. Dotted line—relative heat-resistance.

of leaves are transferred from the temperature of 37.3°C to room temperature, are not connected with the suddenness in the changes of temperature.

To test this assumption the following investigations have been conducted: the thermostat with the pieces of leaves after being kept for 21 hr at the temperature of 37.3°C was gradually cooled down in 7 hr. In such an experiment (Fig. 2) the rate of protoplasmic streaming, just as in the previous conditions, increased within 4 hr after the beginning of the cooling and returned to the initial level within 7–8 hr, but the later decrease did not take place.

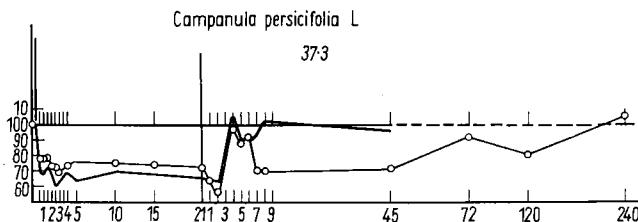


FIG. 2. Changes in the rate of protoplasmic streaming under the influence of heating to 37.3°C then cooling by different methods. Abscissa shows: on the left—time of heating; on the right—time after the cessation of heating (in hr). Ordinate shows: the rate of protoplasmic streaming (per cent of the controls).

Thin line—sudden cooling. Thick line—gradual cooling.

Thermostability at a temperature of 37.3°C quickly increased and attained its maximum at the beginning of heating. It remained at the same level with slight fluctuations during the whole period of heating (21 hr). In case of sudden transfer of the material from the temperature of 37.3°C to room temperature the thermostability after cessation of heating remained unchanged for some time and then gradually decreased for 8–10 days without attaining the level of the controls. In the case of gradual cooling of the pieces which had been heated at the temperature of 37.3°C the changes in thermostability were the same.

In the material which was kept at 39.0°C the spherosome movement slowed down by 40–50 per cent within an hour and remained at this level with slight fluctuations till the end of the heating. After the cessation of heating the rate of spherosome movement remained reduced to the end of the experiment (24 hr). Heat-resistance in such conditions slightly increased (by 0.6–1.7 per cent during 3–4 hr after the beginning of heating). Then the hardening effect decreased down to 0.3°C, remaining at this level till the end of the experiment.

Within 15 min of heating at 41.0°C there were no signs of spherosome movement in the epidermis of a leaf midrib.

Under the influence of heating which caused hardening, an inverse relationship was observed between the initial level of the thermostability and the extent of its increase, which agrees with the data obtained by Shukhtina (1962).

The data obtained show that the changes in the rate of protoplasmic streaming under the influence of moderate heating can be revealed earlier than the changes of viscosity and of photosynthesis. According to Zavadskaya (1963) the increase of protoplasmic viscosity was observed after 16 hr of heating at 37·0°C. The decrease of photosynthesis was observed by Lyutova (1958) at the temperature of 28–30°C.

Thus, the effect of moderately high temperatures on plant cells reveals two types of protective reactions—reparation and heat-hardening. Heat-hardening develops only at temperatures significantly decreasing the rate of protoplasmic streaming. This conclusion confirms the view of V. Y. Alexandrov of heat-hardening as a response of a cell to the injurious effect of heating. 37·3°C can be regarded as the optimal temperature under which the effect of hardening arises in the epidermal cells of the leaf of *C. persicifolia* in the conditions of our experiment.

After the cessation of the effect of hardening temperatures the cells can maintain the increased level of thermostability if the rate of protoplasmic streaming returns to the level of the controls.

The changes in the rate of protoplasmic streaming depend on whether the material is immediately transferred to the conditions of room temperature or is gradually cooled down.

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DISCUSSION

U. HEBER: In which way do you suppose proteins of frost-resistant cells to be more resistant against oxidation than proteins of non-hardy cells?

J. LEVITT: We have only partial evidence on this point and can therefore only speculate. The G—SH oxidizing activity increases during hardening, at least at first. We can therefore only postulate that a link in the electron transport system between G—SH and protein SH is weakened or eliminated.

U. HEBER: How do you explain the fact that addition of SH compounds fails to protect photophosphorylation of isolated chloroplasts, although about 2 per cent of sugar leads to perfect protection? There cannot be much oxidation of SH groups in these experiments since soluble oxidases are removed and soluble SH groups are available in excess which should compete for any available oxygen.

J. LEVITT: Again we can only speculate. We have found that low molecular weight SH substances may be readily oxidized on addition to homogenates. It has also been shown that very high concentrations of low molecular weight SH substances are required to protect protein SH from interacting with each other.

U. HEBER: We know that cell proteins contain SH groups and that in very many cases blocking of SH groups or oxidation of SH groups leads to the inactivation of enzymic activities. How do you explain then the fact that a number of soluble enzymes and perhaps all of them are not affected by the frost-killing of the cell although these enzymes (malic acid dehydrogenase, catalase, transaminases) should be supposed to contain SH groups?

J. LEVITT: (a) Certainly not all enzymes are inactivated by oxidation of SH. Even those that are may have most of their SH groups oxidized without losing activity.

(b) I believe the SH-SS hypothesis is the only one that logically explains the lack of freezing injury in the case of soluble enzymes because of the above facts. One would expect the inactivation in the case of only the elongated proteins because of the greater chance for SH interaction in view of the greater number of junctions between them.

U. HEBER: What is your opinion on the protective effect of sugars? SH groups cannot be protected directly by hydrogen bonding to sugars or to water bound by sugars since SH groups are unable to form hydrogen bonds.

J. LEVITT: SH groups are able to form H bonds although these are weak.

However, the protective effect of sugars may have nothing to do with this. If the sugars penetrate between the proteins, they will certainly form a mechani-

cal barrier between the proteins even on freezing, because they will maintain the proteins apart both by their own bulk and by the bulk of the water molecules which they hold.

S. S. OGANESSION: We studied the heat denaturation of protoplasmic proteins and our results confirm the theory of Prof. Levitt about the role of SH groups in heat-hardiness.

N. A. SATAROVA: In connexion with the interesting report of Prof. Altergott I should like to speak about some investigations in Prof. Genkel's laboratory at the Institute of Plant Physiology. We studied the influence of a short exposure at high temperature (+40; +42°C) and atmospheric drought (relative humidity of air is 30 per cent) on RNA and protein content of potato leaves. It was found that after a short period of heating and atmospheric drought the content of ammonia and amide nitrogen was increased and the proteins were partly hydrolysed. In such conditions the RNA content was higher in the leaves of heated plants and in plants treated with $ZNSO_4$ than in the control ones. The experiments with ^{15}N showed that there is a correlation between RNA content and the intensity of protein synthesis. Owing to relative stability of RNA content the level of protein synthesis in the leaves, stems and roots of potato was rather high after a short period of the above-mentioned treatment. This is the cause of the relatively quick recovery of the normal physiological state.

V. Y. ALEXANDROV (addressed to Dr. Heber): Your report is of special interest because it shows how dangerous it is to transfer the results obtained on isolated particles to the intact cell.

O. A. SEMICHATOVA: Our work on mitochondria gave us the conviction that the estimation of P/O ratio on isolated mitochondria is not an adequate method of evaluating the energy efficiency of respiration. In most cases the results obtained by this method need a special control.

U. HEBER: In our experiments at low temperatures results of experiments *in vitro* with isolated chloroplasts and isolated mitochondria agreed reasonably well with results of experiments *in vivo* with intact leaves. Can it be possible to explain the different results of your experiments *in vivo* and *in vitro* by the reversibility of uncoupling when the intact seedlings were exposed to high temperature?

O. A. SEMICHATOVA: We consider it more likely that in the seedlings no uncoupling of phosphorylation and oxidation occurred. In order to detect uncoupling in the intact cells under the high temperature conditions we applied ^{32}P . But incorporation of inorganic ^{32}P into nucleotides took place even at 42–43°C.

A SURVEY OF EXPERIMENTS ON RESISTANCE-ADAPTATION

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IN POIKILOTERMAL organisms we distinguish two homeostatic mechanisms which can be independent of each other, first, capacity adaptation (Leistungsadaptation) in the normal range of temperatures, and second, resistance-adaptation to temperature extremes, which can be either a heat- or cold-adaptation (Fig. 1). The shifting of the maximum of the rate-temperature curves according to the temperature of adaptation prior to the measurements can also be considered as a problem of heat-adaptation. To distinguish between capacity and

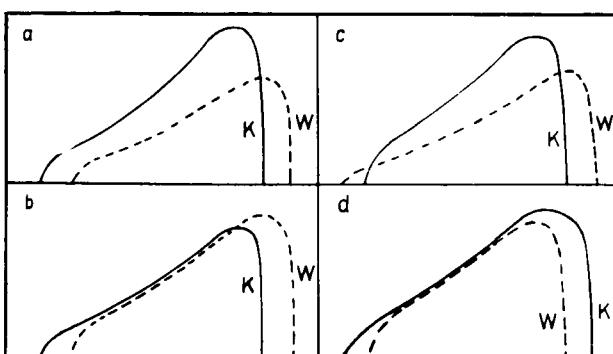


FIG. 1. Different types of temperature adaptation (scheme). W = warm-adapted organisms, K = cold-adapted organisms. Ordinates: the measured capacity (for instance the oxygen consumption), abscissae: experimental temperatures.

- (a) Compensation in the normal range of temperature, reasonable heat- and reasonable cold-adaptation; (b) no compensation, reasonable heat- and reasonable cold-adaptation; (c) compensation, reasonable heat- and paradoxical cold-adaptation; (d) no compensation, paradoxical heat- and reasonable cold-adaptation.

resistance-adaptation may be difficult in those cases where the turning point or optimum lies within the "normal" temperature range, and where the decrease of the rate values beyond this point is very slow. We speak of a reasonable resistance-adaptation when heat-resistance increases and cold-resistance

decreases with rising adaptation temperature; if the changes are in the reverse direction we call it a paradoxical phenomenon.

While botanists mainly deal with temperature-resistance and adaptation of cells, zoologists are usually more interested in the resistance and adaptation of organ functions; these may determine survival limits, at least in higher animals. Intact animals are in the main less resistant to extreme temperatures than is cell metabolism. However, zoologists also come across animals which survive a freezing of their tissues, as some intertidal animals do (Kanwisher, 1955). In these cases, as with many plants, the resistance of the tissues to ice

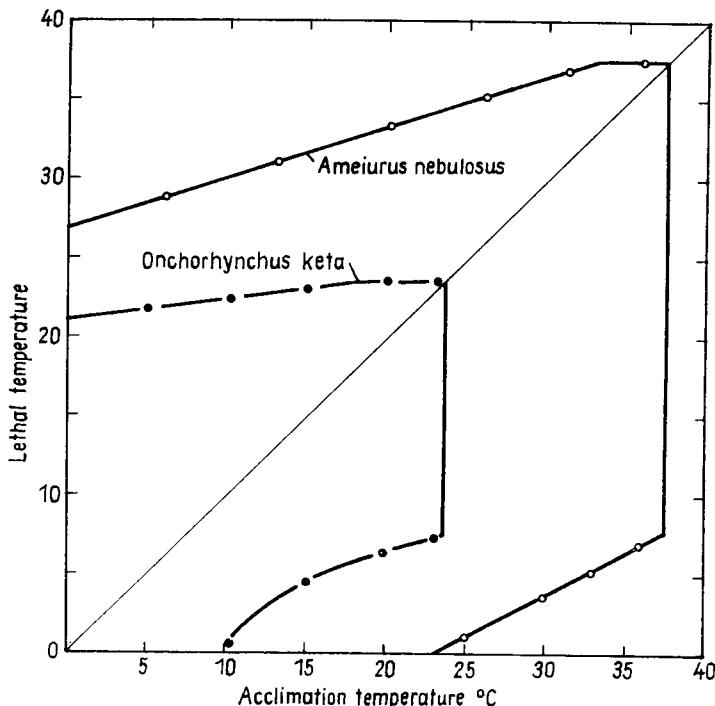


FIG. 2. Lethal temperature relations for two species of fishes. The bullhead, *Ameiurus nebulosus*, is a highly tolerant species in contrast to the chum salmon, *Onchorhynchus keta*. The area enclosed by each trapezium is the zone of tolerance (from Brett, 1956).

formation is decisive. In species which are less cold-resistant, the capacity of the body fluid to withstand supercooling may determine the survival of the species at extremely low temperatures. The resistance of a specific organ function, essential for life, or the interplay of organ functions, seems to be important for cold sensitive species. If survival of an animal at extremely high or low temperatures is determined by specific organ functions which show a reasonable resistance-adaptation, it does not matter for the survival of the individuals and species, whether other more resistant organ functions and the

very resistant cell metabolism adapt at all or show paradoxical adaptation phenomena.

In regard to the survival of intact animals, some groups of animals such as fishes, show almost without exception a reasonable resistance-adaptation to both temperature extremes (Fig. 2). It seems to be sufficient for some other organisms to adapt reasonably in respect to survival resistance to either cold or heat. These organisms may show no resistance adaptation to the other extreme temperature (e.g. not to heat but only to cold as in the bug *Ischnodemus sabuleti*), or they may show a paradoxical adaptation. Organisms, normally endangered by heat, as the yeast *Torulopsis kefyr* possess a reasonable heat- and a paradoxical cold-adaptation (Christophersen and Precht, 1952); other organisms, normally endangered by cold, can show a reasonable cold- and paradoxical heat-adaptation, e.g. several plants (Alexandrow, 1960; Levitt, 1958). It is interesting that this is true, according to Lange, often only for frost-

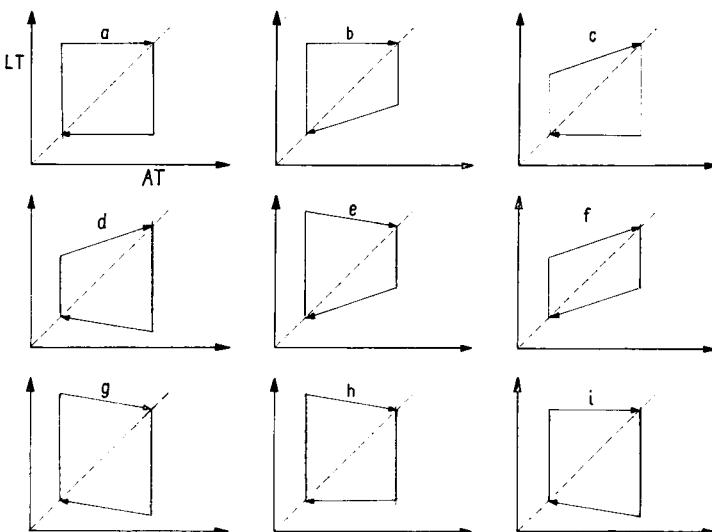


FIG. 3. Possibilities of the dependence of heat- and cold-resistance on the adaptation temperature (scheme). (a) No heat-adapt., no cold-adapt. (b) no heat-adapt., reasonable cold-adapt. (c) reasonable heat-adapt., no cold-adapt. (d) reasonable heat-adapt., paradoxical cold-adapt. (e) paradoxical heat-adapt., reasonable cold-adapt. (f) reasonable heat-adapt., reasonable cold-adapt. (g) paradoxical heat-adapt., paradoxical cold-adapt. (h) paradoxical heat-adapt., no cold-adapt. (i) no heat-adapt., paradoxical cold-adapt. AT = adaptation temperatures, LT = lethal temperatures or another index for the measured heat- and cold-resistance (from Thiede, 1965). Examples: (a) *Dixippus morosus* (Precht); (b) *Ischnodemus sabuleti* (Precht); (c) summer animals of *No-dilitorina granularis* (Ohsawa), tropical *Uca* (Vernberg and Tashian), some plants in summer experiments of Lange; (d) *Torulopsis kefyr* (Christophersen and Precht); (e) several winter plants (Levitt and Lange); (f) fishes (Brett), *Homarus americanus* (McLeese and Wilder); (g) succinodehydrogenase activity of *Xiphophorus helleri* (Precht).

hardened winter plants; in summer time, heat-resistance can reach a second maximum, probably due to a reasonable heat-adaptation without any increase in cold-resistance. Figure 3 shows all possibilities of the dependence of heat- and cold-resistance on the adaptation temperature. (In some fishes no further increase in heat-resistance can be observed when a high value of the adaptation temperature is exceeded (see Fig. 2); such cases are not to be considered here. An example is given in the figure and similar cases are known.)

The previously mentioned experiments of Lange concerning heat-adaptation in plants correspond to ours in animals insofar as the organisms had been adapted to different normal temperatures for a moderately long time and had not been pretreated with super-optimal temperatures for a short time as in the experiments of Alexandrow. Therefore, we examined adjustments in the sense of Alexandrow and not heat-hardening (which can also be observed in animals as shown in this symposium). The terms heat-adjustment and heat-adaptation are synonyms. For simplicity the expression adaptation is used by us in those cases when the adaptation temperature before the experiments has an influence on the measured capacity in the normal range of temperature or on the resistance to extreme temperatures, independent of the question whether these changes possess a biological advantage (resulting from selection) or not; often this question can only be answered with difficulty.

Reasonable resistance-adaptation to one temperature extreme, and paradoxical adaptation to the other extreme, may be due to *one* mechanism of regulation for both. This is because several factors have been postulated for the explanation of resistance-adaptation (e.g. changes in the water content of the cells; formation of protective substances as glycerol or sorbitol in insects (Salt, 1961); special linkages in the molecules, etc.) which increase the resistance to many influences, including *both* temperature extremes (see also Irvine and co-workers, 1957). When one of these factors, which change heat- and cold-resistance in a similar direction, is influenced by the adaptation temperature, resistance-adaptation must be reasonable to one temperature extreme and paradoxical to the other, because both heat- and cold-resistance change with rising adaptation temperature; when they increase, heat-adaptation is reasonable and cold-adaptation is paradoxical; when they decrease, heat-adaptation is paradoxical and cold-adaptation is reasonable. The paradoxical adaptation has no importance, when the mechanism which is involved determines an organ function or reaction which is important for life resistance only to that extreme temperature with a reasonable adaptation; on the paradoxical side a second mechanism, influencing another more sensitive organ function or reaction, may have the effect that the adaptation of survival resistance also becomes reasonable on this side. In these cases reasonable resistance-adaptation to both extreme temperatures is caused by *different* mechanisms. However, also in organisms with both reasonable heat- and reasonable cold-adaptation the postulation of *one* mechanism for both may sometimes be sufficient, because factors exist which influence heat- and cold-resistance in *different* ways.

Besides the effects of hormones, which will be discussed later, an observation of Henckel and Badanova (1956) will be mentioned. They found that the cells of some plants, as *Elodea canadensis* and *Helianthus annuus*, become more resistant to heat and show a higher viscosity after some cations are added to the medium, while cold-resistance decreases.

Also in the case of a reasonable resistance-adaptation to both heat and cold, a rise of adaptation temperature can be combined with a general increase of the resistance of animals to many influences like poisons, lack of oxygen, osmotic influences (Sumner and Wells, 1935; Sumner and Doudoroff, 1938; Schlieper *et al.*, 1960, 1961) and, according to E. v. Buddenbrock (1960) to narcotics as well. This general rise of resistance in these cases does not include resistance to the other temperature extreme, that is to cold. The general resistance can also be combined with cold-resistance. Pitkow (1960) found that guppies (*Lebistes reticulatus*), adapted to 23°C, survived not only cold but also a lack of oxygen better than fishes adapted to 30°C. The conditions of the natural habitat of a species give indications whether cold- or warm-adapted animals are more resistant to other factors (see Vernberg, Schlieper and Schneider, 1963). Also the season may play a role, for instance in plants.

Organ Functions

The resistance-adaptation of single organ functions will be considered first.

We have examined the warm-water fish *Xiphophorus helleri*, the survival of which depends on the resistance of the respiratory centre, as registered by the movements of the opercular gill-cover, or on the ventilation volume of the fish (Precht, 1963; Thiede, 1965). The opercular movements show a reasonable resistance-adaptation to both cold and heat; a similar reasonable resistance-adaptation to both extreme temperatures is found for other organ functions with a wider range of tolerance, as for instance the movements of the pectoral fins, the movements of the mouth initiated by electric stimulus of the midbrain, and strokes of the tail elicited by direct stimulation of the muscle. *Lebistes* shows the same in regard to the contraction of the ventricle of the heart. The throat and the snapping respiration of the frog (*Rana temporaria*) show a reasonable adaptation to heat. Decapitated frogs failed to show any evidence of a heat-adaptation for convulsions induced by electric stimulation; a slight reasonable adaptation was observed, however, with direct stimulation of the thigh muscle. Preparations of *N. ischiadicus*-*M. gastrocnemius* were examined in regard to the conductivity and irritability of the nerves, to the transmission at the end-plate, and the contractibility of the muscle to direct stimulation. The end-plate had the narrowest temperature range and showed a reasonable cold- and a paradoxical heat-adaptation; the contractions of the muscle had a small reasonable heat- and a clearer reasonable cold-adaptation, as was also found for transmission at the end-plates below 0°C. The conduction of the

nerves adapts paradoxically to heat; the cold-resistance of the nerve could not be measured.

The resistance-adaptation of functions of single organs of an animal can be different and need not correspond to survival of the intact animal (as in frogs). If the animal has narrower limits and adapts reasonably, the lack of or the paradoxical resistance-adaptation of organ functions with wider temperature range would be of no importance, as mentioned before. The resistance-adaptation of single organ functions may be caused directly by the adaptation temperature, or superposed factors may play a role in the following ways: (1) Because of the different resistance-adaptation of various functions in the same animal, the question arises whether a certain conformity in the intact animal is brought about by such factors as central nervous activity and hormones. These superposed factors may be influenced by the adaptation temperature in a process which may last for days. (2) On the other hand the change of resistance of the subordinated organ functions in the intact organism occurs by direct "commands" given by the superposed factors, the effects of which do not appear when isolated organs are examined (*direct-effects*). Up to now, cases in which the co-operation of superposed factors (like hormones) is assumed, have been explained in the following way: First, the adaptation temperature influences the superposed factors in a process often lasting some days. These factors also change the resistance of the organ function during this time; these changes can be measured after having isolated the organs. We speak of *after-effects* which must be distinguished from the direct-effects mentioned above; these are only effective in the intact animal and cannot be observed in isolated organs.

In the case of after-effects the superposed factors must not influence the resistance of all functions in the same way (as for example in the nerve-muscle preparation of frogs†) so that reasonable and paradoxical adaptations can exist side by side. Therefore, one should examine the influence of hormones on resistance-adaptation for those organ functions or mechanisms which determine life resistance. (We know more about direct- and after-effects in regard to capacity-adaptation than in regard to resistance-adaptation).

After-effects of hormones on the resistance of isolated organ functions have been examined less than the participation of hormones in the resistance-adaptation of intact animals or their non-isolated organ functions (movements of gill-covers in fishes, etc.). The hormones of the thyroid gland are believed to increase cold-resistance of fishes and, especially for cold-adapted animals, to decrease heat-resistance (Hoar and Eales, 1963). An increase of hormone production with decreasing adaptation temperature might explain a reasonable adaptation to both cold and heat, as well as several aspects of capacity-adaptation (partial and ideal compensation). However, the experimental results are not

† Provided that in this example the resistance-adaptation is caused by superposed factors and not by a direct effect of the adaptation temperature.

clear. Hoar and Robertson (1959) found that day-length also plays a role; furthermore, treatment with thiourea sometimes unexpectedly had an effect similar to thyroxine (see also Hoar and Eales, 1963). In our laboratory Suhrmann (1955), Auerbach (1957), and recently Thiede (1965) worked on this problem. Thiede examined the fish *X. belleri*. Thyroxine increased the cold-resistance in the first few days, at least in cold-adapted animals, and decreased heat-resistance. Especially in warm-adapted fishes, thiourea decreased cold-resistance and, mainly in cold-adapted animals, heat-resistance too. The treatment with thiourea probably had a toxic effect; it generally decreased the resistance to extreme temperatures, in addition to its blocking of the production of thyroxine (see also Bodine, 1950). Especially the resistance of those animals which already had a lowered resistance to extreme temperatures by the preliminary thermal treatment (cold-adapted fishes in experiments concerning heat-resistance and warm-adapted in measurements of cold-resistance) is decreased by the treatment with thiourea.

Another field of research seemed to add contributions to the problem of hormonal regulation of cold-resistance of insects, namely the examination of the rest phase at diapause; but new experiments with the bug *I. sabuleti* showed that caution is necessary. The expression diapause refers to the period of inhibition of growth and development. These periods of rest are very often characterized by further adaptations to unfavourable external conditions, as for instance a decrease in the enzyme activity, regulation of the water economy, and also an increase of resistance to extreme temperatures, which is of special interest in relation to our problems. The question arises whether these regulations and especially the change of the often examined cold-resistance are correlated with the inhibition of growth and development. This might give an indication of hormonal regulation of cold-resistance because there is hormonal regulation in the inhibition of development as has been shown in the detailed work of Williams (1960) and his co-workers. It is outside the scope of this manuscript to discuss all the data presented in the literature which speak for or against such a correlation (see for example Asahina, 1959). We have examined this problem in *Ischnodemus*. Survival of the whole animal showed a limited cold-adaptation during both summer and winter. We did not find capacity-adaptation in the form of a compensation of the oxygen consumption. Towards summer, oxygen consumption increased independently of adaptation temperature, while cold-resistance decreased very much. Neither seasonal regulation is obviously correlated with the beginning of the production of eggs in females. The absence of correlation between the changes in resistance to extreme low temperatures and the period of diapause could be observed when the insects were kept under unnatural conditions, as at high temperature and short day. Therefore, hormonal explanations for diapause cannot be applied in a simple way to the seasonal change of cold-resistance.

The central nervous system can determine life resistance (as for example the respiratory centre of fishes) and can show a resistance-adaptation in its

functions. As a superposed factor in a direct-effect it can also influence the resistance of organ functions, as is seen in experiments of Benthe concerning the irritability of isolated foot muscles of the snail *Limnaea stagnalis*. Heat-resistance (seen by the turning point of the excitability-temperature curves) was greater when the pedal ganglion remained in the foot preparation and was smaller when it was removed.

A further question is whether the alteration of heat-resistance and cold sensitivity of organ functions or of life resistance with the adaptation temperature take a parallel course or not? If they do we can postulate one mechanism for both regulations. According to Tsukuda these changes are parallel in guppies, but this does not apply to all cases (see his Fig. 1 and 2, also Doudoroff). Thiede found for the cessation of opercular movements in *X. belleri* that the gain (or loss) of heat-resistance and the loss (or gain) of cold-resistance are processes which last a similar time beginning approximately on the 4th day after the change of temperature; both processes are completed at the same time. But the course of the curves was not identical. In the ciliate *Zoothamnium biketes* which shows a reasonable heat- and cold-adaption, Vogel (unpublished) could find no corresponding change.

Cell Functions

The problems which have so far been discussed can also be applied to cell metabolism with its complex series of reactions, such as oxygen consumption or to single enzyme reactions. For measurements of the heat-resistance of oxygen consumption of tissues (scraped from the surface of a sieve through which it had been pressed) with the Warburg apparatus, we used the following method: The experimental temperature of the first measurement was 25°C. In the second period the temperature was slightly higher than that for maximum oxygen consumption, for example at 41°C. In a third period the temperature corresponded to that of the first measurement (25°C). When the temperature of the second period was not too far above the maximum of the rate-temperature curves, the oxygen consumption remained nearly constant throughout long experiments. The decrease of the respiration at temperatures above the maximum is initially caused by a reversible denaturation of enzymes, not by irreversible denaturations which would lead to a progressive decrease of the values. Nevertheless, irreversible denaturations of other enzymes occurred in this period but they did not become apparent because of a surplus amount of enzyme. In the third period of the experiment the values may be less than in the first because of after-effects of irreversible denaturations of other enzymes, effects which do not appear in the second part. Therefore, different denaturation processes could be detected with this method. (For details see Precht 1960, and Ohlenbusch and Precht, 1960). Unfortunately, the cold-resistance of the tissue cannot be investigated in a corresponding way. For these measurements we exposed the tissue for certain periods to low temperatures and then

we observed the decrease of the oxygen consumption in comparison to controls.

In the above way we determined the dependence of the heat- and cold-resistance of the oxygen consumption of muscle tissues on the adaptation temperature in *X. helleri* and *R. temporaria*. Various races of the fish behaved differently. In a red race (1) the reversible and irreversible denaturations did not show heat-adaptation, in a green iridescent race (2) the irreversible denaturation adapted paradoxically to heat and the reversible denaturation showed no heat-adaptation. In a grey race (3) the heat-resistance of the irreversible denaturation did not depend on adaptation temperature but the reversible denaturation adapted to heat in a reasonable way. In this race the cold-resistance of the oxygen consumption, the measurement of which corresponded to that of the irreversible denaturation in the determination of heat-resistance, showed a paradoxical adaptation. Naturally it is not certain that, despite of the same method for measuring cold- and heat-resistance of the oxygen consumption, we observed effects on the same enzyme in the chain of reactions. Therefore, we have tried examining the dependence of the resistance of specific enzyme activities on the adaptation temperature. The succinic dehydrogenase activity of muscle tissue in races (1) and (3), determined by the Thunberg method, showed a paradoxical resistance-adaptation to cold and heat. But in these cases we cannot prove that also we have measured the heat- and cold-resistance of the same enzyme reactions and not secondary processes, as for instance the liberation of the enzyme from mitochondria (see Egger and Rapoport, 1961). It is not possible to examine other enzymes such as catalase or aldolase of the muscle of *Xiphophorus* which can be measured in a more exact way, because these enzymes are very cold-resistant. After freezing them 20 times at -38°C , their activity did not decrease but rather increased a little.

The heat-resistance of the oxygen consumption of frog muscle tissue (*R. temporaria*) showed no dependence on the adaptation temperature in the third part of the experiment; in the second part, however, a reasonable heat-adaptation apparently existed.

The question must be asked in the case of cell metabolism whether the adaptation temperature directly influences the tissues or whether superposed factors play a role in the form of after-effects, visible as resistance-adaptation of isolated tissues or in the form of direct-effects, effective only in the intact animal. In cases of a very different resistance-adaptation in isolated tissues (as has been mentioned for the races of *Xiphophorus*) direct-effects could lead to a certain conformity in the intact animal. But we have only a slight knowledge of the working of superposed factors. Direct-effects may not be necessary because of the great resistance of tissues to extreme temperatures; as mentioned above it is unimportant for the maintenance of the species whether, or in which way, the cell metabolism depends on the adaptation temperature when the survival of the animals has narrower limits. But specific temperature-sensitive cell reactions may finally be responsible for the resistance of

organ functions and for survival in cold and heat. However, it is nearly impossible to elucidate these specific reactions by the usual methods of determining the metabolism of scraped or homogenized tissues.

As in capacity-adaptation (see Rao) direct-effects may be caused by the blood of animals. The serum of cold-adapted carps decreases the heat-resistance of the oxygen consumption of muscle tissue of the fish *Idus idus* *in vitro*, while the serum of warm-adapted carps has a less decreasing or even an increasing effect. The decrease by the blood of cold-adapted carps is more pronounced when the heat-resistance of the influenced tissue is high by its own heat-adaptation. The serum of carps has a similar effect on the oxygen consumption of its own muscle tissue (Precht, 1964).

The paradoxical resistance-adaptation of tissues may be caused by the above-mentioned mechanisms which change heat- and cold-resistance in a corresponding manner. This always has the consequence that resistance-

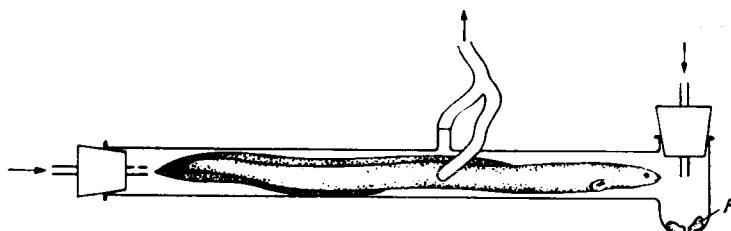


FIG. 4. Experimental method to keep the anterior end and posterior end of an eel at different temperatures. Water inflow at the ends, outflow in the middle.
F = food.

adaptation at one extreme temperature is reasonable, at the other paradoxical (see p. 309). Further studies must be made to clarify this. Also in those cases in which we investigate secondary effects unimportant for the survival of the animal, the examination of the resistance-adaptation of isolated tissues may give information about the general mechanism.

Until now we have only investigated whether adaptation temperature can directly influence the resistance of the tissues. As in the experiments with *X. helleri* we measured the heat-resistance of the oxygen consumption of eel muscle tissue (*Anguilla vulgaris*) and its dependence on the adaptation temperature in three experimental periods with temperatures of 25, 46 and 25°C. The muscle tissue was taken from the anterior and posterior ends of the eel. After these control experiments we exposed the anterior end of an intact eel to low temperature and the posterior end to a higher temperature for eight days or more (Fig. 4).

Our conception was that nervous influences and the amount of hormones present were equal in the whole animal.† A different behaviour of the anterior

† However see Prosser, Precht and Jankowsky (1965), also Schultze (1965).

from the posterior end must be caused by a direct influence of the adaptation temperature on the muscle tissue of both ends; certain objections to this method may be made (see Precht, 1961). The paradoxical heat-adaptation of the consumption of the controls (the muscle of cold-adapted eels is more heat-resistant than that of warm-adapted fishes) seems to be caused mainly by a direct effect of the temperature on the tissue, because the paradoxical heat-adaptation can also be demonstrated on the differently adapted ends of the same animals (Table 1); but this conclusion should be taken with some

TABLE 1. PER CENT INCREASE (+) AND DECREASE (-) OF THE MEASURED VALUES OF THE 2ND AND 3RD PERIODS OF THE EXPERIMENTS IN RELATION TO THE OXYGEN CONSUMPTION OF THE EEL MUSCLE TISSUE IN THE FIRST PERIOD

Experimental temperatures of the three periods: 25, 46, and 25°C. AT = adaptation temperatures. Time of the experiments: June-July. The standard error of the mean is indicated as well as the number (in brackets) of eels used.

Experimental section	Muscle tissue of the:	Controls (AT: 13-14°)	Controls (AT: 23°)	Anterior end: 13-14° posterior end: 23°
2	anter. end	+84 ± 8·7 (4)	+9 ± 6·8 (8)	+64 ± 12·0 (6)
2	post. end	+45 ± 9·3 (4)	+16 ± 8·4 (7)	+17 ± 3·9 (5)
3	anter. end	-8 ± 5·6 (4)	-46 ± 6·9 (9)	-16 ± 9·3 (6)
3	post. end	-29 ± 9·8 (4)	-51 ± 5·2 (9)	-46 ± 4·1 (6)

reservation since not all experimental series gave clear results. The great variability of the values is not astonishing because surely the heat-resistance of the muscle tissue does not influence the resistance of the intact eel and can be regarded as a secondary effect (see also Leloup and Fontaine, 1960). We have not measured the heat-adaptation of intact eels, but probably the adaptation is reasonable as this is true of other fishes.

Ushakov (1959, 1960) and his co-workers examined the heat-resistance of the muscle tissue of many animals. They found almost no heat-adaptation in winter, seldom in summer, but did find changes of the heat-resistance during ovulation, caused by hormones. Changes of the hormonal balance along with the adaptation temperature should, therefore, cause (as a secondary effect) changes of the heat-resistance of the measured tissue. Since the heat-adaptation of the tissue is often lacking, this should mean that neither the capacity-adaptation nor the resistance-adaptation of the intact animals have a hormonal (even thyroidal) basis. This problem should be examined in the future.

Botanists have contributed more than zoologists to the question of the means by which resistance of cells and enzyme reactions are changed during the adaptation to different temperatures. In the yeast *T. kefyr* the water content of the cells decreases with rising adaptation temperature (Christophersen and

Precht, 1951, 1952). This seems to increase the resistance of the succino-dehydrogenase activity to both heat and cold. Therefore, the heat-adaptation of this enzyme reaction is reasonable and the cold-adaptation paradoxical. A shift in the turning point of the curves with increasing adaptation temperature to higher experimental temperatures can be found in succinodehydrogenase activity, which decreases with increasing adaptation temperature (compensation) as also in peroxidase activity which behaves inversely in the normal range of temperature (inverse compensation).

Proteins and Enzymes

The examination of single enzyme reactions in the cells leads to the question whether isolated proteins or enzymes can show a resistance-adaptation. According to the theory of denaturation of Alexandrow, the sensitivity of proteins is of great importance in the problem of heat-resistance of plants, and, according to Ullrich (1962), Heber and others, for cold-resistance too. Two experimental methods are to be distinguished. Either the isolated proteins are adapted to different temperatures or the organisms are adapted and the proteins or enzymes are isolated a short time before the measurement. In the first case the adaptation temperature directly changes the resistance of the proteins; in the latter case it cannot be excluded that during the adaptation the organism synthesizes a new protein with a changed resistance to extreme temperatures. Not only isolated cell proteins and enzymes can be taken but also digestive enzymes, because they are secreted in a purer state. Their change of resistance to extreme temperatures cannot be influenced by so many secondary factors as when we work with enzymes of intact cells (for example yeast cells). Many years ago Frankel (1931, 1932) observed that the time until solidification of gelatin at high temperatures increases when this protein has been kept for some time at higher temperatures; this also causes a decrease of the molecular weight. Christophersen and Thiele (1952) found that a corresponding change of the heat-resistance of commercial pancreatin is reversible. Experiments with proteins, isolated after having adapted the whole organisms, have been carried out by Campell with amylase isolated from a bacterium, and in our laboratory by Mews (1957) with the proteolytic enzymes of the intestinal juice of *Helix pomatia*. Both authors found a reasonable heat adaptation. Recently with a Folin method ($\text{pH} = 4.5$) we examined the proteolytic activity of the gastric juice of *Astacus fluviatilis*; no heat-adaptation could be found as was also the case for the lipase of *Helix* (Mews, 1957). The cold-resistance of the proteolytic activity could not be measured since the examined enzyme was so resistant that an often repeated freezing at -38°C did not decrease its activity. The change of heat-resistance and as far as possible, also cold-resistance of proteins and isolated enzymes must be examined more thoroughly in the future, using both mentioned methods.

Summary

Resistance-adaptation is called reasonable when heat-resistance increases and cold-resistance decreases with rising adaptation temperature; if changes are in the reverse direction we call it a paradoxical phenomenon. The possibilities of the dependence of heat- and cold-resistance on the adaptation temperature are given in a diagram (see Fig. 2).

Reasonable resistance-adaptation to one temperature extreme, and paradoxical adaptation to the other extreme, can be due to one regulation mechanism for both, when this mechanism is influenced by the adaptation temperature and changes in heat- and cold-resistance are in the same direction. A reasonable resistance-adaptation to both extreme temperatures can be caused by two mechanisms or by one mechanism which influences heat- and cold-resistance in different ways (as some cations). Problems of general resistance are discussed.

Functions of isolated organs and cell metabolism can show a resistance-adaptation which has no importance for survival when the life resistance of the intact animal has narrower limits to extreme temperatures. In functions of isolated organs (end-plates of nerve-muscle preparations of frogs), in the oxygen consumption of muscle tissue (of *X. helleri* and of eels) or in enzyme activities (succinodehydrogenase activity of the muscle of *Xiphophorus*) paradoxical adaptation phenomena can be observed.

Resistance-adaptation of isolated organ functions or tissues may be caused by a direct influence of the adaptation temperature (muscle tissue of the eel, Table 1) or superposed factors may play a role. Two possibilities are discussed for an influence of such factors: (1) Adaptation temperature can change hormone production and this can have an influence on heat- and cold-resistance of the intact animal and on the resistance of the organ functions and tissues. These changes can be measured after isolation of the organs or tissues (after-effects). (2) A hormone or nervous activity can influence the resistance of organ functions and cell metabolism only in the intact animal, which cannot be measured in isolated organs or tissues (direct-effects). These effects may cause a certain similarity in the resistance-adaptation of all organ functions and tissues which often behave differently when measured after isolation.

Heat-adaptation of pure proteins or enzymes has also been studied, using two methods: Either the isolated proteins are adapted to different temperatures or the organisms are adapted and the protein is isolated a short time before the measurement of the resistance. Examples for a heat-adaptation of pure proteins or enzymes are mentioned. A cold-adaptation of the extremely resistant macromolecules has not been observed.

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THERMOSTABILITY OF CELLS AND PROTOPLASMIC PROTEINS IN POIKILOTHERMIC ANIMALS IN RELATION TO THE PROBLEM OF SPECIES

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INVESTIGATIONS on the thermostability of cells and protoplasmic proteins in individuals of different populations belonging to one species and taxonomically closely related species are of interest for a physiological analysis of the problem of species and speciation. It is particularly important to study hardly reversible or irreversible heat injuries of cells which make compensating processes impossible. However, at the same time they permit us to judge the properties of protoplasmic protein complexes.

Such investigations were carried out on the cells of poikilotherms by a group of workers of the Laboratory of Comparative Cytology (Amosova, Andronikob, Chernokozheva, Dregolskaya, Dzhamusova, Glushankova, Kiro, Kusakina, Makhlin, Shlyachter, Vinogradova and Zhirmunsky).

However, ontogenetic fluctuations make it difficult to compare cell thermostability of animals from different populations and species. Our experimental data show that ontogenetic shifts can be observed during metamorphosis and reproduction, upon transition to hibernating state and during stress reaction (Schlachter, 1961; Pashkova, 1962; Dregolskaya, 1962, 1963; Kusakina, 1963; Ushakov, 1963a). These changes in thermostability are not usually related to changes in the environmental temperature. Hence, changes in cell thermostability during the individual life of an animal cannot be regarded as a manifestation of thermal adaptations of this organism.

Although ontogenetic changes in cell thermostability make the comparison of populations difficult they, however, cannot be considered as a serious obstacle to investigations. With an exception of stress-reactions which cause momentary changes, ontogenetic fluctuations in cell thermostability are strictly cyclic and are repeated in a standard way, from year to year, and from generation to generation. This means that a comparison of cell thermostability in different populations and species should be made in the same season and at the same stages of development. As has been noted by Professor Schlieper, certain species before the experiment, must be kept at the given temperature, and

marine eurhaline poikilo-osmotic animals must be kept additionally in water of definite salinity.

In these conditions it is possible to reveal certain regularities discussed below.

Studies of the thermostability of cells and proteins in different populations of animals belonging to one species were carried out by our group on 195 populations of 55 poikilothermic species. Experiments have been made on Coelenterates, molluscs, arthropods, echinoderms, fishes, amphibians and reptiles. The majority of the investigated species have not exhibited any significant differences in cell thermostability in representatives of different populations from one species. In 47 species out of 55, i.e. 85 per cent of the investigated cases, cell thermostability proved to be identical in different popu-

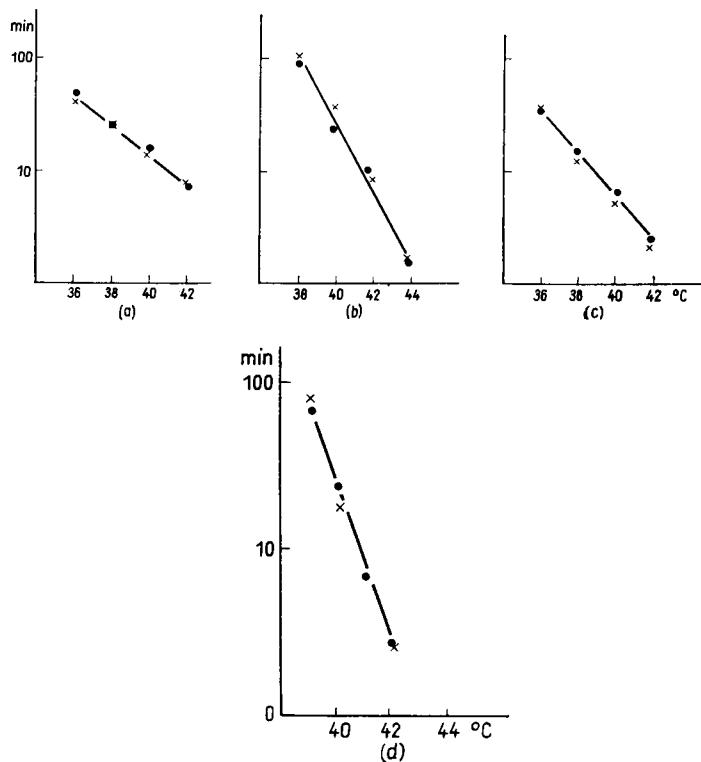


FIG. 1. Relative constancy of muscle thermostability of poikilothermic animals adapted to warm waters (points) and from waters of normal temperature (crosses). a—*Rana ridibunda* from Bulgaria (Svinkin, 1962); b—*Radix ovata* from Baikal region (Dzamusova and Shapiro, 1960); c—*Cobitis taenia* from Baikal region (Kusakina, 1962); d—*Carassius auratus* from Baikal region (Kusakina, 1962). The abscissa gives the temperature in °C; the ordinate, the time of development of complete non-excitability of muscles to electric stimuli (in min).

lations. The corresponding examples were presented in the reports of Dzhamusova and Dregolskaya. Here we shall add to this material some most interesting cases.

For instance, cell thermostability has been found identical in species, some populations of which inhabit warm springs. The experiments were performed on 4 species: frogs (*Rana ridibunda*), molluscs (*Radix ovata*) and fishes (*Carassius auratus* and *Cobitis taenia*). Muscle thermostability of these populations is expressed by a single line (Fig. 1).

We have found no reliable differences in the cell thermostability of populations isolated for a long period of time from one another. This fact is confirmed by the experiments with the Far East frog *Rana temporaria* inhabiting the continent and various islands of the Pacific (Putyatin, Sakhalin and the Kuril Islands).

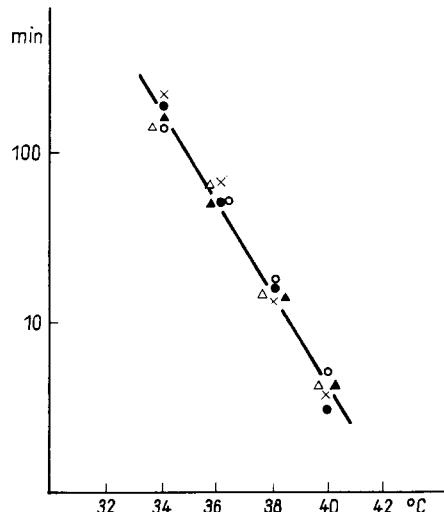


FIG. 2. Relative constancy of muscle thermostability from isolated populations of the frog *Rana temporaria* from the Far East continent (crosses), Putyatin Island (black circles), Sakhalin (white circles), Kunashir Island (black triangles) and Shikotan Island (white triangles). Designations for axes the same as in Fig. 1 (Ushakov, 1959 b).

Figure 2 shows that muscle thermostability of animals from 5 populations which had been isolated for thousands and millions of years also coincided. Similar results were obtained for 5 species of molluscs, amphibians and reptiles.

The comparison of cell thermostabilities made in different races and subspecies of polytypic species gave the same results. In the 16 polytypic species studied, 11 of them showed no difference in cell thermostability within a species.

Figure 3 presents two examples demonstrating a coincidence of muscle thermostability in 4 subspecies of the crayfish *Astacus leptodactylus* and 6 subspecies of the lizard *Lacerta saxicola*. These experiments did not reveal significant differences between the subspecies, and the points obtained in both cases lie on one straight line. In the report of Astaurov similar data have been presented showing that various races of the silkworm exhibit the same cell thermostability.

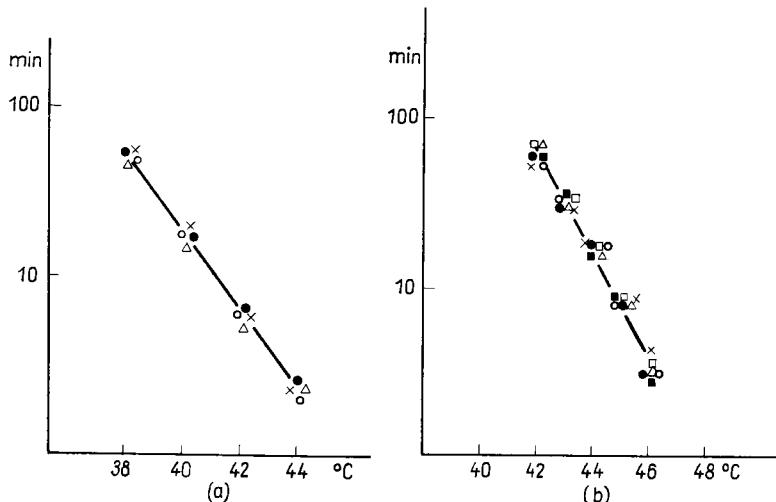


FIG. 3. A relative constancy of muscle thermostability in different subspecies of poikilothermic animals belonging to one species: (a) *Astacus leptodactylus*: *A.L. leptodactylus* (white triangles), *A.L. cubanicus* (black triangles), *A.L. caspicus* (white circles) and *A.L. pilzowi* (black circles) (Ushakov, 1956 b); (b) *Lacerta saxicola*: *L.s. armeniaca* (white circles), *L.s. defilippii* (black circles), *L.s. portschinskii* (white triangles), *L.s. dahli* (white squares), *L.s. terentsevi* (crosses), *L.s. rostombecovi* (black squares) (Ushakov, 1963 a). Designation for the axes the same as in Fig. 1.

The material obtained permits the conclusion that cell thermostability in different populations from most of the species investigated is rather a conservative characteristic. We believe that the conservatism of thermostability of cells is associated with that of their proteins. Some data of the intraspecific stability of protein heat-resistance are reported at the Symposium by Kusakina and Vinogradova.

The comparison of the thermostability of cells and proteins in taxonomically close species (species of one genus) was carried out in our laboratory in more than 250 species of animals, from coelenterates to reptiles.

The thermostability of the cells and proteins of closely related species, generally, is different and is in conformity with the environmental conditions of a species. The greater the difference in the temperature of the environment and reproduction, the greater is the difference in the thermostability of the cells and

proteins. Some of these data are reported by Zhirmunsky, Dzhamusova and Dregolskaya. Therefore, we shall give here only one example which was thoroughly studied.

Table 1 represents our own and other data characterizing the thermostability of the whole organism, sex cells, specialized cells of the adult organism and protoplasmic proteins of two species of frogs, *R. temporaria* and *R. ridibunda*. *R. temporaria* is a more cold-living species: its body temperature in nature is lower, and under the condition of thermopreferendum it actively chooses lower temperatures. Heat shock in *R. temporaria* occurs at 32°C, which is 4° lower than in *R. ridibunda*. In accordance with these data the thermostability of its cells also proved to be lower. The difference in the thermostability of the eggs and spermatozoa of *R. temporaria* and *R. ridibunda* is also 2–3°. The same may be referred to different cells of the adult organism: nervous, muscle, epithelial, and connective. In all the cases, variations in cell thermostability was 2–4°.

Similar results were obtained in the experiments on the comparison of the enzymic thermostability of tissue homogenates. Dehydrogenase of cornea, succinic dehydrogenase of muscles, ATP-ase of muscles and erythrocytes, as well as cholinesterase of muscles and liver have been studied in this respect. The differences in the thermostability of protein complexes between both species were of the same sign and order as in cells. It should be noted that studies on purer protein complexes also reveal differences in protein thermostability of the frog species compared. This refers to the difference found in the thermostability of actomyosin, myosin and haemoglobin of both species.

This example shows that there is a difference between the thermostability of the organism, cells and proteins of closely related species of poikilotherms. This difference concerns the main bulk of homologous cells and proteins and is therefore universal. Apparently, these interspecific difference are genetically determined and are presumably associated with differences in the primary structure of proteins.

Thus two kinds of facts are considered in this report:

1. In most poikilothermic species (85 per cent) no differences in the thermostability of cells and proteins have been found between intraspecific populations.
2. Differences do exist in the thermostability of cells and proteins between taxonomically close species.

The comparison of both these facts enables us to conclude that the thermostability of cells and proteins of poikilothermic animals is a conservative species characteristic (Ushakov, 1959a).

Extensive experimental material established that the thermostability of cells and proteins in different though taxonomically close species correlates with the environmental temperature of the animals compared. This evidence suggests that the level of thermostability of cells and proteins is related to

TABLE 1. THERMOSTABILITY OF DIFFERENT CELLS AND PROTEIN PROTOPLASMIC COMPLEXES OF *Rana temporaria* AND *R. ridibunda* AS RELATED TO ENVIRONMENTAL CONDITIONS OF THE SPECIES

Organism	Object and index of its relation to temp.	Time of temp. action (min)	Temp. (°C)		Author
			<i>R. temporaria</i>	<i>R. ridibunda</i>	
1	2	3	4	5	6
Cells	Body temp. in nature		6·0–26·0	11·0–29·5	Rjumin, 1939 Bannikov, 1943
	Temp. optimum in Herter apparatus		13·0–26·0	18·0–28·0	Bannikov, 1943
	Loss of reflex excitability	30	30·0–32·0	35·0–36·0	Ushakov, 1956b
	Ova, stopping of cleavage	30	34·6	37·6	Andronikov, 1963
	Spermatozoa, loss of motility	30	39·2	41·4	Svinkin, 1959
	Neurons of spinal ganglion, initial increase in vital dye absorption	30	36·0–38·0	38·0–40·0	Schlachter, 1960
	Neurons of spinal ganglion, threshold of paranecrosis	30	36·0–38·0	38·0–40·0	Ushakov, 1960
	Somatic muscles, loss of excitability	30	36·3	40·4	Ushakov, 1955
	Somatic muscles, initial increase in vital dye absorption	5	36·0	38·0	Ushakov, 1955
	Myocardium explants, loss of excitability	30	42·0	44·5	Rumyantsev, 1960
	Myocardium explants, threshold of paranecrosis	5	41·0–44·5	43·0–47·5	Rumyantsev, 1960
	Ciliated epithelium of the gullet, cessation of ciliary movement	5	41·7	44·4–44·8	Alexandrov, 1952
	Cornea epithelium, threshold of paranecrosis	30	39·0–41·0	44·0–46·0	Ushakov, 1960
	Cornea epithelium, initial increase in vital dye absorption	30	36·0	42·0	Makhlin, 1963
	Cartilage cells, threshold of paranecrosis	30	41·0–42·0	45·0–46·0	Ushakov, 1960
	Erythrocytes, paranecrosis	15	44·0	48·0	Braun and Fizhenko, 1963

TABLE 1 (continued)

1	2	3	Temp. (°C)		Author
			<i>R. temporaria</i>	<i>R. ridibunda</i>	
Proteins	Glycerated gullet tissue from pharynx of frog, cessation of ciliary movement	5	40·0	44·0	Alexandrov and Arronet, 1956
	Dehydrogenase activity of cornea <i>in vivo</i> , a 50% decrease	30	38·0	44·5	Makhlin, 1963
	Succinic dehydrogenase activity of muscle homogenates, a 50% decrease	30	42·0	46·0	Vinogradova, 1961
	Cholinesterase activity of homogenates of the liver, 50% decrease	30	41·0-42·0	44·0-45·0	Kusakina, 1961
	ATP-ase of erythrocytes, 50% inactivation	15	46·0	49·6	Braun and Fizhenko, 1963
	ATP-ase activity of muscle homogenates, full inactivation	5	40·0-42·0	46·0-50·0	Komkova and Ushakov, 1955
	Actomyosin, a 50% decrease in ATP-ase activity	15	40·5	44·5	Braun, Nsvetayeva and Fizhenko, 1959
	Myosin, a 50% decrease in ATP-ase activity	30	17·0	20·0	Vinogradova, 1964
	Haemoglobin, 50% denaturation	15	60	64	Braun and Fizhenko, 1963

thermal adaptation of the species. How can this conclusion be linked with the assumption that the thermostability of cells and proteins does not play any role in individual thermal adaptation of the adult organism?

In considering this question we drew the conclusion that cell thermostability is a manifestation of thermal adaptation of poikilotherms at the earliest stages of ontogenesis. At that moment the organism undergoes cellular stages of development (gametes, zygote and early stages of cleavage). Natural selection, which forms adaptations, directly influences cells solely at this very moment (Ushakov, 1963c).

As can be seen from the table and is considered in detail in Andronikiv's report, the thermostability of sex cells is a conservative species index. A similar relation has been found by Runnström on the embryos of marine animals and by Moore on the embryos of amphibians. These authors also detected a surprising intraspecific constancy in the thermostability of the embryos of different populations from a number of species at various parts of their natural areas. These data might be connected with the well-known fact that even eurythermic species reproduce themselves and undergo early embryonal stages in stenothermal conditions.

It should be noted that the cell thermostability of an adult organism is in good conformity with the reproduction temperature of animals. All these facts enable us to suggest that the level of the thermostability of specialized cells in the adult organism is a rudiment of the thermal adaptation of sex cells and embryos at early developmental stages. Since all the cells of the organism arise from a sole zygote, interspecific differences in the thermostability of cells and proteins are universal and characteristic of all or a majority of the cells of the organism. Now a question arises how are these universal differences in the thermostability of cells and proteins formed during micro-evolution? The answer is given by a study on those 15 per cent of species which show differences in the thermostability of cells and proteins in different populations. These species are believed to be undergoing a stage of intraspecific divergence. We have investigated 8 species of poikilothermic animals which are in the process of formation of new species.

Let us consider two examples. The thermostability of cells and proteins in three races of the Baikalian fish *Coregonus autumnalis* was investigated. Two races from Posolsk and Selenga exhibited no differences either in the thermostability of the myocardium or in that of the cholinesterase of muscle homo-

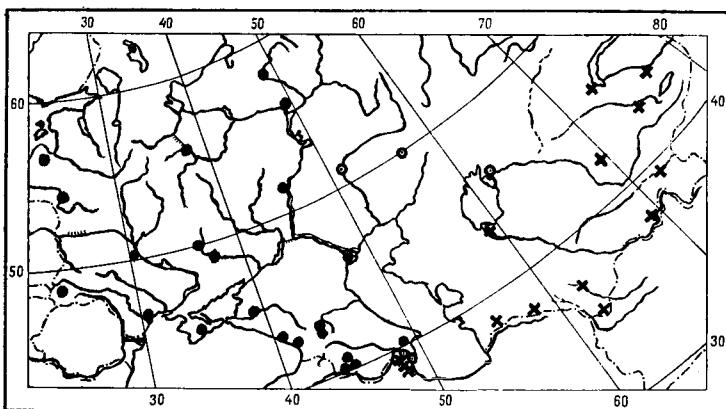


FIG. 4. Habitats of 42 investigated populations of *Rana ridibunda*: populations characterized by high (black circles), low (crosses) and intermediate (white circles) muscle thermostability (Ushakov, 1963 b).

genates. The thermostability of cholinesterase and myocardium in the third race (North Baikalian) proved to be significantly different. It is interesting that, according to Taliev (1941), there are serological differences between the North Baikalian and the other two races of the fish.

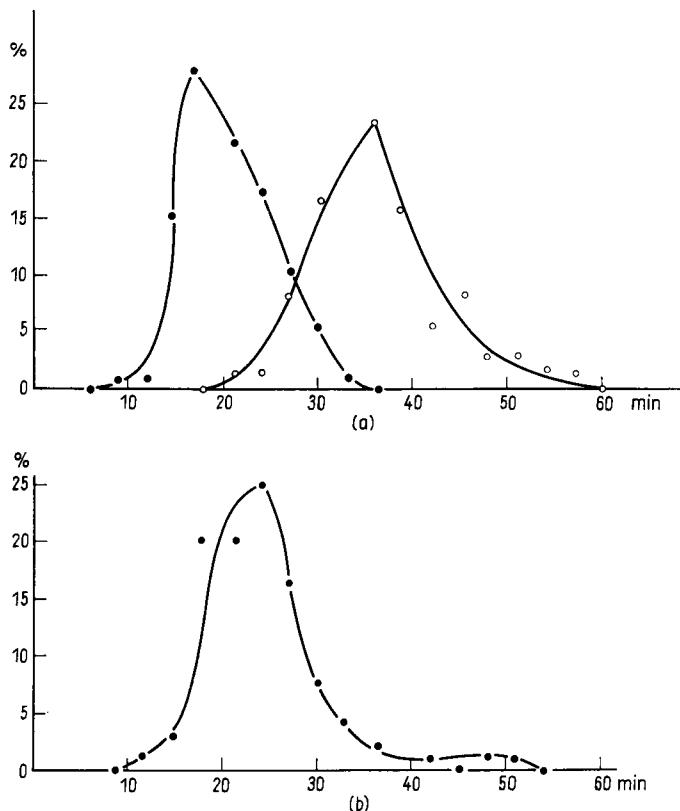


FIG. 5. Distribution curves of muscle thermostability in *Rana ridibunda*: (a) from Central Asia and Talysh (on the left) and European part of the U.S.S.R. (on the right); (b) 5 populations from Salyan and Port Iljic in Azerbaijan, and from Uralsk, Aktubinsk and Aralsk in Kazakhstan. The abscissa gives the time of development of muscle nonexcitability at 40°C (in min); the ordinate, the number of experiments (in percentage of the total number) (Ushakov, 1963b).

However, the resistance of the ATP-ase from muscle tissue was found to be identical in all the three races (Ushakov, Vinogradova and Kusakina, 1962). It means that in the process of divergence changes in the thermostability occur gradually but not simultaneously in all the proteins.

The intraspecific divergence in the lake frog *R. ridibunda* is best studied (Ushakov, 1963b). The muscle thermostability was investigated in 42 populations (Fig. 4). This species is widely spread throughout the territory of the

Soviet Union. By their muscle thermostability the frogs inhabiting the European part of the Soviet Union and Bulgaria differ from the frogs of Central Asia and Talysh (in the Eastern part of Transcaucasia). The upper row in the Fig. 5 represents the distribution curves for muscles, according to their thermostability within both groups of frogs. The intermediate territory is inhabited by frog populations with intermediate thermostability. Detection

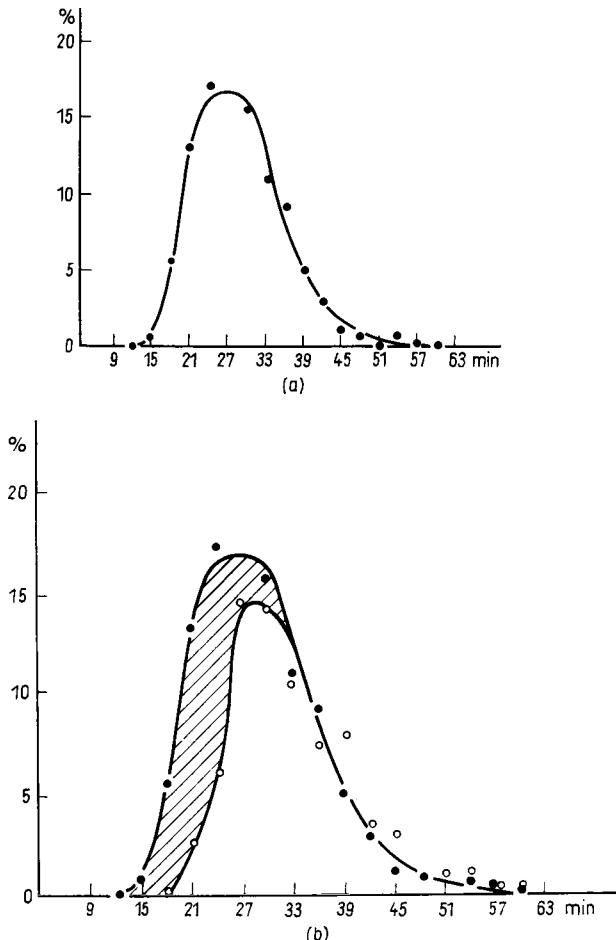


FIG. 6. Changes in the distribution curve of muscle thermostability of *Rana temporaria* tadpole tails as a result of spermatozoan selection: (a) Distribution in control series; (b) Changes of the curve in experimental series. Control data given by solid points, data for tadpoles from zygotes formed with heated sperm given by open circles. The hatched area indicates a decrease in a relative number of individuals with lowered muscle thermostability as a result of spermatozoan selection through thermostability (Ushakov and Chernokozheva, 1963).

of frogs with intermediate thermostability shows that there is a hybridization zone between two groups of frogs.

In accordance with these data in both groups, differences were detected between thermostability of cholinesterase in muscle and liver homogenates and an actomyosin ATP-ase (Vinogradova and Kusakina, 1963). But these groups still do not show difference in the thermostability of spermatozoa (Svinkin, 1959). Differences in haemoglobin thermostability are of another sign compared to the other proteins and whole muscles (Braun and Fizhenko, 1963).

The example also shows that changes in the thermostability of various cells and proteins do not occur simultaneously in the process of speciation.

Assuming that at earlier stages of ontogenesis cell thermostability is of adaptive significance it was important to ascertain whether it is possible, by means of gamete selection, to change the cell thermostability of multicellular organisms developed from these gametes. Such an experiment has been made on *R. temporaria* (Ushakov and Chernokozheva, 1963). The frog sperm was exposed to high temperatures causing selection only of most resistant spermatozoa. Then the ova were fertilized both by the selected and control spermatozoa. The thermostability of the tadpole tail muscles were compared in both groups. It was discovered that the muscle tissue of the tadpoles grown from the ova fertilized by spermatozoa, which had been previously exposed to heating, was more resistant than that of the control samples. It should be noted that an increase in muscle thermostability took place not at the expense of the development of individuals with more resistant muscles as compared to the control, but at the expense of elimination of specimens with less resistant muscle tissue. Figure 6 represents distribution curve of the thermostability of the tadpole tail muscles in the control series and its change resulting from spermatozoan selection. The hatched area corresponds quantitatively to the decrease in the relative amount of individuals with low muscle thermostability resulting from selection of more thermoresistant spermatozoa. This experiment shows that by exposing gametes to high temperatures it is possible to alter the thermostability of specialized cells of the organism. Evidence has still to be presented whether this fact can be considered as a model of speciation.

The material discussed shows that a study on the thermostability of cells and proteins is significant for the physiological analysis of the species problem and microevolution.

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SPECIES DIFFERENCES IN THE HEAT-RESISTANCE OF PROTOPLASMIC PROTEINS OF MULTICELLULAR POIKILOTHERMIC ANIMALS

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THERE is no doubt that the adaptive reactions of cells result from the interaction of many protoplasmic components. However, it is desirable to evaluate the significance of separate protoplasmic components relative to the thermostability of cells. For this purpose it was useful to take into consideration the results obtained from direct experiments on the determination of protein thermostability in representatives of the species differing in their environmental temperature conditions.

But before discussing the experimental data obtained chiefly in the Laboratory of Comparative Cytology, we shall describe the limits within which we confine ourselves. In the present communication we shall discuss only the data on the thermostability of intracellular proteins which are more susceptible to injury than extracellular ones. The constancy of the properties of such proteins is strictly maintained through natural selection by means of the cell homeostasis mechanism (Anfinsen, 1959). The data obtained concern poikilothermic animals for which environmental temperature conditions are especially important. We shall discuss "resistance-adaptation" and not "capacity-adaptation" according to the terminology of Professor Precht, and, finally, we shall discuss only phylogenetic changes in thermostability, i.e. genetically determined differences in the thermostability of protein protoplasmic complexes formed by natural selection in the process of species evolution.

Accordingly, taxonomically closely related animal species were chosen as the subject of our investigation. The experiments were performed on molluscs, the fishes, amphibians and reptiles. The investigations were carried out mainly on the protein preparations showing specific enzymic activity. The thermostability of these preparations was determined by the decrease observed in enzymic activity due to heating and the relationship of residual activity to inactivation temperature was established following a constant heating time, usually 30 min. In some cases for each given temperature, we also determined

the time for 50 per cent inactivation and made a curve indicating a relation of these times to temperature.

Thus far, the cholinesterase activity of muscle, brain and liver homogenates (Kusakina, 1963), endogenous dehydrogenase spontaneous activity of cornea tissue (tetrazolium salt reduction) (Makhlin, 1963), succinic dehydrogenase of muscle homogenates (Vinogradova, 1961), ATP-ase activity of muscle-homogenates, actomyosin and myosin, and haemoglobin have been investigated (some experimental results are given in the report of Ushakov, 1966).

The thermostability of enzymes was investigated for 36 species of poikilotherms. Species of the same genus were studied in 6 cases (14 species belonging to 6 genera). No coincidence has been detected in the thermostability of homologous proteins in representatives of different species. These statistically reliable differences were always in correlation with the environmental conditions of a species. Table 1 represents the data showing the degree of thermo-

TABLE 1. ENVIRONMENTAL TEMPERATURE CONDITIONS OF RAYS AND THEIR ACTOMYOSIN THERMOSTABILITY

	<i>Raja radiata</i> Donov	<i>Raja clavata</i>	Authors
Geographic boundaries	In latitude from 55 to 80° N	In latitude from 35 to 70° N	Nicolsky, 1950; Andriyashev, 1954
Seasonal temperature fluctuations of the habitat	From -1.7 to +7.5°C	From +7.8 to 20°C	Knipovich, 1932; Andriyashev, 1954
Water temperature during egg development	From +1 to +5°C	From +10 to +20°C	Deryugin, 1915; Knipovich, 1932
50% actomyosin inactivation at 30 min heating	34°C	36°C	Vinogradova, 1963

philia in the rays: *Raja radiata* Donov. from the Barentz Sea and *Raja clavata* L. from the Black Sea. The ray *R. radiata* inhabits waters of lower temperatures, which fact is especially significant during reproduction. In this cold-loving ray the thermostability of actomyosin is lower than that in the thermophilic Black Sea species *R. clavata* (Vinogradova, 1963).

The data on the cholinesterase thermostability of muscle of fishes, inhabitants of estuaries in the Baikal region and of the open waters of Baikal, (Kusakina, 1963) demonstrate the relationship between the protein thermostability and the environmental temperature (Fig. 1). At the same time fish belonging to taxonomically different groups were compared. The fish investigated can be divided into three groups on the basis of environmental temperatures: (1) inhabitants of shallow, well-warmed bodies of water; (2) representatives from the bays of Lake Baikal; (3) inhabitants of the open waters of Lake Baikal. In order of cholinesterase thermostability, the fishes can be ordered in conformity with the environmental temperature conditions of different species.

The cholinesterase thermostability proved to be highest in *Phoxinus percnurus* and *Cobitis taenia*, which inhabit shallow, well-heated bodies of water. In the region of Goryachinsk on the eastern shore of Lake Baikal, Cobitis inhabits a warm spring with a water temperature of 30–34°C. Intermediate

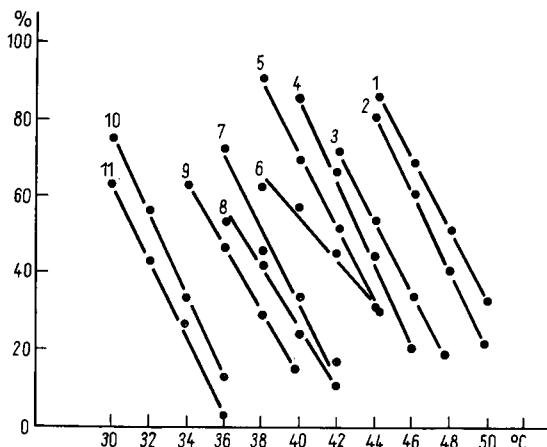


FIG. 1. Residual activity of cholinesterase of muscle homogenates vs. inactivation temperature. 1—*Phoxinus percnurus*; 2—*Cobitis taenia*; 3—*Rutilus rutilus*; 4—*Perca fluviatilis*; 5—*Cottocomephorus grawinksi*; 6—*Batrachocottus baicalensis*; 7—*Procottus jeitelesi*; 8—*Asprocottus megalops*; 9—*Abyssocottus godlewskii*; 10—*Comephorus dybowskii*; 11—*Comephorus baicalensis*.

cholinesterase thermostability is found in representatives of the bay fauna. In the Posolsky Bay, where *Perca fluviatilis* and *Rutilus rutilus* were caught, the water temperature in the summer reaches 19–23°C. A completely different temperature is characteristic of the open waters of Lake Baikal where even in the warmest time of the year, the temperature of the surface layers does not exceed 10°C and in the deep bottom layers, the temperature is about 3°C throughout the whole year. The cholinesterase thermostability of Cottus and two species of Comephorus which inhabit this region, proved to be the lowest of the fishes tested.

The correlation between the temperature conditions of the environment and the cholinesterase thermostability is clearly apparent not only from comparison of representatives of different ecological groups but also of taxonomically close species, for example the inhabitants of the open waters of Lake Baikal such as the suborder Cottoidei. The amplitude of the seasonal temperature variations and the maximum summer temperature of the water decrease as the depth increases. Based on the depth to which a given species is limited and, in particular, the depth at which spawning occurs we can judge to some extent the degree of the thermophilic nature of a given species. The deeper water species are evidently more cryophilic. Table 2 shows Taliev's data (1955) on the verti-

cal distribution and temperatures of thermal narcosis of the intact animal, as well as the results of the study of cholinesterase thermostability in the investigated Baikalian Cottoidei. To determine the temperature of thermal narcosis, the fish was placed in water at 3–4°C which was gradually heated up to the temperature at which responses to a stimulus ceased and respiration became spasmoidic and arrhythmic. The criterion of cholinesterase thermostability was the temperature at which 30-min treatment caused a 50 per cent decrease in activity. It is evident from the table that more cold-loving deep-water species

TABLE 2. THERMOSTABILITY OF ORGANISM AND MUSCLE CHOLINESTERASE OF BAIKALIAN COTTOIDEI

Species	Zone at which spawning occurs (after Taliev, 1955)	Temperature of narcosis of fishes (after Taliev, 1955)	Temp. of 50% inactivation of cholinesterase
<i>Cottocomphorus grawinkii</i>	Littoral	20·1	42·2
<i>Batrachocottus baicalensis</i>		20·6	40·8
<i>Procottus jeitelesi</i>	Sublittoral	19·0	38·2
<i>Asprocottus megalops</i>	Deep-water	17·5	36·8
<i>Abyssocottus godlewskii</i>		17·0	35·6
<i>Comephorus dybowskii</i>	Cannot be associated with a definite bathymetric zone; are found from sublittoral to abyssal (below 500 m)	9·8	32·4
<i>Comephorus baikalensis</i>		8·7	31·2

are characterized by low thermostability both of the organism as a whole and of the cholinesterase. The species are arranged in the table according to their vertical distribution, and this arrangement coincides with the order of thermostability of the protoplasmic proteins. The temperatures of thermal narcosis of the fishes are also in accord with our data.

We shall confine ourselves to two examples indicating a correspondence between the environmental temperature conditions of the species and the thermostability of protoplasmic proteins (for literature see Kusakina, 1963 a). It must be noted, however, that the proteins of the representatives of more thermophilic species always proved to be more thermoresistant. We do not have precise information in all the cases on the temperature conditions of the investigated species, but we have never observed instances in which a more heat-loving species had proteins less resistant to temperature. This coincidence cannot be regarded as accidental. We could not determine beforehand our choice of subjects under the field conditions. Thus, our experiments were influenced by the material we would obtain. In many cases determination of species was made after the experiments with enzymes had been done.

According to the protein theory of heat injury we may suppose that the thermostability of intracellular proteins can be judged by the heat-resistance of cells. To scores of species for which there is a correspondence between the thermostability of the intracellular proteins and the environmental temperature, we may now add hundreds of species for which such a correspondence is revealed by a comparison of cell thermostability. Thus species differences in the thermostability of protein protoplasmic complexes are related to the differences in the environmental temperature conditions.

A genotypical determination of these differences is expressed in the conservatism of the thermostability of protoplasmic proteins (Ushakov, 1966). We

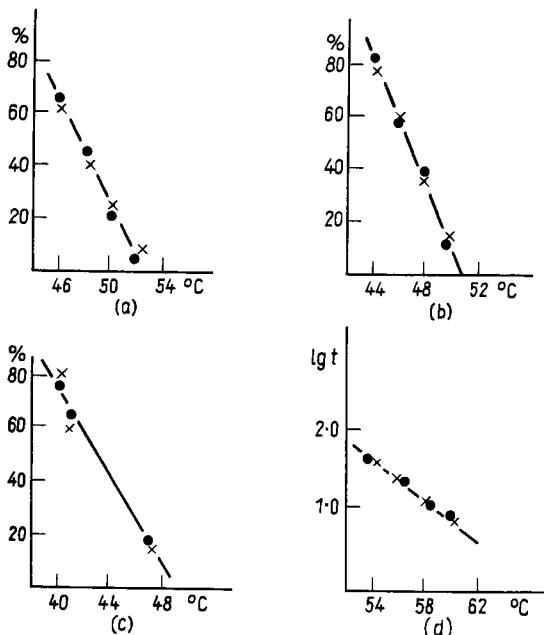


FIG. 2. Protein thermostability of *Bufo bufo bufo L.* (crosses) and *B.b. virucosissima* (points). Abscissa—inactivation temperature (the inactivation time is 30 min); ordinate—(a) cholinesterase activity of liver homogenates, (b) cholinesterase activity of muscle homogenates, (c) ATP-ase activity of muscle homogenates (according to Panteleyeva and Ushakov, 1956), (d) haemoglobin, logarithm of time for 50 per cent coagulation (from solubility).

shall give here several examples of this conservatism (Fig. 3). One of the two subspecies of *Bufo bufo virucosissima* from the North Caucasus lives in a milder climate than *Bufo bufo* from the environments of Leningrad. It is evident from the curves in Fig. 2 that despite this fact, the thermostability of the ATP-ase and cholinesterase of muscle homogenates and the cholinesterase of liver homogenates and blood haemoglobin in these two species is identical.

Points indicate the data for the Caucasian and crosses for the Leningrad subspecies; both subspecies coincide on the same straight line. Even a greater difference characterizes the environmental temperature conditions of *Carassius auratus gibelio* from a warm spring (Coryatchinsk on the eastern shore of Lake Baikal) and those of normal waters in which temperature varies through the year from about zero to a high level. *Carassius* from a thermal spring lives at the temperature of 25–30°C during the whole year. Nevertheless the cholinesterase thermostability of muscle homogenates in both of these groups of *Carassius* coincides (Fig. 3).

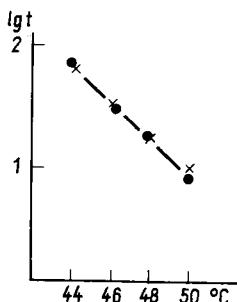


FIG. 3. Cholinesterase thermostability of muscle homogenates of *Carassius auratus gibelio* Bloch. from the hot spring (points) and the water of normal temperature (crosses). Abscissa—inactivation temperature; ordinate—logarithm of time of 50 per cent inactivation.

The content of non-protein components of intracellular media changes in the process of acclimation, and, probably, differs in specimens of one species taken from different points of an area. However thermostability of cells and proteins, even such contaminated ones as cholinesterase of liver homogenates, is practically the same. Therefore, Ushakov (1958) suggested that cell thermostability is not determined only by non-protein admixtures. Glushankova (1963) extracted lipid admixtures from actomyosin preparations of muscle of *Trachurus* from the Black Sea. In "large" and "small" *Trachurus* from one shoal actomyosin thermostability differed (Altukhov, 1966) while the iodine numbers of the lipids coincided. There are some more arguments denying the leading role of admixtures in determination of protein thermostability (Ushakov and Glushankova, 1962a, b, 1963; Glushankova, 1963; Amosova, 1963; Kusakina, 1963c; Sleptsova, 1963). This concerns, for example, the same range of differences in the thermostability of different protein preparations extracted from the tissues of the same pair of species. The table presenting data on the protein thermostability of grass and lake frogs was given in Ushakov's report. But there is no doubt that various concentrations of protein preparations isolated from different tissues and differently purified are not equally contaminated with lipids and other non-protein substances. However, a range of species differences in thermostability is actually the same for such well-purified pro-

teins as myosin and haemoglobin and such maximally contaminated proteins as the cholinesterase of liver homogenates or succinic dehydrogenase of muscle homogenates.

The experiments of Gustavson and Takahashi (Gustavson, 1956) are most convincing arguments confirming the existence of real species differences in the thermostability of proteins. These authors not only described differences in the collagen thermostability in 25 fish species from the waters of Sweden and Japan. They showed a correspondence between the "shrinkage temperature" of collagen and the environmental temperature of this fish. They gave a detailed analysis of the nature of species differences in the collagen thermostability which was found to be based on the peculiarities in the primary structure of a molecule.

Figure 4, drawn according to the data of Gustavson and Takahashi, shows the correlation they detected between the level of collagen thermostability

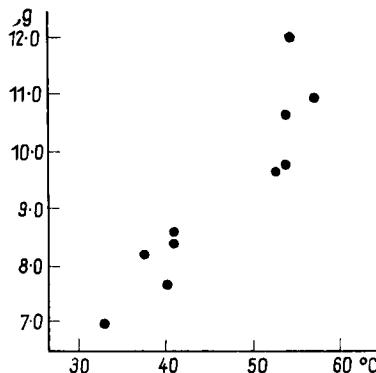


FIG. 4. The relation of collagen thermostability of some teleosts to oxyproline content in a molecule. Abscissa—collagen shrinkage temperature; Ordinate—oxyproline content in g per 100 g collagen (according to Gustavson, 1956).

and the amount of oxyproline in its molecule. Apparently, a higher collagen thermostability in thermophilic fish is provided due to an increase in hydrogen bonds at the expense of oxyproline OH groups. In denying the leading role of admixtures in determination of protein thermostability, we mean only the admixtures which contaminate protein complexes separated from tissues. But the non-protein components of protein protoplasmic complexes seem to be significant for the thermostability of cells and proteins. Thus differences in the thermostability of homologous intracellular proteins in representatives of taxonomically closely related poikilothermic animals cannot be regarded as artifacts for they exist in reality. Their biological significance was noted by Ushakov (1966) and Andronikov (1966).

Therefore, we shall only speak about possible biochemical mechanisms by means of which a correspondence between protein thermostability and

environmental temperature conditions of a species is obtained. At the present time, we know almost nothing about these mechanisms, but nevertheless we can formulate the demands required for any attempt to interpret the correspondence. Firstly, it should be noted that species differences in the protein thermostability are rather considerable, 20°C for the cholinesterase of muscle homogenates (Fig. 1), 26°C for actomyosin of skeletal muscles, 24°C for collagen (Fig. 4). Secondly, these differences are universal, i.e. they concern various proteins isolated from the tissues of the same pair of species (see the table in Ushakov's report) and are approximately of the same amplitude for all the proteins of a species.

The work of Gustavson and Takahashi is the only investigation we know which gives a biochemical analysis of species differences in the thermostability of homologous proteins in poikilothermic animals. In the case of collagen, these differences proved to be related to the peculiarities in the primary structure of the protein molecule. However, we have no reason to suppose that for all the species and proteins investigated this mechanism is the only possible one. It is not excluded that the heterogeneity reported for many proteins and for enzymes, in particular, has some significance for this problem. In the case of enzymes these multiple forms are known as isozymes. In the case of lactate dehydrogenase there were found isozymes which sharply differed in their thermostability according to Plageman, Gregory, and Wroblewski (1961). Had such isozymes been detected in different proportions in different species they could have accounted for species differences in the thermostability of a given enzyme.

We hope that a further analysis of the mechanisms proving species differences in the thermostability of homologous proteins will contribute to the evolutionary biochemistry.

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THE RELATION BETWEEN RESISTANCE OF CELLS AND TISSUES TO DAMAGE AND DENATURATION CAPACITY OF PROTEINS

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PHYSIOLOGY and biochemistry present a great deal of evidence demonstrating the differences in the resistance of living systems at various levels of organization to the effect of injurious factors. There are numerous examples showing different resistance of homologous tissues in different species of animals as well as within one species (Alexandrov, 1952; Ushakov, 1956; Kusakina, 1963). Variations in resistance depending on the season, sex, stage of ontogenesis, physiological state (Svetlov, 1943; Ushakov, 1963) and also changes in resistance of tissues, cells and intracellular structures in relation to their morphology and the character of metabolism (Hunter *et al.*, 1959; Braun and Ganelina, 1961; Ganelina, 1962) were previously described.

Now the question arises: what is the reason for the different stability of protoplasm to injury? At the present stage of investigation the protein theory evidently gives the best-founded concept of the problem. According to this theory, the reactivity of the cell and substantial changes which occur therein in response to the action of stimuli are due to the denaturation of cell proteins (Nassonov and Alexandrov, 1940; Nassonov, 1959). Hence, the stability of protoplasm is the resistance function of intracellular proteins. In this connexion the existence of mechanisms regulating the stability of these proteins has been postulated. The process of proteinization can be regarded as one of such mechanisms (Engelhardt and Venkstern, 1943; Braun, 1960). Structural and chemical (conformational) changes of the protein macromolecule causing the change in its resistance are temporal and appear as a result of intermolecular binding of the protein with other substances—sucrose, glycerin, nucleic acids, ATP, etc. It is known that the addition of such substances to the protein *in vitro* prevents it from denaturation (Putnam, 1953). The other mechanism is the synthesis of protein, the structure and properties of which are fixed hereditarily. In modern protein chemistry one can find numerous examples of protein isomers and protein-variants possessing similar functions but differing in the composition of their primary structure conformation and in

their physico-chemical properties (Tomkins *et al.*, 1961; Kushner, 1963). The question as to which way the cell selects to provide the stability of its proteins must be experimentally proved. It is the topic of numerous investigations begun by Claude Bernard. However, the data obtained are rather miscellaneous and contradictory. Alongside with the works demonstrating the existence of a correlation between the resistance of tissues and extracted proteins (Hammarsten, 1908; Mirsky, 1938; Korzhuyev and Koshtoyanz, 1934; Alexandrov and Arronet, 1956; Panteleyeva and Ushakov, 1956) there is also negative evidence to this correlation. Moreover, there are (Siebert *et al.*, 1960) many works establishing the relation of cell resistance to injury to antidenaturant content (Bate-Smith and Bendall, 1947).

This question is far from being clear and needs further experimental research.

We investigated contractile muscle proteins of lake (*Rana ridibunda* Pall.) and grass (*Rana temporaria* L.) frogs (Braun, Nesvetayeva and Fizhenko, 1959). According to some authors, muscles of the lake frog are more resistant to the injurious effect of heating: thermal contracture of muscles and accompanying signs of damage in lake frogs is observed at temperatures 2·5–3° higher than in grass frogs. In agreement with these data the actomyosin of the lake frog proved to be more resistant to heat denaturation: a 50 per cent inhibition of ATP-ase actomyosin activity at 15 min heating occurs at 44°C for *R. ridibunda* and at 40·4°C for *R. temporaria* (Fig. 1).

We have found differences in the resistance of erythrocyte proteins of the same animal species (Braun and Fizhenko, 1963). The erythrocytes of the lake frog are more resistant to heat injury as compared with those of the grass

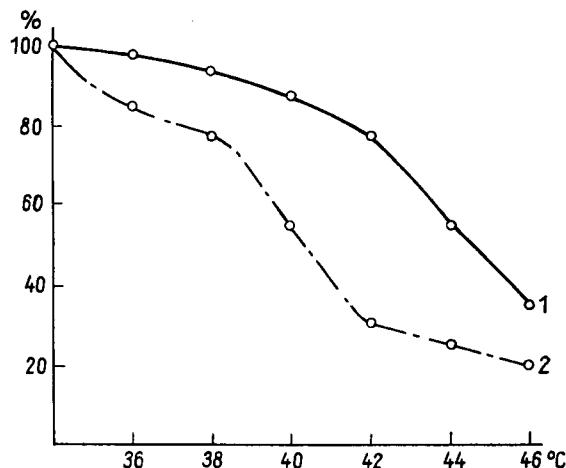


FIG. 1. Heat denaturation of actomyosin of skeletal muscles in *Rana ridibunda* (1) and *Rana temporaria* (2). The abscissa gives the temperature (in °C); the ordinate, ATP-ase activity of actomyosin after a 15-min heating (in percentage of the activity of unheated actomyosin).

frog, the inhibition of the formation of dye granules and other signs indicating injury of erythrocytes of the lake frog are observed at a temperature 4° higher than in the grass frog. According to these data, proteins of erythrocytes (fixed ATP-ase and haemoglobin) are more resistant in *R. ridibunda* than in *R. temporaria*: a 50 per cent inhibition of erythrocytic ATP-ase takes place at 46°C for *R. temporaria* and at 49.6°C for *R. ridibunda*. A 50 per cent haemoglobin denaturation of the grass frog is attained at 60°C, whereas that of the lake frog at 64°C (Fig. 2).

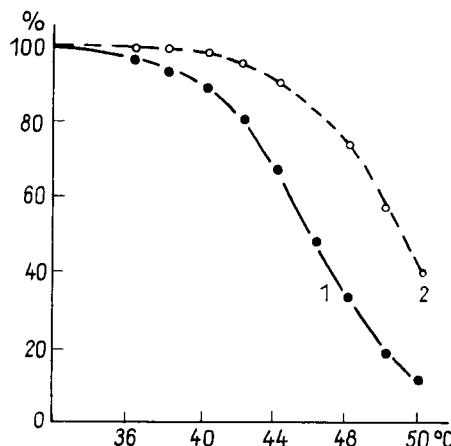


FIG. 2. Heat inactivation of adenosine triphosphatase (ATP-ase) of erythrocytes in *R. temporaria* (1) and *R. ridibunda* (2). The abscissa gives the temperature (in °C), the ordinate, ATP-ase activity after a 15-min heating (in percentage of the activity of unheated erythrocytes).

We have also obtained evidence on the resistance of contractile proteins of the myocardium and skeletal muscles (Braun, Nesvetayeva and Fizhenko, 1963). According to Rumyantsev and others during the same period of heating the cardiac muscle can tolerate a temperature 3.5–4° higher than skeletal muscles (Rumyantsev, 1960). As can be seen from Figs. 3 and 4 the actomyosin of cardiac and skeletal muscles differs similarly: a 50 per cent decrease in ATP-ase actomyosin for the cardiac muscle is observed at 46°C and for skeletal muscles at 40°C.

Figure 5 represents the data obtained from the study of proteins of stretched and unstretched skeletal muscles. Weiss in the U.S.A. and Ganelina in the U.S.S.R. have shown that the removal of the contractures which appear as a result of the action of stimuli on the muscle increases resistance to damage (Weiss, 1933; Ganelina, 1962). It was suggested that the higher resistance of stretched muscles might be due to the greater stability of their proteins through the fact that the stretching of muscles under a small load can change (decrease) denaturation ability of muscle proteins.

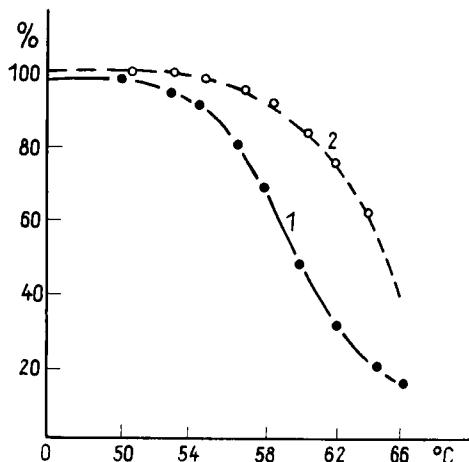


FIG. 3. Heat denaturation of haemoglobin of *R. temporaria* (1) and *R. ridibunda* (2). The abscissa gives the temperature (in °C), the ordinate, the amount of dissolved haemoglobin (in percentage of the haemoglobin concentration in the unheated solution).

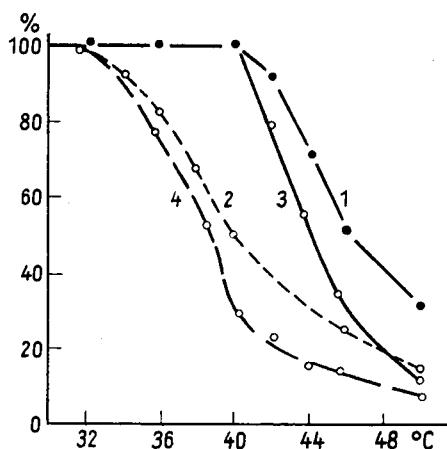


FIG. 4. Heat denaturation of actomyosin from the heart (1,3) and skeletal muscles (2,4) of the rabbit (1,2) and grass frog (3,4). The abscissa gives the temperature (in °C); the ordinate, the ATP-ase activity of actomyosin after a 20-min heating (in percentage of the activity of unheated actomyosin).

Experiments with glycerinated models confirmed the suggestion. It was established that after a spontaneous heat denaturation a stretched muscle model retains greater enzymic (ATP-ase) activity and greater amount of soluble proteins, and the muscle fibres extracted from the model show a higher contractile ability. The data obtained by Ganelina were also supported theoretically: recently it was shown that the stretching of muscles under a small load

is necessary and sufficient for the stabilization of α -helical configuration of fibrillar muscle proteins and can make them more resistant to denaturating effects (Vorobyev and Ganelina, 1963).

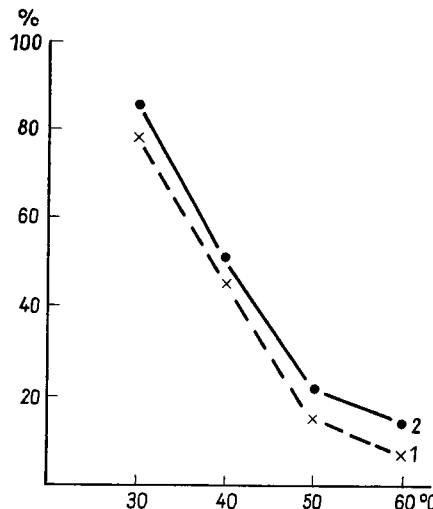


FIG. 5. ATP-ase activity of contracted (1) and stretched (2) muscle models. The abscissa gives the temperature (in °C); the ordinate, the ATP-ase activity in 1 mg phosphorus per 100 mg of the wet weight of muscles.

There are more data demonstrating a very distinct correlation between the stability of tissues and cells on the one hand, and the protein resistance of these tissues and cells on the other. During the last years a vast amount of data were obtained by Ushakov, Markert *et al.* (Ushakov, 1963; Markert and Ursprung, 1962). These data show that in all cases when differences were found between the resistance of the tissues similar difference were also detected between the resistance of the proteins extracted from these tissues. On the contrary, in cases where the stability of various tissues did not differ, no difference was observed in the resistance of proteins. These data suggest that the resistance of the tissues and cells is due to the resistance of the proteins. Hence, the stability of the functional activity of the tissues can be used to judge the resistance of their proteins. A contrary suggestion seems to hold true: the difference between the resistance of proteins can be used as a criterion of tissue resistance. Thus it might be predicted that the myocardial and skeletal muscles should differ in their resistance to ethanol, for the actomyosin of these tissues shows evident differences in resistance to this agent (Braun, Nesvetayeva and Fizhenko, 1963). Probably differences will be found in heat-resistance of the uterine and skeletal muscles since significant divergences have been revealed in the thermostability of their actomyosin (Braun and Mirovitch, 1956).

We can point out the facts contradicting the suggestion that the cell injury is caused by the denaturation of protein components of the cell: in some cases

denaturation of proteins is brought about due to stronger alterations than the injury of the cell. Haemoglobin denaturation, for example, occurs at the temperatures above 55°C, i.e. 10–15° exceeding the temperature of thermal death. However, it should be taken into account that the death of the cell as a system is not necessarily accompanied by the alteration of all its components. The possibility is not excluded that isolated proteins possess properties differing from those in the cell, while in the protoplasm they are components of higher structures or of the complexes with low molecular substances; the breakdown of these complexes occurs under weaker influences.

It should be pointed out that there is evidence indicating the difference in the relation of proteins to the character of denaturating factors: some proteins differing in their stability to heating proved to be equally resistant to alcohol and high hydrostatic pressure. We came across similar adaptation of proteins to the temperature factor while investigating actomyosin and haemoglobin. However, heat-resistant proteins of thermophilic micro-organisms exhibit an increased resistance to different kinds of agents (Koffler, 1961). We are still far from a full understanding of the molecular mechanism of such changes in resistance. It might be only suggested that the stabilization of the protein macromolecule under various conditions is provided at different levels of its structural organization and due to bonds differing in energy and localization; therefore the denaturation of proteins makes it possible to reveal specific and non-specific features in the effects of external agents.

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THE STUDY OF ACTOMYOSIN THERMOSTABILITY OF SKELETAL MUSCLES OF SEVAN TROUT†

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THE conservatism of the thermostability of muscle cells and proteins, and its independence from the area of habitation enabled Ushakov (1959) to use this characteristic as a cytophysiological species criterion of poikilotherms. During recent years much evidence was obtained to confirm this point of view (Ushakov, 1959-1962; Kusakina and Vinogradova, 1959; Braun, Nesvetayeva and Fizhenko, 1959; Dzhamusova, 1960; Kusakina, 1962).

Experiments with partial (sympathectomized) or fully denervated muscles have shown that the thermostability of muscle contractile proteins is independent of nervous influence (Hovanessian and Petrossian, 1961; Hovanessian and Zamenian, 1961). It is probable that the degree of thermostability of cytoplasmic proteins plays a limiting role in the adaptation of the whole organism to fluctuations in its environmental temperatures (Stroganov, 1956).

The endemic complex species of the trout conspecies *Salmo ischchan* Kessler (Lake Sevan, Armenian SSR) is of special interest for the study of problems concerning the influence of environmental temperatures on the species conservatism of thermostability of cytoplasmic proteins. According to the literature (Fortunatov, 1927; Vladimirov, 1944) this species consists of four subspecies or races which differ in biological and morphological indices, chiefly in their spawning time and the optimum spawning temperature. It is very important to note that during the sexual cycle various races approach each other in their morphology (Leshinskaya, 1950).

The thermostability of actomyosin was studied in the two subspecies *Salmo ischchan gegarkuni* (S.i.g.) and *Salmo ischchan aestivalis* Fortunatov (S.i.F.). Evidently both these races have been inhabiting the Lake Sevan since the Quaternary period. According to Vladimorov (1944), S.i.F. spawns in summer and the maximum spawning time occurs when the water temperature rises to +9.6°C. The corresponding seasons for S.i.g. are autumn and winter when

† We wish to thank the workers of the Sevan Hydrobiological Station of the Academy of Sciences of Armenian SSR for their assistance and valuable advice in the preparation of the present work.

the temperature is +7.4°C. The S.i.F. spawns in the lake and in underground rivers, while S.i.g. spawns at the mouth of the river (Pavlov, 1951).

Our experiments were carried out on the shore of the lake and under laboratory conditions. Actomyosin was extracted from spinal muscles for 12 hr at +2°C, 2-3 hr after the fish had been caught. Actomyosin thermostability was determined by thermal inactivation of ATP-ase and cholinesterase activity and changes in the relative viscosity of protein solutions by means of incubation at +29, +31, +33, +35, +37°C ($\pm 0.02^\circ$) (Fig. 1). The results of the preliminary investigation have shown that calcium chloride is an ATP-ase activator for actomyosin and that the optimum pH of the enzyme differs for both species (7.19 for S.i.F. and 9.1 for S.i.g.). Cholinesterase activity is characterized by the same optimum pH.

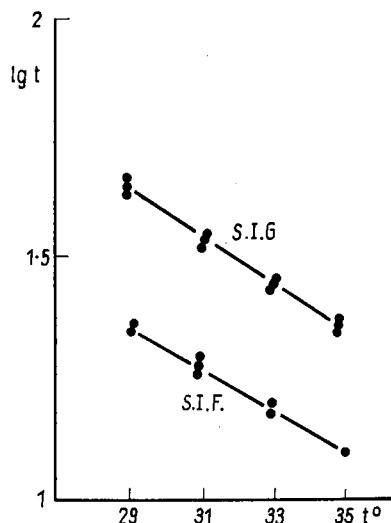


FIG. 1. Thermostability of actomyosin from of skeletal muscles S.i.g and S.i.F. determined by a decrease in viscosity of actomyosin solutions. The ordinate shows the logarithm of the time within which the viscosity of actomyosin is changed. Protein concentration is 1.65 mg per ml.

A study of the relative viscosity of actomyosin solutions has shown that incubation at +29°C leads to some decrease in the viscosity (-2 per cent). This incubation consisted of protein with Tris as a buffer. The buffer contained 2.5×10^{-3} M calcium chloride and 1.5×10^{-3} M ATP. The determination of a degree of ATP-ase inactivation in the both subspecies demonstrated that at 29°C the actomyosin of S.i.g. lost 30-35 per cent of the initial activity whereas S.i.F. 60-70 per cent (Fig. 2). The difference in thermostability was about 2-2.5°.

The cholinesterase activity of actomyosin was determined by the method of Koker *et al.* (1957). Acetylcholine was measured according to Hestrin (1949).

Experimental results have shown that the actomyosin heat denaturation was followed by the increase in cholinesterase activity. This activation probably is due to the fact that cholinesterase is related to some other protein molecule combined with actomyosin. A half-hour incubation of actomyosin at 37°C caused an increase in cholinesterase activity to 125 per cent of the initial value in S.i.g. and to 144 per cent in S.i.F. Hence actomyosin thermostability determined by cholinesterase activity proved to be higher in S.i.g.

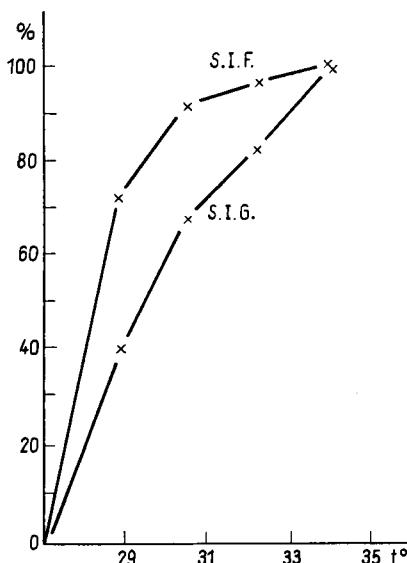


FIG. 2. Thermostability of actomyosin ATP-ase. The abscissa gives incubation temperature; the ordinate, inactivation of the enzyme (in per cent). Incubation—15 min.

The high actomyosin thermostability of S.i.g. is at variance with the optimum temperature of spawning. The high heat-resistance in this subspecies seems to be related to the fact that the growth and development of young fishes coincides with the maximal temperature regimen of water (spring-summer).

The assertion that subspecies of the Sevan trout have been isolated seems rather disputable. A long geological period of living under similar conditions unquestionably drew these subspecies nearer in biological as well as in morphological respects.

There are intermediate forms between the subspecies. Moreover, we know of cases where females of one subspecies were fertilized by males of another (Leschinskaya, 1950). However, there is also evidence indicating the remoteness of these subspecies: the S.i.g. form, for example, finds places for spawning as far as possible from the lake.

Considering the data obtained one must admit that a long existence under identical ecological conditions does not influence the protein thermostability, although there are other data indicating a considerable rapprochement of the species (biological as well as morphological).

Summary

1. Actomyosin thermostability in the subspecies of the Sevan trout *Salmo ischchan gegarkuni* is higher than in the subspecies *Salmo ischchan aestivalis* Fortunatov. The difference in thermostability is about 2–2.5°. This fact seems to indicate the taxonomic isolation of these subspecies.

2. The existence for a long geological period under similar ecological conditions has not influenced the protein thermostability of closely related subspecies of the Sevan trout.

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CYTOPHYSIOLOGICAL AND SEROLOGICAL ANALYSIS OF INTRASPECIFIC DIFFERENTIATION OF THE BLACK SEA HORSE MACKEREL

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THERE is no morphological difference between "large" and "small" horse mackerel inhabiting the Black Sea. Therefore, both these forms are regarded as different local stocks of one and the same species *Trachurus mediterraneus* Stdr. (Aleyev, 1956). Nümann (1956) does not differentiate between these forms and considers "small" horse mackerel to be a young age group of the "large" one. At the same time, divergencies in the biology of these fishes are rather remarkable and allowed some authors to characterize "large" forms as a taxonomic category which is higher than a stock (Tikhonov, 1959; Safyanova and Revina, 1960). However, this supposition has not been supported by taxonomic material. Therefore, the aim of the present investigation was to study the character of differentiation between the Black Sea mackerels by means of the cytophysiological determination of the isolated muscle tissue thermostability (Ushakov, 1959) and by the serological ring-test method (Boyden, 1926).

Material and Method

Investigations were carried out at the Kara-dag Biological Station of the Ukrainian Academy of Sciences and at the Azov-Black Sea Research Institute for Marine Fisheries and Oceanography. Cytophysiological experiments were performed on shore and during marine expeditions. The thermostability of isolated muscle tissues of northern, eastern and southern horse mackerel was compared (Aleyev, 1956). The northern and eastern stocks are presented as "small", while the southern as "large" forms. Experiments were made on fresh-caught fishes by Ushakov's method (Ushakov, 1959a). Gill muscles of the mackerel (*m. transversus ventralis posterior*) were transferred to Ringer's solution heated to the standard temperature maintained with accuracy of $\pm 0.1^\circ$. The retention time of tissue excitability to electrical stimuli served as an index of thermostability. The induction current was generated by Du-Bois-Raymond (the voltage of the primary coil was 2.7 V). Serological analysis was carried out by the ring-test method described by Altukhov and Apokin

(1963). Polyvalent water-saline extracts from the dorsal muscles of the "large" and "small" horse mackerel *T. mediterraneus*, specimens of *Trachurus trachurus* Linné from the South-East African coastal areas and of *Sarda sarda* Bloch served as antigens. After the immunization of rabbits, active antisera were obtained in dilution 1 : 128,000 for "large" forms and 1 : 1,024,000 for "small" ones. The protein content of antigens was determined by Roberts-Stolnikov and micro-Kjeldal methods, both being used before the onset of immunization and in the process of reaction. The reaction was followed for one hour and the appearance of rings in different antigenic dilutions was recorded. In each series, two controls were made: normal rabbit serum + antigen, and anti-serum + 85 per cent sodium chloride.

Results

The thermostability curves of the isolated muscle tissue of the horse mackerel are shown in Fig. 1. The muscles of "small" horse mackerel of both northern and eastern stocks have been found to possess the same thermostability, whereas the muscles of "large" forms are characterized by much more pronounced heat-resistance.

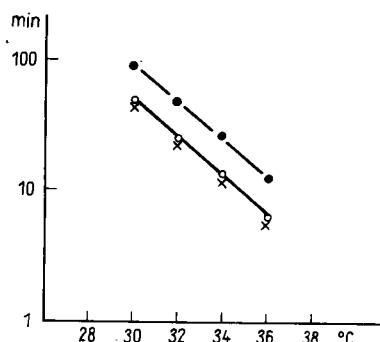


FIG. 1. Curves of thermostability of muscles of the Black Sea horse mackerel. The abscissa gives the temperature (in °C); the ordinate, the retention time of excitability (in min, logarithmic scale). Crosses—"small" horse mackerel of the eastern stock. Circles—"small" horse mackerel of the northern stock. Black circles—"large" horse mackerel.

The revealed difference is statistically significant (Table 1) and amounts to as much as 2°C.

Higher thermostability of the "large" horse mackerel muscles corresponds to its greater thermophilic characteristics—the fish hibernates in the Turkish and Caucasian inshore areas, i.e. in the warmest regions of the Black Sea (Aleyev, 1956, 1957, 1959; Safyanova and Revina, 1960; Altukhov, 1963).

Thus, the data available indicate a distinct cytophysiological difference between the "large" and "small" forms of the Black Sea horse mackerel. This difference bears an adaptive character.

TABLE 1. RETENTION TIME OF EXCITABILITY OF *M. TRANSVERSUS VENTRALIS POSTERIOR* OF THE BLACK SEA HORSE MACKEREL

	Temperature (°C)											
	30			32			34			36		
	<i>n</i>	$M \pm m$	<i>P</i>	<i>n</i>	$M \pm m$	<i>P</i>	<i>n</i>	$M \pm m$	<i>P</i>	<i>n</i>	$M \pm m$	<i>P</i>
"Small" horse mackerel of the northern local stock	21	51.4 ± 1.5	0.01	18	27.6 ± 0.9	0.50	15	13.4 ± 0.5	0.10	20	6.0 ± 0.1	0.25
"Small" horse mackerel of the eastern local stock	17	51.0 ± 1.9	0.999	15	26.6 ± 1.1	0.999	16	13.3 ± 0.5	0.999	15	6.1 ± 0.2	0.999
"Large" horse mackerel	10	87.2 ± 4.5		10	46.5 ± 1.9		10	26.2 ± 1.7		10	11.6 ± 0.4	

Note: *n*—number of tests; $M \pm m$ —the arithmetic mean and its error in the second power; *P*—probability of difference reliability.

The comparison of these differences with the data on the cell thermostability of various groups of poikilotherms and fishes in particular (Ushakov, 1959a, 1959b; Kusakina, 1959, 1960, 1962; Altukhov, 1963) shows that these differences are of the same range as those observed between taxonomically close species.

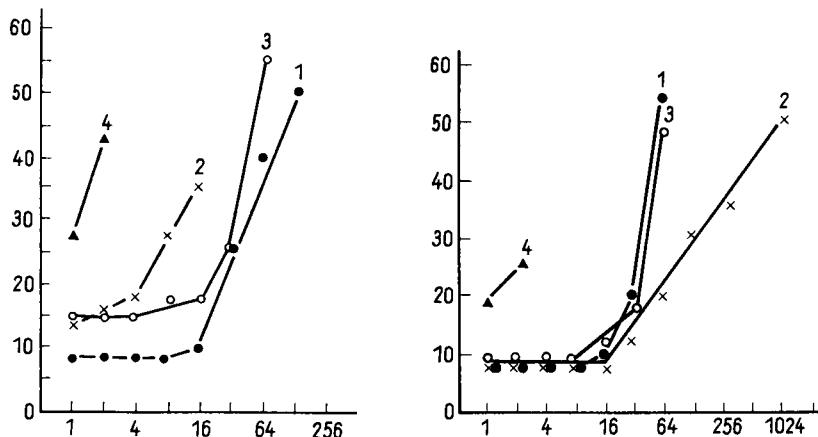


FIG. 2. Results of the precipitation reaction of different antigens with anti-serum of "small" (a) and "large" (b) horse mackerel, diluted in the ratio 1 : 6 by NaCl solution. Each point on the graphs represents a mean value obtained from two to three tests. 1. "Large" horse mackerel, 2. "Small" horse mackerel, 3. *T. trachurus* L., 4. Bonito (*Sarda sarda* Bloch). Unit of dilution—1 part of protein per 1000 parts of 0·85 per cent NaCl solution. The abscissa gives the dilution of antigens, the ordinate, the time of appearance of precipitation—ring.

Now let us dwell on the serological part of the work. The experimental results presented as graphs in Fig. 2 indicate that serological differences are observed between all the investigated forms and can be determined both by the rate and the dilution of the reactions. The least intensive reactions with the horse mackerel antiserum were observed with *S. sarda* antigens; this is due to the fact that these species belong to different families. Considering the character of differentiation of the horse mackerel, special attention should be paid to reactions of the antigens of the species *T. trachurus* which exhibits a closer approximation to the "large" horse mackerel than does the "small" one. On the other hand, the experiments with antiserum of the "small" form show that the species *T. trachurus* differs from "small" horse mackerel just as the "large" forms differ from the "small" ones. Thus the material shows that the degree of serological differentiation between the "large" and "small" horse mackerel is higher than their divergence, by this feature, from *T. trachurus* whose systematic position is indisputable.

On this basis it seems possible to draw conclusions about the interspecific character of the serological differences between "large" and "small" forms of the Black Sea horse mackerel.

Summary

A cytophysiological analysis has been carried out to reveal intraspecific differentiation of the Black Sea horse mackerel. The data obtained testify to the existence of distinct divergences between a "large" horse mackerel on the one hand a "small" horse mackerel of the northern and eastern local stocks on the other; these differences are considered as interspecific.

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CHANGES IN THERMOSTABILITY LEVEL OF *RANA TEMPORARIA* TADPOLE MUSCLE TISSUE AS A RESULT OF THE ACTION OF TEMPERATURE ON SPERMATOZOA

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A COMPARISON of the experimental material obtained in the Laboratory of Comparative Cytology with the published evidence had shown that the level of cellular heat-resistance in higher poikilotherms is a form of temperature adaptation which is presumably brought about at the early stages of ontogenesis when the organism consists of one or many slightly differentiated cells (see Ushakov and Chernokozheva, 1963). At this time the organism exhibits minimal adaptive possibilities which are wholly limited by cellular adaptations. On the basis of the above it can be suggested that natural selection, which constitutes adaptation, controls the thermostability level of the cells at these early stages of ontogenesis and that more stable gametes give rise to animals with more resistant and specialized cells.

We studied muscular heat-resistance of the tadpole tail (*Rana temporaria* L.) in control animals and those developed from the zygotes after the fertilization by spermatozoa exposed to preliminary heating (36°C for 30–40 min). Muscular heat-resistance was estimated by the time required for the loss of excitability at 38°C.

A study of heat-resistance in the muscle tissue of the control animals revealed a considerable intrapopulation variability which cannot be due to methodical reasons only. The minimum time for the loss of excitability is 15 min, whereas the maximum is 57 min. The mean value for 405 experiments is 29.31 ± 0.38 min.

It should be noted that the curve, indicating time distribution for the progression of muscular non-excitability at 38°C, was non-symmetrical ($g = 0.86$, $p = 0.001$), while the curve of the time logarithm distribution corresponds to the classical symmetrical Gauss–Laplace curve ($g = 0.007$, $p = 0.400$). The same evidence was obtained for other species of animals (e.g. *Rana ridibunda* Pall.). It shows that on comparing experimental results according to the

method of Student and Fischer one must use the geometrical mean time (arithmetical mean of time logarithm), but not the arithmetical mean time and its square error as usual. The logarithm of the geometrical mean time of non-excitability progression was 1.4530 ± 0.0050 .

Muscular heat-resistance of tadpoles which grew from the ova fertilized by the spermatozoa specially selected according to a thermostability level increased, which was in agreement with our supposition. The arithmetical mean time of the non-excitability progression for 110 experiments was 32.70 ± 0.26 min, the logarithm of the geometrical mean time was 1.5004 ± 0.0105 min. Differences between the control and experimental series were statistically significant ($p = 0.001$). A more detailed examination of the material showed that the distribution curve alters at the expense of the lessening, in the experimental series, of the number of individuals possessing a lower level of muscular thermostability, while the right-hand portion of the curve practically remained unchanged.

Thus, the data obtained confirm the suggestion that the selection of gametes may affect the thermostability of the specialized cells of the multicellular organism. But this question is far from being settled and requires a species analysis which we hope to undertake in the nearest future.

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ON THE ABSENCE OF CONSTANT CORRELATION BETWEEN HEAT-RESISTANCE OF CELLS AND THE MELTING POINT OF THEIR LIPIDS

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1. According to the lipid theory of Heilbrunn (1924) and Bělehrádek (1931) the heat damage of cells is determined by the melting point of protoplasmic lipids. However, up to the present time, no special investigations have been made to study the correlation between the heat-resistance of cells and the melting point of their lipids. We studied the dependence of cellular heat-resistance and extracted protein complexes on the basis of the iodine number of their lipids, since the melting point of lipids can be determined by means of their iodine number.

2. Experiments were conducted on muscle tissues as well as on homogenates and enzymic complexes prepared from muscle and nerve tissues. The heat-resistance of muscle tissue was checked by the survival time of isolated muscles in heated Ringer solution. The temperature required for a 50 per cent inactivation of the enzymes was taken as a criterion of the heat-resistance of the tissue homogenates and the enzymic complexes. The iodine numbers of lipids extracted from muscles and enzymic complexes were measured by Kauffmann's semimicro method.

3. In experiments with frog muscle tissues (*Rana temporaria* L.), muscles which had lost excitability were used for the extraction of lipids and the determination of their iodine numbers. This permitted the calculation of the correlation coefficient between the shifts of muscular heat-resistance within one population and the iodine number of the lipids extracted from these muscles. In four sets of experiments the correlation coefficient varied from 0·12 to 0·31; this fact indicated the absence of any constant correlation between the iodine number of protoplasmic lipids and their cellular heat-resistance. In addition, it was shown that a decrease in the heat-resistance of muscle tissue associated with the reproduction of frogs in nature, was not accompanied by changes in the iodine number of their lipids. In the spawning period (experimentally induced by injections of hypophyseal extracts) a rise in the cellular heat-

resistance results not in an increase but in a decrease of the iodine number of the lipids (Ushakov and Glushankova, 1961).

4. A study of frogs (*Rana ridibunda* Pall.), dwelling in normal and warm basins with a water temperature of 30-39°C, has revealed that the lipids of animals from thermal springs, according to the theory of Heilbrunn and Bělehrádek, have lower iodine numbers compared to the lipids extracted from frogs populating water with a normal temperature. This difference, however, does not affect the heat-resistance of the cells, and both groups of animals show the same muscular heat-resistance.

Moreover, the correlation coefficient of the iodine number of lipids and the heat-resistance of muscle tissue in 10 different populations of frogs equals 0.59 whereas according to Heilbrunn and Bělehrádek it must possess not a positive but a negative value (Ushakov and Glushankova, 1962).

5. In experiments with rats (*Rattus norvegicus* Berk.) the iodine numbers of the lipids changed due to different fat diets (butter and vegetable oil). Despite significant differences in the iodine numbers of the lipids from muscle tissue, the heat-resistance of both groups of rats appears to be identical (Glushankova, 1963 b). No difference was detected in the heat-resistance of actomyosin from muscles and acetylcholinesterase from muscle and brain homogenates in the two groups of rats (Kusakina, 1963; Sleptsova, 1963).

6. The actomyosin heat-resistance and the iodine numbers of lipids extracted from this enzymic complex were studied in two races ("small" and "large") of one species which most authors refer to as *Trachurus mediterraneus* Stdr. According to Altukhov (1962), "large" and "small" races differ in their muscular heat-resistance and serology. In accordance with these data, heat-resistance of actomyosin was found to be different, though the iodine numbers of the lipids extracted from actomyosin practically coincided (Glushankova, 1963 a).

7. The data obtained indicate the absence of constant correlation between the heat-resistance of the cells and that of the protoplasmic proteins, on the one hand, and the iodine number of their lipids on the other. This means that the thermal death of cells cannot be due to the melting of protoplasmic lipids. The evidence presented confirms the protein theory of thermal death of cells.

Numerous data showing the dependence of iodine numbers of animal and plant lipids on environmental temperature conditions apparently are to be associated not with the melting point of the lipids but with an increase in their chemical activity due to enriching of lipids with double bonds.

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DISCUSSION

Discussion of B. P. Ushakov's paper

B. L. ASTAUROV: Are there any individual variations in the thermostability of tissues and how wide can they be? If they exist, as I understand from the examples with the spermatozoa, then I wonder whether it means that you examined only the average survival time of the spermatozoa at the lethal temperature without use of statistical constants, such as the variability degree or some other?

B. P. USHAKOV: First I shall dwell on the methodical aspect of the question. I cannot but agree with you that the position of points in the semilogarithmic graph cannot be regarded as a criterion of similar heat-resistance of cells. I must acknowledge that in our early work we made a mistake but in all the communications of our workers at this Symposium it was pointed out that the statistical treatment of the experimental material by the least squares and other methods and not the coincidence of the curves in the graph was the criterion for the reliability of the results. Recently we not only applied statistical methods to our new data, but also recounted the bulk of our experimental material from the previous work and made necessary corrections. The work we have undertaken was reflected in my paper published in 1960 in "Cytology", the dissertation of Dr. Dzhamusova etc. That is why your comment in respect to our experimental technique today is mainly of historical significance.

B. L. ASTAUROV: What is the reason for the individual variations of the thermostability? Is it an error of the method or is it a real variability? If it is a real variability, what factor is responsible for it?

B. P. USHAKOV: It is not a mistake of the method. It is a real variability, and it is as real as the variability of the other indices. Today it is difficult to say what it depends on. We observed different values of the thermostability in tadpoles obtained from different parents. All the tadpoles obtained from a pair of parents formed a tadpole family and showed one and the same thermostability of their cells. Some such tadpole families showed a high cell thermostability, while one other has revealed a low thermostability. These experiments allowed us to suggest that the variability of heat-resistance of the cells should be not only a phenotypical but also a genotypical one. Now we are engaged with the analysis of the variability of the cell thermostability.

B. L. ASTAUROV: What moment in the development of the sex cells do you think to be most suitable for the determination of thermostability? Sex cells

usually develop within the animal body for a long time. Then in the animal with external fertilization the sex cells exist in the environment, outside the body for a very short time. Then the zygote begins developing and the embryo at the early stages possesses a very changeable thermostability. At what moment do the sex cells reveal the thermostability which reflects the thermostability of somatic cells?

V. B. ANDRONIKOV: I would like to answer this question as it concerns my experiments. Spermatozoan heat-resistance was determined in adult animals. The sperm used was able to cause a fertilization which was followed by a normal development of embryos. The sperm was taken both from testicles and spermatic vesicles, the resistance in both cases was found to be identical.

We estimated the thermoresistance rise as a test object both in unfertilized mature cells-eggs (the fertilization occurred after heating of different intensities) and fertilized ones taken in certain periods of time after fertilization up to the appearance of the first furrows of cleavage. Further, heat-resistance was examined at 2, 4 and so on blastomere stages. Heat-resistance did not appear to change till the 32 blastomere stage.

In some experiments we used some other tests, besides the loss of the ability to split. The inhibition of the ability to form gastrula and normal embryo (up to the stage of hatching) was determined. It has been found that when all these three tests were used the curves of resistance change were parallel to abscissa up to the 32 blastomere stage. After this a more or less simultaneous decrease in the thermoresistance occurs (according to all three tests). Thus, the heat-resistance can be identified by either of the three methods at any early developmental stage mentioned above.

B. L. ASTAUROV: The facts you speak of differ greatly from much data on thermoresistance fluctuations at early stages of development of sea-urchins, fishes and silkworms. Such a surprising stability of the thermoresistance at the early stages of ontogenesis deserves further investigation.

V. B. ANDRONIKOV: Facts are facts. The discrepancies can be partly explained by the fact that we studied the most resistant cell—the egg, as in our experiments we tried to find out the death conditions for 100 per cent of eggs, but not for 50 per cent as it is usually the case with the majority of studies of this kind.

V. Y. ALEXANDROV: How must we understand species differences in thermoresistance found in such proteins as collagen, which have nothing to do with the early stages of ontogenesis, if these very early stages are just the object of selection?

B. P. USHAKOV: It is quite evident that in selecting the germ cells, we do not act upon those proteins which will come into being in the adult organism. However, the selection of the most heat-resistant spermatozoa caused the differences in heat-resistance of the cells of tadpoles. This question is difficult and needs further examination.

I must answer one more question: whether the temperature can influence the germ cells when they are in either maternal or paternal organism. We treated with a high temperature the pupae of flies. The flies hatched from those eggs laid eggs in turn, and in the first laying of the first generation the change of cell heat-resistance could be found. Thus, the question can be answered affirmatively.

V. B. USHAKOV, JR.: In the reports of Dr. Braun with co-workers, Dr. Oganesyan and Dr. Petrosyan, interesting data have been presented which indicate a certain parallelism between muscle thermostability and that of the actomyosin. Hence the question arises as to what extent thermal damage of the actomyosin may account for the thermal death of muscle fibres.

Figure 1 presents changes in the affinity of frog sartorius muscle for the neutral red after treatment of the muscle with heated Ringer solution for 15 min. The affinity was used as an indirect test of denaturational changes in muscle proteins after heat treatment, since it has been demonstrated (Alexandrov and Nasonov, 1939; Braun, 1948a, b) that the uptake of biological dyes is much greater in denatured proteins than in native ones. The uptake of neutral

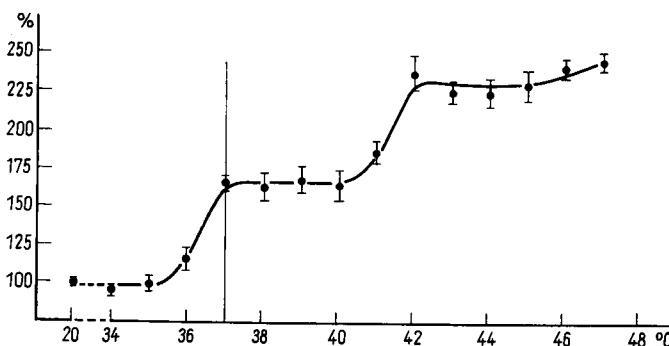


FIG. 1. Quantity of neutral red taken by muscles after their heat treatment for 15 min vs. temperature of Ringer solution.

red increases in a stepwise fashion, and both of the steps are presumably associated with denaturational changes in two protein fractions which differ in their thermostability. In this case the threshold lethal temperature was 37° (vertical line in Fig. 1), i.e. the temperature which causes the first increase in the affinity to the dye. Therefore in this set of experiments the thermal death of muscles is paralleled by the first step in the dye uptake. The same coincidence has been observed in studies on the effect of a constant temperature for different times (Ushakov, 1963).

Similar results have been obtained with other durations of heat treatment, and an inverse correlation was found between temperature and the time of its effect, i.e. the longer the time, the lower is the temperature which causes both the death of muscle and its first increase in the affinity to the dye (Fig. 2).

The inverse time-temperature relation is clearly seen from a semilogarithmic plot (Fig. 3) from which a temperature coefficient can be approximately calculated which equals 20,000. The high value of the coefficient presumably

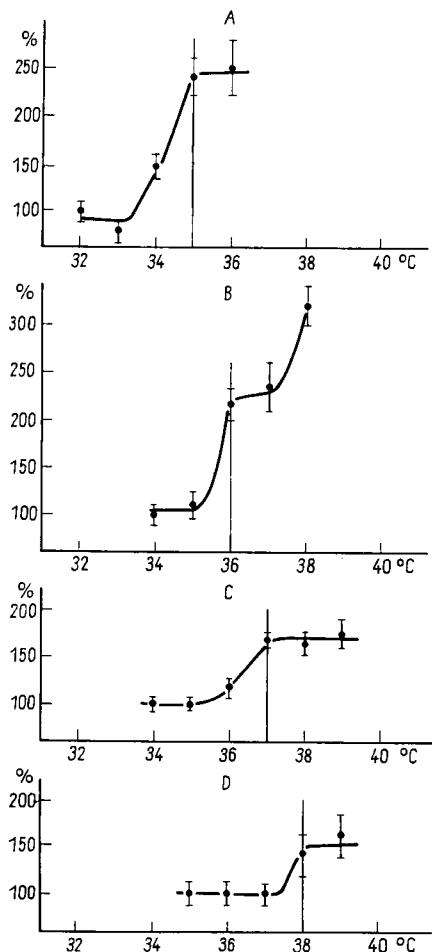


FIG. 2. Quantity of neutral red taken by muscles after their heat treatment of various durations vs. temperature of Ringer solution. A—heat treatment lasted for 100 min. B—for 30 min. C—for 15 min. D—for 5 min.

indicates that denaturation of some protein fraction accounts for the first step in the increase of the dye uptake as well as for the thermal death of muscles.

For the present it is not possible to tell what this fraction may be, although some evidence can be presented that it is not the actomyosin. For instance, the affinity of the glycerated muscles (from which most of the soluble proteins

are removed) increases in one step only (Fig. 4B), this step coinciding with the second increase in non-glycerated muscles (Fig. 4A). This is true when glyceration has been made at -17°C . If it is made at 0°C , the actomyosin

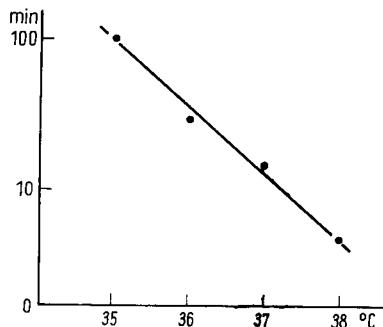


FIG. 3. Retention time of muscle excitability and the time of heat treatment (in min, logarithmic scale) necessary to bring about the first step in the increase of the dye uptake vs. temperature.

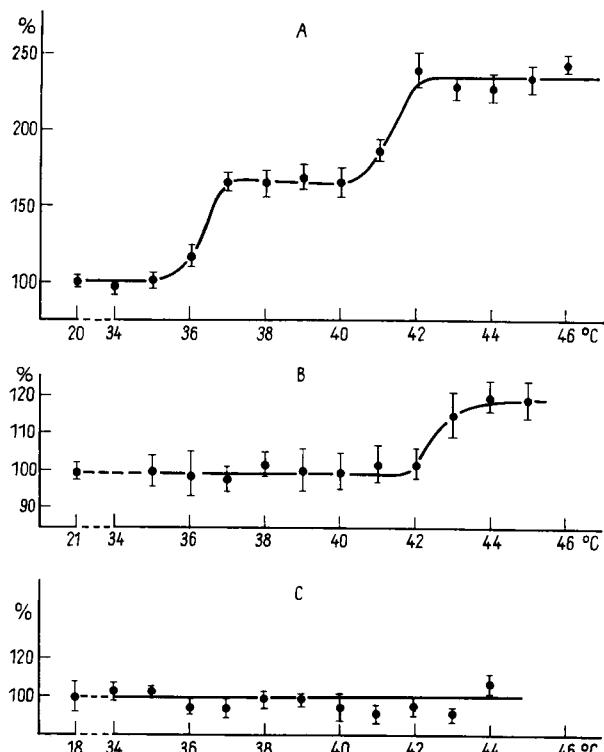


FIG. 4. Quantity of neutral red taken by muscles after their heat treatment within 15 min vs. temperature of Ringer solution. A—non-glycerated muscles. B—muscles glycerated at -17°C . C—muscles glycerated at 0°C .

system becomes inactive, and muscles do not contract any more on the addition of adenosine triphosphate. In this case heat-treatment of muscles does not result in any increase of the dye uptake (Fig. 4C). Therefore, it may be concluded that the second step in the uptake of neutral red by non-glycerated muscles is due to the denaturation of actomyosin. Since the death of muscles coincides with the first increase, which disappears after the glyceration, it may be suggested that the thermal death of muscles results from heat-denaturation of some soluble fraction which is removed by glyceration.

In conclusion, similar results have been obtained in the course of the studies on the opacity of muscles which changed under the influence of temperature in a stepwise fashion as well. Hence the possibility is excluded that a stepwise increase in the dye uptake is due to permeability changes but not to denaturation of different protein fractions.

METABOLIC AND CENTRAL NERVOUS ACCLIMATION OF FISH TO COLD

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TEMPERATURE acclimation in poikilotherms such as fish is of adaptive advantage for their survival in an environment with a fluctuating temperature, also at the geographic limits of the range of a species. The net effect of acclimation is to permit activity to be maintained at similar levels at different temperatures, that is, to compensate for cold or warm. Precht (1955, 1958) classified patterns of rate functions to cold as follows: (1) overcompensation so that metabolism (or any other rate function) is higher in the cold than at the initial temperature, (2) perfect compensation with the same metabolic rate at each temperature, (3) partial compensation, (4) no compensation, the metabolism continuing to follow the van't Hoff relation, and (5) inverse or anomalous adaptation with metabolism reduced below that due to the van't Hoff relation. Prosser (1958, 1961) described rate functions at different temperatures as changing with acclimation by either a shift in the rate curve (translation) or a rotation (change in Q_{10}). A translation indicates changes in the levels of the enzyme activity whereas rotation implies changes in the temperature characteristics and could be due to either an alteration in enzymes or in co-factors (qualitative changes or quantitative changes in relative amounts in parallel paths).

Acclimation can be measured in terms of the metabolism after complete adjustment to each of two (or more) temperatures. It can also be measured as metabolism at one intermediate temperature for animals acclimated to different temperatures. In the latter measurements, the cold-acclimated animals have a higher metabolic rate than warm-acclimated ones when both are measured at the same intermediate temperature.

Examples of various patterns of acclimation have been summarized for the metabolism of various poikilotherms (Prosser, 1961). Temperature acclimation is more evident in fish than in arthropods. Most fish show a partial compensation for temperature changes, although a few species show inverse acclimation. For example, *Carassius gibelio* follows Precht's type 3 and *Carassius carassius* type 5 (Roberts, 1960). Races of the tropical *Xiphophorus helleri* differ in acclimation pattern (Precht, 1962).

When oxygen consumption by isolated tissues is measured, the gills of goldfish show good temperature acclimation, the muscles less, the liver still

TABLE 1. ACTIVITY OF ENZYMES FROM POIKILOTHERMIC VERTEBRATES ACCLIMATED IN DIFFERENT TEMPERATURES

(Values, inverse acclimation, or Precht's Type 5). (n.s., not significant) temperature of measurement usually 20°C except where stated. Enzyme activities in different units of measurement, hence of relative value only.

Oxidative enzymes	Temp. of measurement	Acclimation temp. and enzyme activity per g wet weight or mg protein		Per cent by which cold exceeds warm
		Cold	Warm	
Succinic dehydrogenase: eel liver (Precht, 1951) goldfish liver (Murphy, 1961)	15°	11°	26°	
		0·43	0·24	79
		5°	30°	
	25°	15·9 $\mu\text{l. O}_2/\text{g}_w/\text{min}$	14·3	n.s.
		17 $\mu\text{l. O}_2/\text{g}_w/\text{min}$	19·9	n.s.
		8·11 $\mu\text{l. O}_2/\text{g}_w/\text{min}$	6·64	21
Rhodeus muscle (Krüger, 1962)	30°	8° 4·9 min	24° 8·6 min	75
Cocarboxylase: eel liver (Carlsen, 1953) eel muscle (Carlsen, 1953)		440	345	28
		390	367	n.s.?
Malic dehydrogenase: goldfish liver (Murphy, 1961)	15°	5°	30°	
		9·15 $\mu\text{l. O}_2/\text{g}_w/\text{hr}$	16·2	-44
	20°	14·7 $\mu\text{l. O}_2/\text{g}_w/\text{min}$	22·5	-34·5
Cytochrome c oxidase: goldfish liver (Murphy, 1961)	15°	5°	30°	
		15·0 $\mu\text{l. O}_2/\text{g}_w/\text{min}$	15·9	n.s.
	25°	23·4 $\mu\text{l. O}_2/\text{g}_w/\text{min}$	22·8	n.s.
		0·236 $\mu\text{l. O}_2/\text{mg}_{\text{pt}}/\text{min}$	0·183	29·5
		13·9 $\mu\text{l. O}_2/\text{g}_w/\text{min}$	12·3	n.s.
DPNH cytochrome reductase: goldfish liver (Murphy, 1961)		5° 23·5/g _w	30° 29·9/g _w	n.s.
TPNH cytochrome reductase: goldfish liver (Murphy, 1961)		5° 4·13/g _w	30° 4·97/g _w	n.s.

TABLE 1. (continued)

Oxydative enzymes	Temp. of measurement	Acclimation temp. enzyme activity per g wet weight or mg protein		Per cent by which cold exceeds warm
		Cold	Warm	
Catalase:				
carp gill (Ekberg, 1961)		5° 219.7	25° 223.7	n.s.
eel liver (Precht, 1951)		50	77	-35
CN inhibition:				
goldfish gill (Ekberg, 1958)		10° 79.1% inhib.	30° 57.5	n.s.
goldfish liver (Ekberg, 1958)		85.9%	81.2	
goldfish liver (Kanungo and Prosser, 1959)		No significant difference in inhibition by CO, CN, azide.		

Hexose Monophosphate Shunt Enzymes

Hexose monophosphate shunt enzymes	Acclimation temp. and enzyme activity per g wet weight or mg protein		Per cent by which cold exceeds warm
	Cold	Warm	
Glucose-6-PO ₄ dehydrogenase:			
Crucian carp gill (Ekberg, 1961)	5° 44.2 arb. units	25° 43.9	n.s.
goldfish liver (Murphy, 1961)	5° 0.298	30° 0.548	-46
6-PO ₄ -gluconic dehydrogenase:			
Crucian carp gill (Ekberg, 1961)	5° 11.0 arb. units	25° 4.7	134
goldfish liver (Murphy, 1961)	5°	30°	

Glycolytic enzymes

Aldolase:			
Crucian carp gill (Ekberg, 1961)	5° 96.3	25° 64.3	50
Rhodeus:			
muscle 25° (Krüger, 1962)	10° 3.21	20° 5.11	-59

TABLE 1 (continued)

Hexose monophosphate shunt enzymes	Acclimation temp. and enzyme activity per g wet weight or mg protein		Per cent by which cold exceeds warm
	Cold	Warm	
Anaerobic acid production: Crucian carp gill (Ekberg, 1961)	5° 263 (7 hr day) 326 (17 hr day)	25° 176 (7 hr day) 194 (17 hr day)	49 68
Rhodeus: muscle 25° (Krüger, 1961)	10° 28.2	20° 30.2	-6
Lactic dehydrogenase: goldfish liver (Murphy, 1961)	5° 1893/g _w 19.10/mg _{pr}	30° 1415/g _w 11.3/mg _{pr}	n.s. 65
IOA inhibition: goldfish gill (Ekberg, 1959)	10° 52.6% inhib.	30° 77.4% inhib.	

less and the brain and heart virtually none (Ekberg, 1958; Murphy, 1961). In *Salmo gairdneri* there is slight compensation of the whole fish, none in the gills, complete acclimation in the brain and overcompensation in the liver (Evans *et al.*, 1962).

Data on metabolic enzymes are very confusing (Table 1). Among the electron transport systems, succinic dehydrogenase showed compensation in eel liver (Precht, 1951) and *Rhodeus* muscle (Krüger, 1962) but not in these same tissues of goldfish (Murphy, 1961). Cocarboxylase shows compensation in liver but not in eel muscle (Carlsen, 1953). Malic dehydrogenase of goldfish liver shows an inverse adaptation (Murphy, 1961). Cytochrome oxidase of goldfish liver showed no significant effects (Murphy, 1961) but recent observations (Freed, 1963) revealed compensation in goldfish muscle. The gills are more sensitive to cyanide after cold acclimation (Ekberg, 1958).

Activity of the pentose phosphate shunt is low in carp (Brown, 1960) and of two shunt enzymes, glucose-6-PO₄-dehydrogenase showed either no effect or inverse acclimation while 6-PO₄-gluconic dehydrogenase showed overcompensation in crucian carp gills (Ekberg, 1961) or no effect in goldfish liver (Murphy, 1961). Recent observations by Hochachka and Hayes (1963) on the liver and the muscles of *Salvelinus fontinalis* showed more ¹⁴CO₂ from glucose ¹⁴C₁ than from glucose ¹⁴C₆, also greater incorporation into fat in cold- than in warm-acclimated trout. This supports the suggestion of Kanungo and Prosser (1959) of the greater use of the pentose phosphate shunt in cold-acclimation.

Fish rely very much on glycolysis and in the cold, crucian carp can survive anaerobically by energy from glycolysis and conversion of carbohydrate to fat (Blazka, 1958). Lactic dehydrogenase shows some temperature compensation in goldfish liver (Murphy, 1961) but not in bitterling muscle (Krüger, 1962). The total acid production by crucian carp gills was elevated in cold-acclimation but CO_2 production was not (Ekberg, 1961). Aldolase activity was markedly increased in carp gills in cold-acclimation (Ekberg, 1961). The iodoacetate sensitivity of goldfish gills (Ekberg, 1958) and of *Salvelinus* muscle was increased (Hochachka and Hayes, 1963).

It may be concluded that there are marked differences among tissues and also among enzymes of intermediary metabolism. No single pattern of enzymic changes is universal. This indicates that the acclimation is primarily at the cellular level. We have recently found that the uptake of uniformly labelled glucose is in about the same proportion as the enhancement of oxygen consumption by cold-acclimation (Table 2). In these experiments, liver from 30°C-acclimated fish had higher \dot{Q}_{O_2} and glucose uptake as did those from 5°C-acclimated fish than from 15 and 20°C fish.

TABLE 2. RELATION BETWEEN ACCLIMATION TEMPERATURE AND OXYGEN CONSUMPTION OF GOLDFISH AT 16°C, AND O_2 CONSUMPTION AND GLUCOSE UPTAKE BY GOLDFISH LIVER AT 20°C

Acclimation Temp. (°C)	O_2 consumption, starved fish ml $\text{O}_2/\text{g}_w \text{ hr}$	O_2 consumption liver, ml $\text{O}_2/\text{mg}_d \text{ hr}$	Glucose uptake counts/min mg dry
30	0.086 ± 0.005	1.49 ± 0.30	363 ± 60
25	0.10 ± 0.012	1.10 ± 0.11	231 ± 53
15	0.10 ± 0.013	1.31 ± 0.31	245 ± 16
5	0.13 ± 0.0036	1.95 ± 0.39	314 ± 37

Attempts to find hormonal participation in the acclimation process have also led to conflicting results. The thyroid as judged histologically, is stimulated by cold in a few species and not in most species. Thiourea is said to eliminate metabolic differences between warm- and cold-acclimated crucian carp (Suhrman, 1955) but to increase the differences in *Leuciscus* (Auerbach, 1957). Figure 1a, b shows a few of the results obtained on our laboratory by Klicka (1962). Thyroidectomy by radioiodine, treatment with thyrotropic hormone, and treatment with thiourea failed to alter the metabolic compensation of goldfish to either low or high temperatures. The time-course of the compensation attained a steady level in 6 to 12 days. Adrenocorticotropic hormone likewise had no effect on the time-course of the acclimation (Klicka, 1962).

Among other biochemical changes which may occur in temperature acclimation, the most commonly reported are in the lipids. In trout, three times

more $^{14}\text{C}_1$ from radioactive glucose was incorporated into fat in cold- than in warm-adapted fish (Hochachka and Hayes, 1963). Acclimation of goldfish to cold was accompanied by increased unsaturation of the tissue lipids and

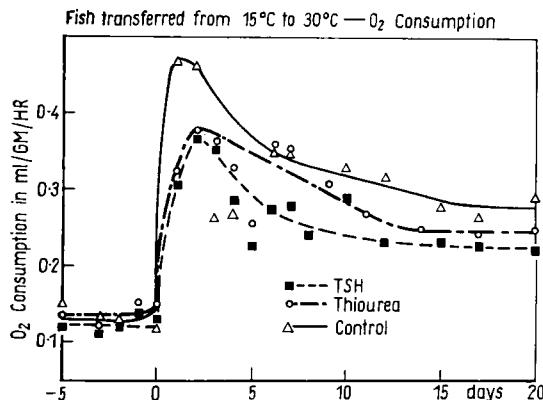


FIG. 1a. Oxygen consumption of goldfish before and after transfer to temperatures indicated. Data for controls, thyroidectomized by means of radio-iodine and treated with adrenocorticotropic hormone (from Klicka, 1962).

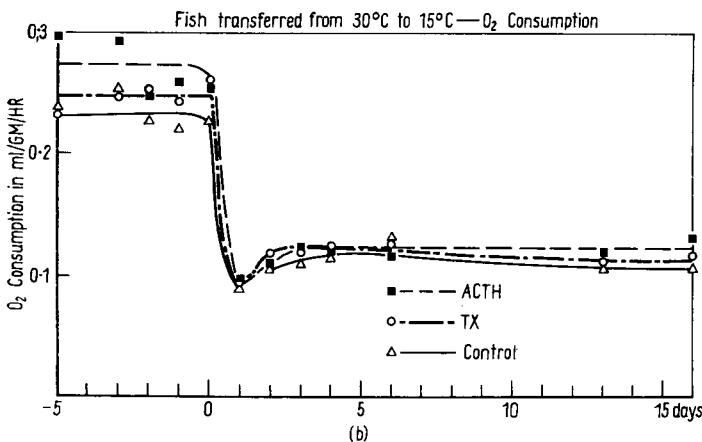


FIG. 1b. Oxygen consumption of goldfish before and after transfer to temperatures indicated. Data for controls, thyroidectomized by means of radio-iodine and treated with adrenocorticotropic hormone (from Klicka, 1962).

acclimation to heat by decreased unsaturation (Hoar and Cottle, 1952). In cold, the tissue phospholipids increased relative to cholesterol. Preliminary observations by Murphy and Johnston (1961) in our laboratory indicate a higher percentage of stearic and palmitic acids in the liver of 30°C-acclimated than of 5°C-acclimated goldfish. Livers of goldfish from 5°C had 1.76 per cent

of lipid, from 30°C 3·97 per cent of lipid. The iodine numbers were as follows: 30°C, 97·5; 15°C, 100·3; 5°C, 102·3: hence the liver lipids were more unsaturated in the cold-acclimated state.

In addition to biochemical changes associated with temperature acclimation, there are behavioural changes which indicate compensations of the central nervous system. The oxygen consumption as measured during maximum swimming activity rises more rapidly with temperature and flattens off sooner than does standard metabolism. The difference indicates that the maximum energy available for activity at an intermediate temperature and this "optimal" temperature varies with acclimation. Similarly, the maximum possible swimming speed is greatest at about the same "optimal" temperature. One view is that metabolic energy limits the swimming speed; a converse view is that the oxygen consumption reflects the amount of muscular work permitted by the nervous system (Fisher, 1958).

We have examined the lowest temperature for various nervous functions in fish that have been differently acclimated. Conduction in peripheral nerve continues down to lower temperatures than do spinal reflexes and these have lower blocking temperatures than the maximal speed of swimming (Table 3) (Roots and Prosser, 1962). Also the blocking temperatures of the more complex nervous functions are more readily modified by acclimation. In recent

TABLE 3. RELATION BETWEEN ACCLIMATION TEMPERATURE AND MINIMAL TEMPERATURE FOR NERVOUS FUNCTIONS

Acclimation temp. (°C)	Nerve block temp. (catfish) (°C)	Spinal reflex block (goldfish) (°C)	Minimum cruising temp. (sunfish) (°C)	Conditioned response block (goldfish) (°C)
30	—	10	15–20	20–21
25	3–4	5	10–15	15–16
15	2–3	1	5–10	10–12
10	1			
5	0	1	No swimming	1–2

experiments, goldfish were conditioned to interrupt their breathing to a light as the conditioned stimulus. After conditioning, the temperature was lowered and the conditioned response dropped out; it usually returned on rewarming. The temperature of forgetting varied according to the temperature at which conditioning occurred. For example, fish conditioned at 30°C lost the response at 20–21°C, those from 25°C at 15–16°C, those conditioned at 15°C lost it at 10–11°C and those conditioned at 5°C were blocked at 1–2°C. Furthermore, the lowest temperature at which conditioning could occur depended on the temperature of prior acclimation. The minimum temperature for conditioning was 17–18°C after 30°C acclimation, 12–13°C after 25°C acclimation, and 6–7°C after acclimation at 15°C.

The molecular mechanisms underlying the preceding nervous and metabolic changes with temperature acclimation are not known. However, it is probable that numerous biochemical alterations—particularly in enzymes and lipids—are involved. We may speculate as to how a physical factor such as temperature can cause biochemical changes which become evident after some days of acclimation. The microbial biochemists have elucidated the control

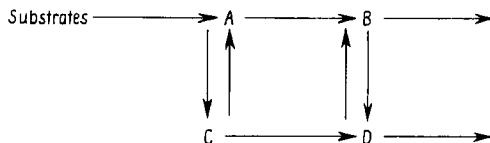


FIG. 2. Diagram of parallel and shunt pathways of metabolism.

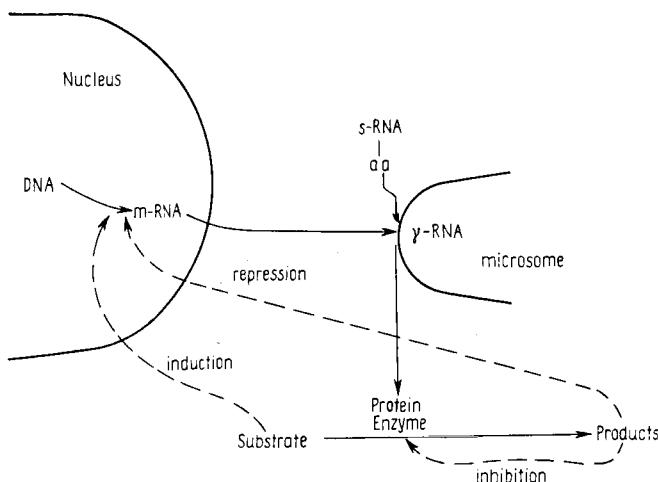


FIG. 3. Diagram of sequence in protein synthesis and its control.

of enzymic proteins by substrates but they have paid little attention to such control by physical factors such as temperature. One possible explanation in terms of enzyme induction is diagrammed in Fig. 2. If there are alternate and cross-linked pathways having different temperature characteristics, one may be slowed more than another in the cold. Thus an intermediate such as pyruvate or lactate may accumulate because its degradative enzymes have a higher Q_{10} than those enzymes forming it. A given intermediate may reach concentrations which induce synthesis of enzymes in an alternate pathway. For example, if $A \rightarrow B$ has a high Q_{10} , A accumulates in the cold and may induce the enzyme catalysing $A \rightarrow C$.

Figure 3 suggests that the action of an intermediate or product of a biochemical reaction may show feedback control by direct inhibition, by repression of synthesis of a particular messenger RNA or by induction as by a

substrate. The concentration of the controlling products or substrates would be dependent on the temperature characteristics of the particular steps. Thus temperature can cause adaptive changes of a biochemical nature and these changes can be not only in specific enzymes but in the nucleotides necessary for enzyme synthesis. Thus the cellular basis for temperature acclimation depends upon the genetic capacity (DNA) of the system for stimulation or repression.

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ONTOGENETIC ADAPTIVE CHANGES IN HEAT-RESISTANCE OF SILKWORM EGGS IN RELATION TO THE SEASONAL CHANGES IN THE PERIODS OF ACTIVE DEVELOPMENT AND REST (DIAPAUSE)

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AFTER considerable intraspecific stability of cellular heat-resistance and a correlation between the level of resistance and temperature conditions of the normal environment of a given species were found (Adensamer, 1934; Alex- androv, 1952; Battle, 1926; Patzl, 1933; Runnström, 1927, 1930, 1936), many investigations on several hundreds of metazoan species strengthened and multiplied the original data and gave them the significance of a general rule (Ushakov, 1958, 1959, a, b, 1961). High constancy of protoplasmic heat-resistance of any given species ("conservatism of heat-resistance") and the possibility of using this cytophysiological characteristic as a diagnostic criterion of species were especially stressed (Ushakov, 1958, 1959a, b, 1961). Not the cellular but the total or organismal thermostability has been proposed also by Fry (1957) as a taxonomic characteristic.

It should be expected *a priori*, however, that along with intraspecific uniformity, instances of intraspecific differentiation of heat-resistance must also occur. Without such a differentiation, adaptive evolution of heat-resistance in the course of speciation could not be explained. Likewise, since it is evident that there can be no stages in the individual development of any organism which are not adapted to the environment, it should be possible that ontogenetic changes in cellular thermoresistance would be observed at least in those multiple cases when the ontogenesis of a poikilotherm proceeds under the conditions of orderly changing temperatures.

One could hardly expect for instance to find constancy of thermoresistance in the ontogenesis of those terrestrial poikilotherms whose life cycle is adapted to wide seasonal fluctuations of temperature sometimes reaching dozens of degrees centigrade above and below zero.

Little by little an abundance of facts (some of them presented in the papers at this Symposium) has been accumulated which extend our knowledge of

cellular thermoresistance and put those or other restrictions on the idea of its nearly absolute intraspecific stability.

Intraspecific differences in thermoresistance are by now known to be rather trivial (Ushakov, Vinogradova and Kusakina, 1962; Ushakov, 1963, 1966); many representatives of lower types of Metazoa such as Coelenterata and Vermes are characterized by their ability to show adaptive changes in their heat-resistance, sometimes resembling those observed in unicellular organisms and plants (Gorodilov, 1961; Kamshilov, 1960; Dregolskaya, 1962, 1963); seasonal changes in heat-resistance were also observed (Schlachter, 1961; Pashkova, 1962; Dregolskaya, 1962; Altukhov, 1963) as well as reactive responses to the action of high temperatures (Schlachter and Chernokozheva, 1966). Age differences have also been shown recently (Kusakina, 1963). Distinct ontogenetic changes in heat-sensitivity still have attracted little attention.

This paper analyses the very distinct ontogenetic changes in heat-resistance observed in the course of the embryonic development of *Bombyx mori* L.; data are also presented on clearly seen rises of heat-resistance in response to the action of super-optimal temperatures, resembling changes found on the so-called "heat-hardening" of plants (Alexandrov and Feldman, 1958; Alexandrov and Yazkuliev, 1961).

These data deserve attention in several respects as follows:

1. The changes in heat-resistance observed are considerable, clear and regular.
2. Rather reasonable ideas can be put forward regarding known biochemical changes in the development of silkworm eggs which may determine these variations in heat resistance.
3. Up to now cytoecology of insects has received attention quite inconsistent with the relative importance of insects in the animal world and in the life of mankind; the data presented concern one of the best studied species of these animals.

When turning to the experimental data it should be mentioned that, due to the peculiarities of the material (the embryo surrounded by a hard egg shell), in the course of observations one is dealing not directly with cellular heat-resistance but with the general resistance of the embryo as a whole. However, the temperature dependence of the eggs shows the same peculiar regularities as the process of heat denaturation of cellular proteins. Although it can not be taken as proven, there are ample grounds to believe that the total thermoresistance of the eggs essentially reflects either the average (integral) heat-resistance of the cellular proteins of the embryo or that of the proteins of its most sensitive or vitally important cellular components (limiting total viability).

The action of super-optimal temperatures (40–60°C) upon silkworm eggs has been studied repeatedly. It was thoroughly studied in the experiments

on artificial heat parthenogenesis in unfertilized eggs, while in fertilized ones it was studied in the experiments on thermal stimulation to the non-diapause development, the so-called "artificial hatching" (Astaurov, 1940, 1943, 1957, 1958; Emme, 1946a, 1947, 1952). Ontogenetic changes in heat resistance of fertilized eggs at the very beginning of development (during maturation divisions, fertilization and cleavage) were studied in great detail in the experiments on the induction of thermal androgenesis (Astaurov and Ostrikova-Varshaver, 1957, Astaurov, Ostrikova-Varshaver and Strunnikov, 1958).

At more advanced developmental stages, during the course of embryogenesis which takes approximately 10 months and includes the short pre-diapause period, aestivation and hibernation or summer-autumnal and winter rest (diapause) and finally the active development in the spring, the ontogenetic changes of heat-resistance were studied in detail while elaborating the technique of thermal cure of the silkworm eggs infected with Nosema (Astaurov, Bedniakova, Vereiskaya and Ostrikova-Varshaver, 1962).

Thus, heat-resistance has been determined in the course of the whole embryogenesis, starting from the unfertilized egg to the time of hatching. Investigations have been carried out both on the eggs developing with diapause and hibernation and on those devoid of diapause and made to hatch by stimulation with hydrochloric acid ("artificial hatching").

The percentage of successful embryonic development (percentage of survival) is taken as the measure of the damaging action of hot water (correspondingly, of heat-resistance). On the basis of dose survival curves reflecting the dependence of survival upon the exposure time at a constant high temperature, the semi-lethal dose of heating, LD_{50} , was determined. The dependence of the effect as a function of the exposure time at a constant high temperature follows an S-shaped curve (cumulative) which mirrors the random variation of the eggs in the sample with respect to their heat-sensitivity.

It can be seen in Fig. 1 that the dose curves for various egg samples differing in developmental stages and heated at different temperatures in all cases show a fairly good coincidence with the ideal sigmoid curves to be expected if the heat-resistance of eggs shows a random variation which follows the normal binominal Gauss-Laplace distribution.

The LD_{50} that kills 50 per cent of eggs corresponds to the average heat-resistance of the eggs (embryos) in terms of the duration of heating. Small doses of heating (less than 0.25 LD_{50}) are harmless and it is not improbable that they may even slightly stimulate vital activity and increase the viability of the embryo.

Temperature dependence of the exposure time which causes a certain effect, e.g., killing 50 per cent of eggs, or LD_{50} , is exponential and follows the Arrhenius's equation with high values of the temperature characteristics μ , of the order of 110,000–150,000 cal/mol. Corresponding very high values of the van't Hoff's temperature coefficient Q_{10} are peculiar to the process of heat denaturation of proteins. The dependence of the exposure time (ordinate)

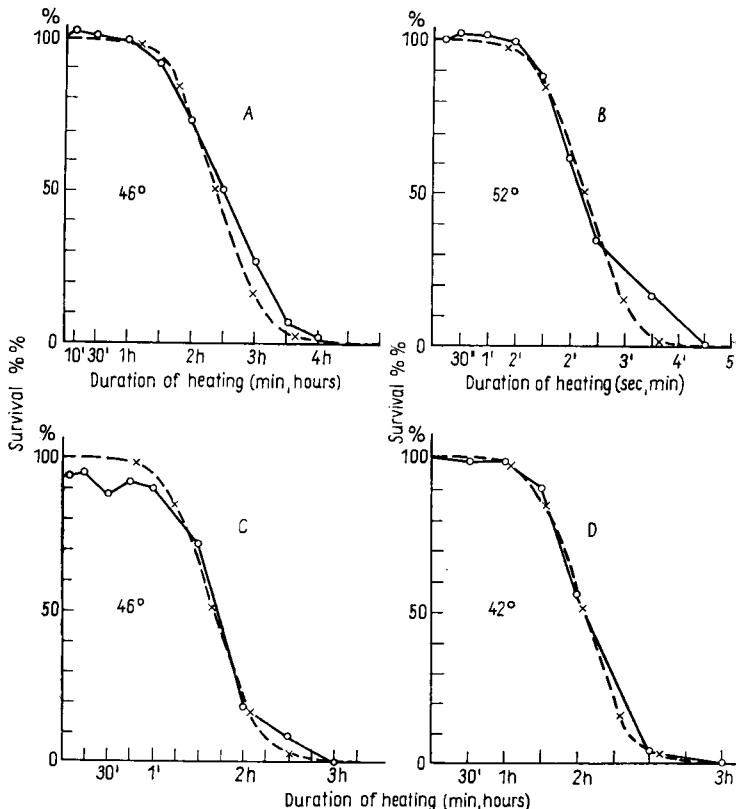


FIG. 1. Dependence of egg survival (percentage of hatching, ordinate) upon the duration of heating (abscissa) at a constant temperature. (Dose-effect curves): solid lines—empirical data; broken lines—theoretical expectation. A, B— F_1 hybrid Bagdad \times Askoli eggs at the very beginning of hibernation; heat treatment at 46°C (A) and 52°C (B); C—the Bagdad-race eggs, age—one day; heating at 46°C ; D—the same eggs caused to develop without diapause by treatment with dilute HCl, 7th day of incubation; heating at 42°C .

plotted logarithmically against temperature (in C°) is linear (Fig. 2). All this evidence strongly supports the conclusion that the sensitivity of silkworm eggs and embryos to the action of high temperatures mainly represents the sensitivity of its cellular proteins to heat denaturation.

Temperature characteristic μ is of the same value: (1) for the eggs at different developmental stages, (2) for the embryos developing either with diapause or without it, (3) for the eggs of different races of silkworm, (4) upon heating with a preparatory warming or without it, and (5) upon both hot-water and hot-air treatment.

Heat-resistance of the eggs does not or almost does not depend upon the race of the silkworm eggs, neither does it depend upon whether they were

obtained from pure races or by interracial hybridization (all other factors, as developmental stage, mode of heating, temperature and exposure time, being equal). It is worth mentioning that our data on the equality of heat-resistance of the embryo in parental races and their F_1 hybrids do not coincide with the results gained in the experiments on the estimation of future larval viability by means of preliminary heating of egg samples (Mametkuliev, 1962). Mametkuliev found that the hybrid eggs ready to hatch are more heat-resistant than the eggs of the parental races. This problem is rather complicated and requires

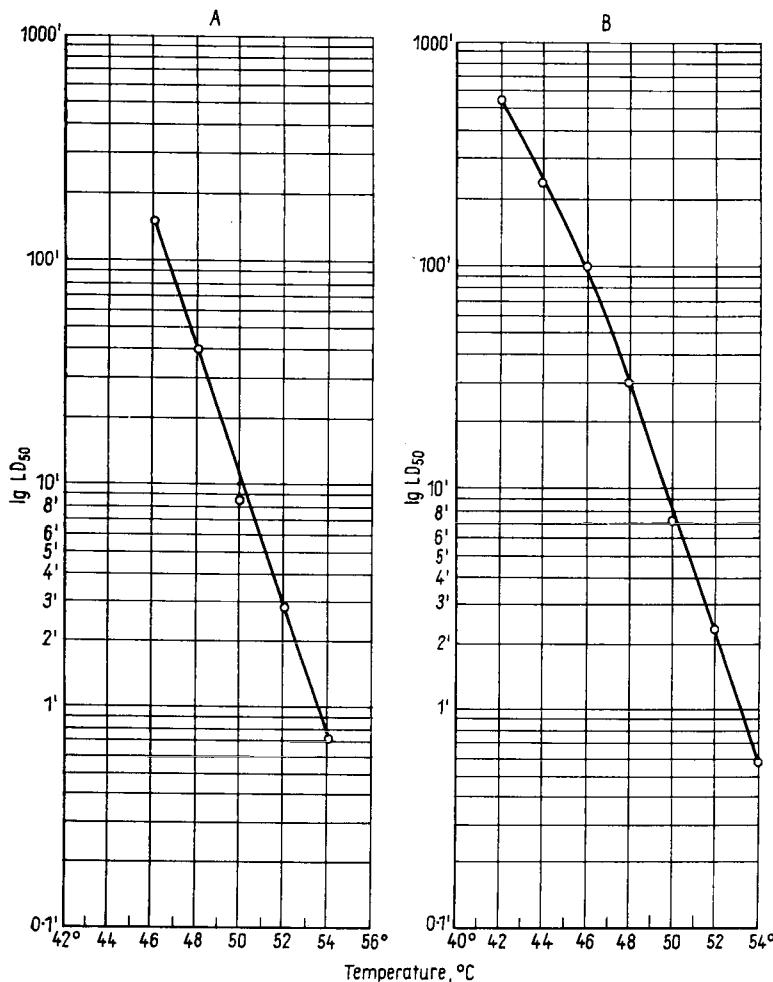


FIG. 2. The logarithm of the semi-lethal exposure time ($\lg LD_{50}$, ordinate) plotted against temperature ($^{\circ}\text{C}$, abscissa). A—According to the experiments with the Bagdad \times Ascoli 180-day-old eggs; B—according to average data of all the experiments in relative units (LD_{50} at 46°C is taken for 100).

further investigation, the more so since higher heat-resistance of proteins in hybrids was found also in interspecific crosses of fishes (Kusakina, 1959).

Heat-resistance shows an adaptive increase when preparative moderate warming at sublethal temperatures is applied ("heat-hardening"). When applying preliminary "heat-hardening" in the form of a gradual air heating up to 40°C and subsequent maintenance of the eggs at 40°C during one or (seldom) two hours, 85 paired comparison trials showed the value of the LD₅₀ for a subsequent injurious thermal heat shock to increase by an average amount of 36·4 per cent with respect to the non-warmed control (100 per cent). For the time being, such an adaptive increase in heat-resistance of *B. mori* embryos seems to be very similar to "heat-hardening" of plants (Alexandrov and Feldman, 1958; Alexandrov and Yazkuliev, 1961).

Since this adaptive increase in heat-resistance seems to be determined by the reactions proceeding at the cellular level, this also supports the inference that the total heat-resistance of the eggs can be taken as characteristic of the heat-resistance of cellular proteins.

The eggs are capable of such an adaptive increase of their heat-resistance at all developmental stages. However, the ontogenetic dynamics of this ability has been studied insufficiently and the possibility is not ruled out that with the transformation of the embryo into the developed larva, systemic (e.g., neurohumoral) regulatory mechanisms of heat-resistance arise which function not at the cellular but at the organismal level.

Ontogenetic changes in heat-resistance are rather great. Figure 3a is a graphical representation of them (abscissa—age of eggs in days, ordinate—LD₅₀ as a measure of the heat-resistance with respect to the exposure time). Both changes taking place during a prolonged development with the diapause (some 10 months) and changes during short (10–15 days) non-diapause development are presented. The figure shows the average results of many parallel and fairly coincident experimental series.

In case of the development with the diapause the total heat-resistance at the transition from the least resistant to the most heat-resistant stage is increased 550 times. So great an increase is due to the fact that during the metaphase of the second maturation division the egg cell (or, more correct, its dividing nucleus) is extremely sensitive to heating and shows at this stage an insignificant heat-resistance (Fig. 3a, within the circle). A similar fall of heat-resistance related to the rhythm of cellular divisions is also known for the developing eggs of other organisms such as sea urchins, grundling (*Mesogurnus fossilis*) and others (Lönnig, 1959; Vakhrameieva and Neufakh, 1959).

After the critical heat sensitive stage, heat-resistance increases rapidly and considerably and, in case of the development with diapause, reaches its maximum at the early part of the diapause period. During the period of summer-autumnal and winter rest (aestivation and hibernation) heat-resistance is maintained at a high and rather stable level slowly decreasing to the moment of reactivation and resumption of the spring development. During active

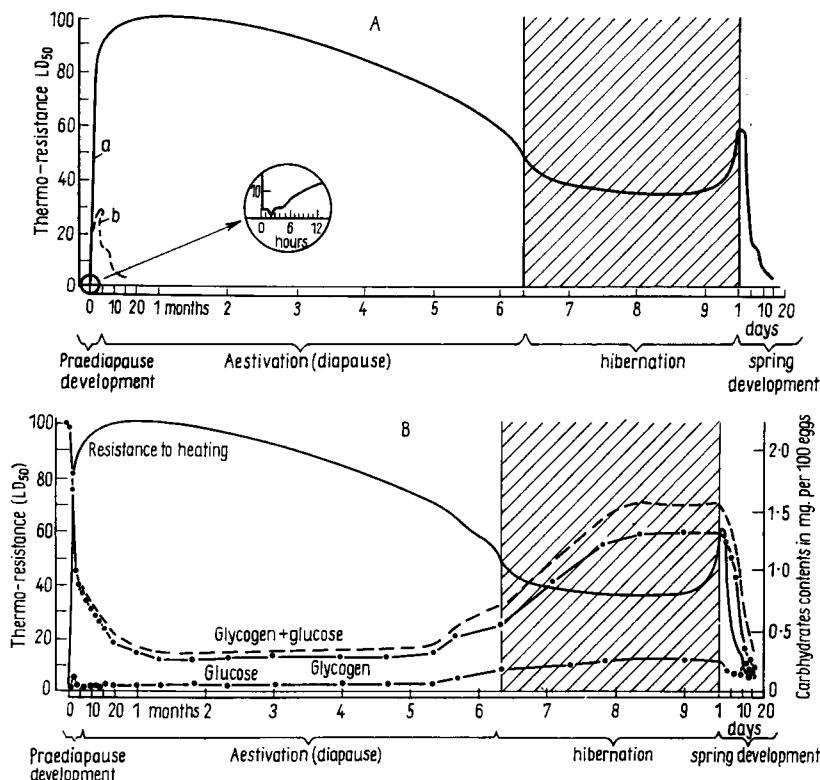


FIG. 3. The course of heat-resistance changes and carbohydrate content during the embryogenesis. Heat-resistance is in terms of LD_{50} (duration of exposure) at 46°C . A—ontogenetic changes in heat-resistance a. development with the diapause; within the circle—details of heat-resistance changes during the first 12 hr of development, on a magnified scale; b. non-diapause development obtained by treatment with dilute HCl or "artificial hatching". B—comparison of ontogenetic changes in thermoresistance (left ordinate) with the ontogenetic dynamics of carbohydrate content (right ordinate); development with the diapause and hibernation, curves of carbohydrate content are presented according to Chino's data (1957).

spring development, heat-resistance rapidly and steadily decreases reaching the second minimum by the moment of hatching when heat-resistance is only 4 per cent of its maximal level during diapause. In case of the non-diapausal development caused by treating with dilute hydrochloric acid the early rise of heat-resistance reached only 28–30 per cent of the maximal level found in diapausing embryos (Fig. 3a, dotted line, Fig. 4). Heat-resistance then rapidly sinks in just the manner as observed during the spring developmental phase of the hibernated eggs (Fig. 4). Unfortunately, the course of the changes in heat-resistance during the natural non-diapause development of the eggs of summer generation of bivoltine races has not been studied.

Apart from brief declines of heat-resistance at the moments corresponding to the metaphases I and II oocyte maturation divisions which are due to the heat disturbances of meiotic divisions, all orderly changes in the heat-resistance of developing eggs are gradual in time course. No periods of sharp drops of resistance to thermal shocks which could be called critical and no sensitive stages of morphogenesis can be found during the embryonic development of the silkworm.

From the biophysico-biochemical viewpoint changes in the heat-resistance of protoplasmic proteins may be due mainly to the content of antidenaturants such as soluble carbohydrates; the content of these substances is in its turn

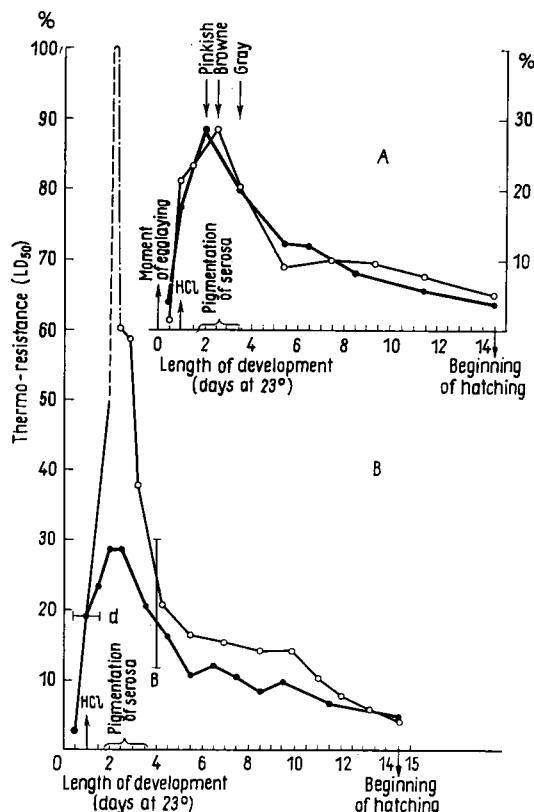


FIG. 4. Thermoresistance at successive stages of non-diapause development, per cent of the maximal thermoresistance during the diapause taken as 100. A—average data obtained in three series of two experiments with the eggs of the Bagdad race. Heating in three series of the first experiment (thick line) at temperatures of 46, 48 and 50°C, and in three series of the second experiment (thin line) at the temperatures of 42, 44 and 46°C; B—average data of two preceding experiments (thick line) are compared with the course of thermoresistance in the pre-diapause and spring development periods of the diapausing eggs (thin line).

closely related to the accumulation and consumption of energy reserves and to the respiratory metabolism. Changes in the carbohydrate composition and content in the course of silkworm egg development have been thoroughly studied in recent years (Chino, 1957, 1958). Figure 3b is a graphical representation of these changes according to Chino's data in comparison with our own ontogenetic curves of heat-resistance, shown in Fig. 3a.

The rise of heat-resistance at the onset of the diapause, and, therefore, during the replacement of the aerobic cyanide-sensitive respiration with the anaerobic cyanide-stable one, runs in parallel with the change of glycogen reserves to polyhydric alcohols and other soluble carbohydrates (mainly glycerol) which play the role of antidenaturants in high cold- and heat-tolerance of aestivating and hibernating eggs. With the recovery of the active state, at the end of hibernation, a resynthesis of glycogen takes place. The drop of the heat-resistance during the active phase of spring development is reciprocal to an increase in the rate of respiratory metabolism and is probably related to the hydration of the protoplasm at the expense of water produced during the oxidation of glycogen reserves consumed in course of spring development. This hypothesis is being tested in our laboratory by V. D. Kiryushina; the data obtained up to the present seem to support the suggestion that during the spring development the egg content actually undergoes some hydration although it is unclear whether this slight increase in water content is sufficient to explain such a abrupt drop of heat-resistance and whether some other causes are also in play.

Seasonal ontogenetic changes in heat-resistance are of a distinctly adaptive character and are closely related to the onset and termination of the state of rest (the diapause). Their biological significance seems to coincide with that of the diapause itself which has been developed as an adaptation to the survival during unfavourable seasons. However, the rise of heat-resistance is probably not a direct adaptation to environmental temperatures but an indirect one: like the onset of the true diapause state, the corresponding tolerance cannot be regarded as a direct response to environmental conditions which do not undergo significant seasonal changes during this time; both the onset of the diapause and abrupt rise of heat-resistance are determined by internal factors. On the other hand, the principal biological significance of these changes consists not in the fact or not only in the fact that they provide resistance to super-optimal injuring temperatures at the denaturation level. Such high temperatures have hardly been met with by the silkworm eggs on the paths of evolution. In the regions of sericulture, especially in natural subtropical ranges of the silkworm, winter eggs are laid by moths of mono-voltine races in mid-late June, while the moths of the second generations of bivoltine races lay them in mid-late August. During aestivation in nature they are exposed to the action of temperatures of the range 25–35°C, more seldom of the range 35–40°C and for a short time maybe even to higher temperatures. Therefore, the possibility cannot be ruled out that an increase in resistance

found with respect to sublethal temperatures exceeding 42°C, i.e. to the temperatures which are almost never met with by the silkworm eggs in natural environment, is correlatively brought about by adaptive mechanisms "hitting further than the mark" that have been developed as a protection against super-optimal summer temperatures of southeast Asia.

It is very likely, however, that the main significance of physiological and physico-chemical changes which are the basis of the increase of heat-resistance consists not in the direct protection against high temperatures but in the performance of other still more important biological tasks of the diapause. These changes must first of all enable the egg to survive the severe winter period. They must provide the egg with high frost-hardiness by lowering the freezing point of the protoplasm. During both the aestivation and hibernation periods the chemical changes must ensure, through the inhibition of metabolism the minimal loss of reserves of energy and essential substances. They must also prevent water losses by decreasing transpiration and evaporation.

There are extensive data which indicate that frost-resistance of animals and plants—this especially concerns plants surviving cold seasons in the state of dormancy—is very often attained through the conversion of starch, glycogen and sometimes lipids stored during the active phase of life into soluble carbohydrates, such as monosaccharides, glucose phosphates, polyhydric alcohols and other compounds that increase osmotic pressure and, consequently lower the freezing point of tissue fluids (Levitt, 1954, 1956a, 1956b, 1958; Precht, Christophersen and Hensel, 1955; Biebl, 1962 and, concerning cold-hardiness of insects in particular, Ushatinskaya, 1957).

For the silkworm *B. mori* all these preceding inferences are in good agreement with some experimental data; it was found in our laboratory by G. A. Pokrovskaya (1959) that changes in the cold-resistance of freshly laid eggs up to the onset of diapause run exactly parallel to the changes just described in heat-resistance.

As to the conversion of glycogen into polyhydric alcohols the clear indication of the frost protective role of glycerol is of a particular interest. Just as it is found in the silkworm (Chino, 1957, 1958), glycerol appears instead of glycogen with the onset of the diapause (in this case of the pupal diapause) in some Saturnid moths, for instance in *Platisamia cecropia* (Wyatt and Mayer, 1959, Wyatt, 1958). An unusually high content of glycerol and consequently, the very low freezing point were found by Salt in several insects compelled to hibernate in the diapause state in the open under the severe climate of Canada (Salt, 1957, 1958).

On the other hand, it is well known that sugars, glycerol and other osmotically active substances act as protectors against heat denaturation of proteins. They increase also the thermal doses required for heat sterilization, when added to suspensions of microbial cells, spores, etc.

V. J. Alexandrov and his collaborators (1958, 1959) have collected many facts showing the non-specific changes (for example, seasonal ones) in the

protoplasmic resistance of plant cells to cooling and heating, and to other injurious agents as well. The same authors have found cold-hardening to be accompanied by a concurrent increase in heat-resistance.

On the basis of the denaturation theory of cell injury and stimulation developed by Nassonov and Alexandrov (1940), which regards different cellular injuries as non-specific denaturation of cellular proteins, these authors tend to regard changes in cold- and heat-resistance also as nonspecific responses which increase tolerance to different injuring agents.

Although the main adaptive significance of the appearance of antidenaturants probably consists in the increase of cold-hardiness, the same physiological mechanism solves two problems: together with the resistance to low temperature it increases that to high temperatures as well. It is very likely that a simultaneous increase in osmotic pressure prevents the egg desiccation as well. Thus, the complex nonspecific increase in hardness during the diapause of the silkworm, may be determined by a common basic physiological mechanism.

It should also be noted finally that in the case of artificial heat parthenogenesis and thermal elimination of the diapause ("artificial hatching"), a rather great individual variation in excitability to heat shocks is striking in different batches and is of hereditary nature (Astaurov, 1940, 1943; Emme, 1946a, b). It seems that a close mutual interrelation does exist between heat-resistance and thermoreactivity; some observations strongly indicate that the range of the variation in heat-resistance of different egg batches and between individual eggs is also very considerable. Individual variability in heat-resistance has also been found recently in frog spermatozoa (Ushakov and Chernokozheva, 1963).

All these fluctuations in heat-resistance are conspicuous and of considerable range and this gives hope to find easier immediate causes of their variability and thus elucidate intimate physiological mechanisms determining heat-resistance of cellular proteins. From this viewpoint the ontogenetic, reactive and individual variability of the heat-resistance of the cell proteins of cold blooded animals deserves much more attention.

It should also be stressed that a thorough analysis of the action of high temperatures upon the cellular heat-resistance of the silkworm is of particular interest in relation to diverse application of heat shocks in the sericultural practis as a means for the control of the developmental processes and vital activity (induction of artificial parthenogenesis and androgenesis or experimental sex control, production of experimental polyploidy, thermal elimination of the diapause or "artificial hatching" and thermal cure of the infected eggs from nosmatosis, etc.).

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HEAT-RESISTANCE OF GAMETES OF POIKILOTHERMIC ANIMALS

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EXPERIMENTS performed on a great number of species of poikilotherms have shown that the level of resistance of somatic cells and cell proteins to injurious temperature, i.e. their heat-resistance, is a definite species characteristic correlated with a degree of thermophilia of a species. The purpose of the present work was to interpret the data obtained by a study of the heat-resistance of animal sexual cells and to compare them with the results of the experiments conducted on specialized animal tissues.

Investigations carried out on the spermatozoa of taxonomically close species of amphibians, molluscs and echinoderms showed that the spermatozoa of different species exhibit different levels of heat-resistance (Svinkin, 1959, 1961; Andronikov, 1963). As can be seen from Fig. 1 a straight line characterizing the dependence of the time of retention of movement of spermatozoa for lake frogs (*Rana ridibunda* Pall.) after heating to different temperatures lies in region of higher temperatures than a corresponding line for grass frogs (*Rana temporaria* L.). It indicates that the spermatozoa of lake frogs, a more thermophilic species, show a higher level of heat-resistance than the spermatozoa of *R. temporaria*. Analogous results were obtained from the experiments with molluscs and sea urchins (Fig. 1b).

A series of investigations performed on the ova of amphibians (Moore, 1942, 1949; Volpe, 1957 and others) showed that temperatures causing damage to the ova are different for different species, and the whole are in conformity with the environmental temperature conditions of a species. We obtained similar evidence in studying the heat-resistance of the ova in frogs and sea urchins (Andronikov, 1963). The level of heat-resistance determined by the loss of the cleavage capacity of the ova, due to heating, is in conformity with the degree of thermophilia of a species. This can be clearly seen from Figs. 1c and d: as in the case with spermatozoa, the straight lines indicating heat-resistance of the ova of more thermophilic species are located in the region of higher temperatures than the straight lines for less thermophilic species. Hence, the heat-resistance of spermatozoa and that of ova of the species investigated are different and correlated with environmental temperature conditions of the species.

Poljansky (Poljansky and Orlova, 1948; Poljansky, 1957) and Sukhanova (1959, 1961) showed that Protozoa, free living as well as parasitic, can quickly change their level of heat-resistance depending on the temperature conditions in which these unicellular organisms (or their hosts) were previously kept.

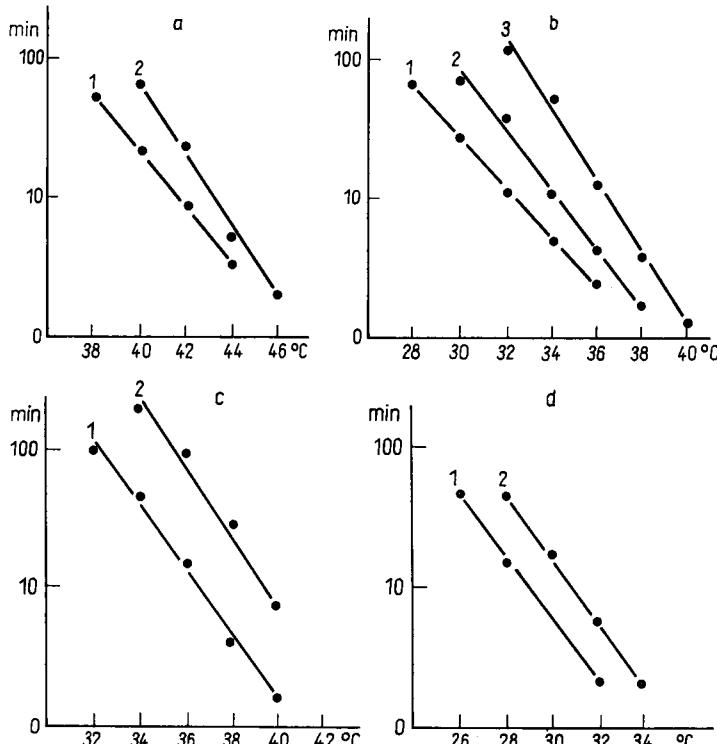


FIG. 1. a. Heat-resistance of spermatozoa of the frogs *Rana temporaria* (1) and *R. ridibunda* (2); b. Heat-resistance of spermatozoa of the sea urchins *Strongylocentrotus polyacanthus* (1), *S. intermedius* (2) and *S. nudus* (3); c. Heat-resistance of ova of the frogs *Rana temporaria* (1) and *R. ridibunda* (2); d. Heat-resistance of unfertilized ova of the sea urchine *S. intermedius* (1) and *S. nudus* (2). The abscissa gives the temperature in °C, the ordinate, the time of heating in min (logarithmic scale) causing the loss of motility in 100 per cent spermatozoa or loss of capacity for cleavage in the ova.

The fact that in the natural state spermatozoa show some independence from the organism suggests that they might show reactions similar to those in Protozoa. We made attempts to change artificially the heat-resistance of spermatozoa. For this purpose two groups of grass frogs were kept at low (2–4°C) and relatively high (23–25°C) temperatures for a month. The tests showed that the maintenance of mature animals under various temperature conditions does not influence the heat-resistance of their spermatozoa which remained the same as in the control freshly caught animals.

Similar experiments with molluscs carried out for 5 months confirmed the results of experiments with frogs. In this case we also failed to induce any shifts in the heat-resistance of spermatozoa (Svinkin, 1959, 1961).

It should be interesting to know the degree of stability of the heat-resistance level of gametes and whether there are differences in the heat-resistance between individuals of one species but taken from different regions of its geographic range. The studies of Runnström (1927, 1929) on marine invertebrates and the experiments of Blair (1941, 1942), Moore (1942, 1949) and Volpe (1957) with amphibians showed that the maximal temperatures the ova and embryos of these animals can tolerate without evident deviation from standard development are identical for a species and do not depend on the regions of the geographic range from which an animal has been taken.

We studied the heat-resistance of spermatozoa of lake frogs from different parts of the Soviet Union (Svinkin, 1959). The level of heat-resistance of spermatozoa in this frog species taken from Moscow, Tashkent, Erevan and Frunze proved to be the same (Fig. 2a), despite climatic differences of these regions. Moreover, according to the data of Ushakov (1963), there are two (Asiatic and European) forms of lake frogs whose differences are so great

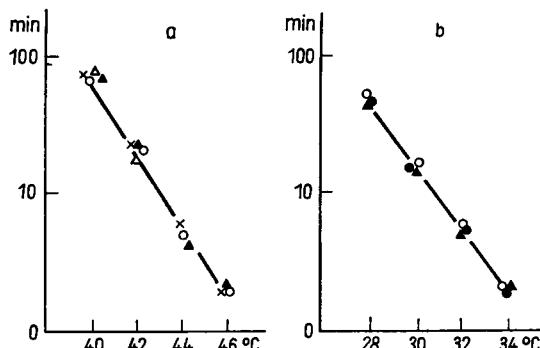


FIG. 2. a. Comparison of heat-resistance of the spermatozoa of *R. ridibunda* from Moscow (solid triangles), Erevan (open triangles), Tashkent (crosses) and Frunze (circles); b. Comparison of heat-resistance of the unfertilized ova (points), zygotes (circles) of *S. nudus* and the zygotes of *S. nudus* × *S. intermedius* (crosses). The abscissa gives the temperature in °C, the ordinate, the time of heating in min (logarithmic scale) causing a complete loss of motility of spermatozoa or loss of capacity for cleavage in the ova and zygotes.

as to include such a very important index as the heat-resistance of somatic tissues. However, these differences are not found in their spermatozoa, this fact being additional evidence which supports the idea of conservatism of the heat-resistance level as a characteristic of the species taken as a whole.

Thus the level of heat-resistance of spermatozoa is constant and independent of temperature of the environmental or maintenance conditions of adult

animals of the given species. In this respect they show similarity to somatic cells of higher poikilothermic animals.

The heat-resistance of ova was investigated in a different way. We compared heat-resistance of unfertilized and fertilized ova. For this purpose one group of the ova was heated and then fertilized, while the other was fertilized before heating. The experiments were performed on frogs and sea urchins. It was discovered that the time of heating at 36°C required for the repression of cleavage capacity of the unfertilized as well as fertilized ova of the grass frog was 14–15 min. In the sea urchin *Strongylocentrotus nudus* at 30°C both the unfertilized and fertilized ova lost their cleavage capacity in 16 min. Thus, despite different functional states of the unfertilized and fertilized ova, the level of their heat-resistance proved to be the same (Svinkin, 1962; Andronikov, 1963).

It is known that the level of the heat-resistance of a mature intact animal is not constant and exhibits a considerable lability (Ushakov, 1956, 1958; Zhirmunsky, 1959 and others). It should be especially interesting to determine the heat-resistance not only of ova but also of a zygote, i.e. practically of a newly formed organism. Studies on the zygotes of frogs and sea urchins showed that the level of their heat-resistance not long before the beginning of cleavage was the same as in the unfertilized ova of the given species (Fig. 2b). It is worth mentioning that the zygote obtained by fertilization of the ova from *S. nudus* by spermatozoa of *Strongylocentrotus intermedius* exhibits the same level of heat-resistance as a normal nonhybrid zygote. It indicates a great constancy of such characteristics of the zygote as its heat-resistance.

Hence, all the cells of Metazoa (sexual, embryo and somatic) show a definite level of heat-resistance (for each type of cell correspondingly) which is typical for each species of animal. Therefore, the cells of multicellular animals differ significantly from those of Protozoa whose heat-resistance can change rapidly due to temperature alterations of the environment.

Thus the regularity reported earlier for somatic cells is also characteristic of sexual cells. It supports Ushakov's suggestion (1961, 1963) that a species specificity of the heat-resistance of the somatic cells results from the difference in the heat-resistance of the sexual cells which give rise to the animals of the given species. In this connexion, a study of the heat-resistance of the sexual cells, zygotes and earlier embryonic stages in the development of poikilotherms will enable us to understand the ways species specificity appears in the heat-resistance of somatic cells and elucidate its role in the adaptation of an organism to the environmental temperature conditions.

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REGULATION OF EXCITABILITY IN NERVOUS AND MUSCLE TISSUES OF VARIOUS ANIMALS DUE TO TEMPERATURE CHANGES

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NASSONOV and Suzdalskaya studied the effect of temperature changes on the excitability of frog nerves from a new point of view (1956a). A nerve or muscle was located on the electrodes in a glass chamber with running paraffin oil, the temperature of oil was regulated. Excitability was determined by the response of the muscles to stimuli of various duration. The experimental values obtained for the threshold of excitability were represented as strength-duration curves (Figs. 1 and 2). A strength-duration curve for a nerve or muscle was determined at the initial temperature; after that the temperature

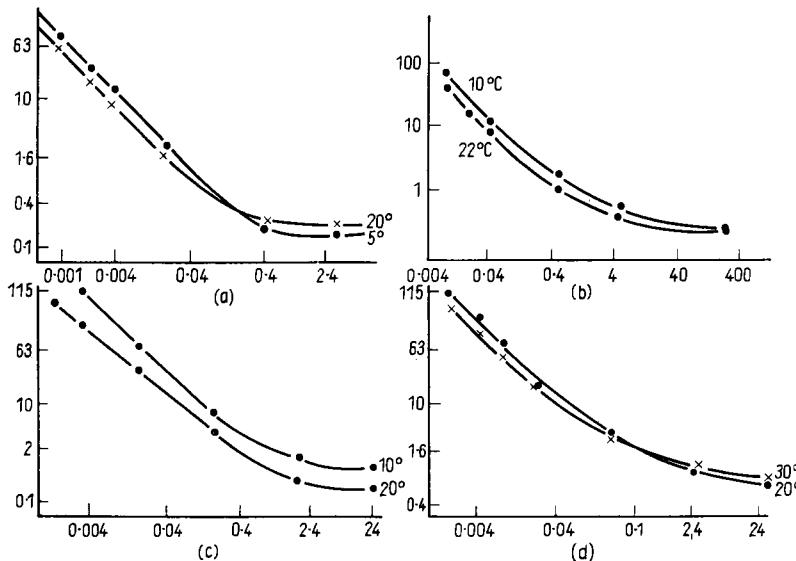


FIG. 1. Strength-duration curves at change of temperature; a—frog's nerve (Nassonov and Suzdalskaya, 1956); b—frog's heart (Nassonov and Rosenthal, 1960); c and d—rat's muscles (Suzdalskaya, 1959), abscissae—stimulus duration in msec; ordinate—threshold of excitability (v); logarithmic scale.

was changed within 3–6 min and all the points of the curve were redetermined. Then the muscle or nerve was returned to the initial temperature and the third measurement of the curve was made. In some experiments we determined only the constants b and a , i.e. the threshold of excitability to prolonged and short stimuli.

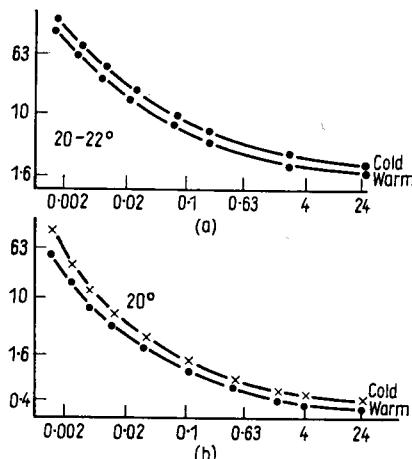


FIG. 2. Strength-duration curves for muscles of animals from high and low temperature habitat; a—frog's muscles (Ushakov and Zander, 1961), b—turtle's muscles (Suzdalskaya and Kiro, 1963); on the axes—the same as in Fig. 1.

In conformity with some published data, on the nerves of frogs and turtles, it was established that a 10° cooling of tissue in the range between $30-50^{\circ}$ is accompanied by an increase of excitability in response to prolonged stimuli and by a decrease of excitability in response to short stimuli. Warming produces the opposite effect: excitability to prolonged stimuli decreases and vice versa. Excitability to the electrical stimuli of average duration (corresponding to physiological stimuli) remained constant. These correlations are expressed by the intersection of the strength-duration curves. The point of intersection indicates the duration of the stimuli at which excitability does not change upon alteration of the temperature. It was established for frog nerves that the temperature influence on excitability varies, depending on the season. In spring, summer and autumn, i.e. in active periods, the excitability of nerves in response to stimuli of various duration changes has been described above (Fig. 1 a). For spring, summer and autumn frogs the intersection of the curves indicates the stabilization of nerve excitability to the stimuli of definite duration. In winter, when the environmental temperature varies insignificantly and the tissue metabolism in frogs tissues is considerably decreased, the nerve response to temperature alteration is different. Cooling caused a fall of the excitability along the whole strength-duration curve; warming caused an increase, i.e.

the constants of excitability a and b change in one direction; the strength-duration curves of frog nerves measured at different temperatures go in parallel and do not intersect. Excitability in each point of the curve depends on the temperature. Inhibition of the nerve glycolysis of "spring" frogs by 2 per cent monooxacetate causes the same effect. Evidently, excitability regulation is an active process which involves some components of the cell metabolism.

The preservation of a constant threshold of excitability of the nerves of summer frogs to the stimuli of intermediate duration with the change of temperature can be regarded as the cells' adaptation to the performance of

TABLE 1. CHANGES IN STRENGTH-DURATION CURVES OF NERVES AND MUSCLES OF DIFFERENT ANIMALS UPON ALTERATION OF TEMPERATURE

	Objects	Strength-duration curves upon alteration of temperature					
		Intersecting		Additional comment	Parallel		Additional comment
		$t^{\circ}\text{C}$			$t^{\circ}\text{C}$		
Nerves	n. ischiadicus <i>Rana temporaria</i>	20°	5°	Spring	20°	5°	Winter or spring at the action of 2% MJA
		30°	20°	Summer			
	n. ischiadicus <i>Emys orbicularis</i>	20°	10°				Without aeration
	n. ischiadicus <i>Rattus norvegicus</i>	40°	35°	Upon aeration	20°	5°	Upon aeration
Muscles	n. ischiadicus <i>Columba livia</i>				30°	21°	Without aeration
	m. sartorius <i>Rana temporaria</i>	21°	7°		40°	35°	
		25°	15°		30°	20°	
	m. cardialis <i>Rana temporaria</i>	22°	10°		30°	20°	
		21°	6°		26°	5°	
	m. retractor capitis	25°	6°		27°	15°	
	<i>Emys orbicularis</i>	30°	20°		27°	15°	
	m. puboischiotibialis	38°	20°		30°	20°	
	<i>Emys orbicularis</i>	26°	5°		32°	22°	
	m. pedis <i>Mytilus gallo-provincialis</i>	27°	15°		39°	29°	
	Isthmus <i>Scorpaena porcus</i>	27°	15°		30°	20°	
	m. soleus <i>Rattus norvegicus</i>	30°	20°		27°	15°	
					32°	22°	
	m. extensor digitorum longus <i>Rattus norvegicus</i>				30°	20°	
	m. flexor metacarpi radialis <i>Columba livia</i>				20°	10°	

their functions under changing temperature regimes. To verify this suggestion the excitability of nerves and muscles of some animals was investigated with the change of temperature (Table 1). The intersection of the strength-duration curves was observed in muscles of poikilotherms upon alteration of the temperature which occurred in the physiological range, i.e. which does not damage the tissue (from 28–20 to 5–6°C). This revealed the capacity of muscle tissue (both smooth and striated) of phylogenetically remote species of cold-blooded animals to regulate excitability under incoming impulses and to support its independence from fluctuations in environmental temperature. It should be noted that the co-ordinates of the region of the strength-duration curve, which does not shift with temperature change, depend largely on the physiological characteristic of one or the other tissue. Thus the point of the intersection of strength-duration curves of the frog's heart (Fig. 1 b) is situated in the region of the great (rheobase) current duration (Nassonov and Rosenthal, 1960). This is in agreement with the fact that the duration of the action potential of the cardiac muscle is considerable (0·1 sec), while the point of intersection of the curves of the skeletal muscles is in the middle region where the duration of a stimulus is about 0·1 msec (Suzdalskaya, 1957; 1959; Suzdalskaya and Kiro, 1957). The temperature environmental condition of poikilotherms in nature may influence the rheobase increase of their isolated muscles due to thermal effects. Thus, according to Ushakov and Zander (1960) in frogs inhabiting cold springs (water temperature differs from that in warm springs by 9–10°C) the threshold of muscle excitability to prolonged stimuli at 30–37°C increased more rapidly in comparison with that of frogs from warm springs. Moreover, at room temperature the muscles of frogs inhabiting warm springs showed a higher excitability to stimuli of any duration than the muscles of frogs from cold springs (Fig. 2a). Suzdalskaya and Kiro (1963) investigated the influence of temperature regimen (two groups of animals were maintained correspondingly at 27 and 4°C) on the functional properties of turtle retractors. Figure 2b represents the strength-duration curves of retractors determined at room temperatures. The distribution of the curves indicates that a decrease in the environmental temperature results in decrease in the absolute value of excitability of retractors to stimuli of any duration. Moreover, in the retractors of turtles from the aquarium with a water temperature of 4°C, under the influence of high temperatures, a fall in the threshold of excitability to both short and prolonged stimuli proved to be rather considerable. In these two groups of turtles, alongside with excitability, the resistance to high temperatures (43°C) and ethanol (Table 2) was determined. As can be seen from the table, heat and ethanol resistance of the retractors of turtles kept in the cold is lower than in the turtles from warmer conditions, i.e. from a temperature of 27°C. It should be noted that the excitability regulation, i.e. the intersection of strength-duration curves of retractors remains in the both groups of animals and is always revealed upon temperature change.

Excitability of nerves of warm-blooded animals was investigated by Nasso-

nov and Suzdalskaya (1956a, 1958). Rat nerves will not conduct impulses at temperatures lower than 5°C, while pigeon's nerve will not do so at a temperature below 10°C. Nerves of warm-blooded animals are very sensitive to oxygen deficiency. In the absence of oxygen, cooling of pigeon's nerves by 10° in the temperature range between 40 and 20°C and for rats in the limits of 40–5°C was continually accompanied by a fall in excitability to stimuli of any duration (Table 1). The warming of nerves in the same temperature range increased their excitability. In this case the strength-duration curves never intersected and, hence, the excitability of the nerve always depended on temperature.

TABLE 2. RESISTANCE TO TEMPERATURE EFFECT AND ETHANOL OF HEAD RETRACTOR OF TURTLES (EACH FIGURE—THE MEAN OF 12 EXPERIMENTS)

Stress	Retractor	Retention time of excitability (in min) of turtle head retractors kept at		Fiducial probability
		27°C	4°C	
43°C	1st pair	55·3	32·1	0·02
	2nd–3rd pair	33·8	20·9	0·04
	1st pair	36·8	26·8	0·02

However, during maintained aeration the rat nerve was cooled from 40 to 35°C and then rewarmed from 35 to 40°C. The strength-duration curves for these two conditions intersect, that is, there was an opposite alteration of excitability in response to short and long stimuli on cooling from that on warming. Thus, the maintenance of metabolic processes at a certain level provides a constancy of excitability not only in the nerves of cold-blooded but also of warm-blooded animals. However, regulation of excitability in the latter instance can be obtained only over a narrow temperature range.

Temperature alterations of muscles of warm-blooded animals (rat and pigeon) (Suzdalskaya, 1957a, 1959a) in the range of 20–10°C induce the same changes of excitability for stimuli of any duration: decrease on cooling and increase on warming (Fig. 1 b). Upon temperature changes in the region of 40–20°C the excitability of the investigated muscles remains constant to stimuli of intermediate duration. In the case of short and prolonged stimuli the excitability changes in the opposite direction (Fig. 1 d). Hence, muscles of warm-blooded animals retain their capacity to regulate excitability only up to a definite temperature level. It is significant that the temperature range, within which excitability regulation is retained, is lower for tissues of poikilotherms than for those of warm-blooded animals. Thus, there is no constancy of excitability in response to stimuli of corresponding duration, when nerves of warm-blooded animals are cooled below 35°C; for muscles of warm-blooded animals this boundary falls to 17–20°C, excitability regulation in nerves and muscles of poikilotherms takes place over a wider temperature range.

Summing up the above, it can be concluded that the nerves and muscles of cold-blooded and warm-blooded animals show a cellular mechanism for retaining excitability at a constant level under the conditions of changing temperature. This mechanism is supported by a definite and sufficiently high level of metabolic processes.

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THE ANALYSIS OF COMPENSATORY PROCESSES IN ISOLATED RODENT MUSCLES DURING HEAT INJURY

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WE INVESTIGATED the reaction of isolated cells of rodents to injurious thermal effects. It was found that compensatory processes delaying development of heat damage of muscle fibres arise in isolated muscles of these animals as a response to the effect of injurious temperatures. Investigation of protoplasmic protein synthesis reveals one of the mechanisms of these processes.

This investigation was carried out on isolated muscles of rodents: *Rattus norvegicus* Berk., *Microtus arvalis* Pall., *Microtus gregalis* Pall., *Lagurus lagurus* Pall. and *Citellus pygmaeus* Pall. We studied the effect of a wide range of temperatures under different aeration conditions on the retention time of excitability and the intensity of protein synthesis. Determination of muscle thermostability was carried out by the method of Ushakov and Gasteva (1953). The retention time of muscle excitability was measured in Ringer's solution at different temperatures. The moment when excitability was lost was determined by the disappearance of contractions in response to stimulation by electrical current. The temperature was maintained with an accuracy of 0.2°C. The solution was aerated by "bubbling" oxygen through it. The intensity of the protein synthesis was determined by the rate of methionine ^{35}S incorporation. Muscles were incubated in Ringer's solution upon addition of 0.001 per cent labelled methionine. In order to extract the protein we used a method which enabled us to eliminate the adsorbed methionine (Melchior and Halikis, 1952).

Thermal reactions of cells are different in different temperature regions. Figure 1 represents the relationship between the temperature and the time of the loss of excitability by rat muscles. In the semilogarithmic graph it is represented as a broken line consisting of two segments with different slopes. In the high temperature range (41–47°C) the increase in the reaction rate due to the temperature rise is very high. Q_{10} of this process is 44. A high and constant value of Q_{10} leads us to suppose that the death of muscles in this temperature zone is due mainly to one process, i.e. to changes in the protoplasmic protein complexes similar in nature to denaturation (Ushakov and Gasteva, 1953). In the lower temperature range (29–40°C) rat muscles retain

their excitability for a shorter period of time than might be suggested by time accounted for by protein heat denaturation. In this case \mathcal{Q}_{10} is equal to 2 or 3. Acceleration of the cell death at these temperatures is due to the influence of non-physiological conditions as a result of their isolation from the organism (Ushakov and Gasteva, 1953).

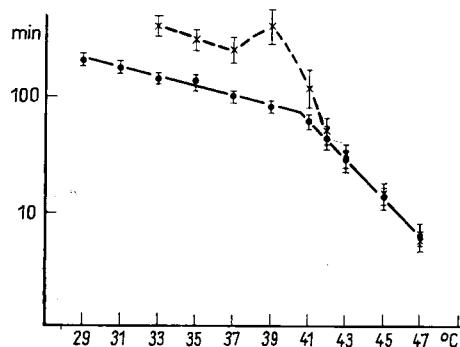


FIG. 1. The thermostability curve of anterior tibialis of the *Rattus norvegicus* in aerated (crosses) and non-aerated (points) Ringer's solution. The abscissa gives the temperature (in °C), the ordinate, the time (in min) in logarithmic scale. Vertical straight lines—confidence intervals of the mean.

The type of relationship described between the time when non-excitability begins to develop and the temperature was noted for many species of poikilotherms (Ushakov, 1955, 1956a, 1956b; Svinkin, 1959). In this case cells behave merely as protein systems and do not show any compensatory processes which can delay the development of non-excitability. However, under the action of some stimuli of a chemical character on muscle tissues, compensatory processes enabling muscle fibres to retain their excitability for a considerably longer time than could be expected from the alteration of protoplasmic protein complexes, were noted in a number of poikilothermic animals (Ilyinskaya and Ushakov, 1952; Lopatina, Ushakov and Shapiro, 1953; Ilyinskaya, 1960; Ushakov and Krolenko, 1960; Zhirmunsky, 1962). As was established by special experiments (Ushakov, 1959), an increase in the survival time of cells in the process of stimulation can be explained by the active protective reactions of the cell directed towards the reduction of the injurious effect and related to changes in the intensity of metabolism. On the basis of the above we suggested that, upon intensification of tissue metabolism, it should be possible to reveal compensatory processes in the cells in relation to the injurious effect of heat. In this connexion a second series of experiments was carried out with additional aeration of Ringer's solution (Fig. 1). As can be seen from the graph of the influence of oxygen, some deviation is observed in the upper portion of the curve representing the alteration rate of cell proteins. This deviation

TABLE 1. INTENSITY OF LABELLED METHIONINE INCORPORATION IN MUSCLE PROTEINS OF RATS IN RELATION TEMPERATURE AND AERATION OF INCUBATION MEDIUM

Number of experiments	Radioactivity of incubation medium (number of counts ml/min)	Aerated Ringer's solution		Non-aerated Ringer's solution		Difference in % $\left(1 \frac{n_1}{n_2} \times 100 \right)$
		Amount of diluted O ₂ (in volume %)	Mean amount of counts for 10 mg of dry protein (n ₁)	Amount of diluted O ₂ (in volume %)	Mean number of counts for 10 mg of dry protein (n ₂)	
39°C (incubation time 60 min)						
I	6971	1.41	152	0.57	47	122
II	695	2.28	32	0.40	7	357
III	569	2.10	26.5	0.40	8	231
45°C (incubation time 25 min)						
I	1030	2.46	3.5	0.66	3.3	6
II	2412	1.97	2.0	0.77	2.0	0

indicates an increase in the retention time of excitability as compared with what we would expect in the case of the death of muscles which has been determined by the denaturation time of the cell proteins. At 39°C the survival time of muscle fibres increased by 407 per cent. In the experiment conducted at higher temperatures (42–47°C) aeration of solutions does not increase the survival time. It appears that the survival time of cells at a moderate intensity of temperature is determined, on one the hand, by an injurious effect of heating and on the other by the compensatory processes inside the cell which partially repair the damage.

On the basis of published data on inhibition of compensatory processes in muscles by metabolic inhibitors and our own experimental data concerning the appearance of these processes under the influence of oxygen, we studied the intensity of muscle metabolism under various temperature and aeration conditions. The rate of protein synthesis was used as a measure of the metabolic rate. In the mean temperature range amino acid incorporation was measured in aerated as well as in non-aerated solutions. However, the rate of radioisotope incorporation into muscle protein in the aerated solution was twice or three times as high as in the non-aerated one (Table 1). This means that in experimental conditions under which muscles are capable of compensatory reactions, one can observe a considerably more intense protein synthesis than in the case when the compensatory reaction was not revealed.

In the high temperature range methionine incorporation into muscles was insignificant in aerated and non-aerated solutions and the same in both cases. It might be suggested that under high temperatures, causing rapid denaturation and breakdown of protein complexes of protoplasm, the protein synthesis in isolated cells is carried out very weakly. The aeration of solutions neither accelerates this process nor increases the retention time of muscle excitability at this temperature.

Thus, due to additional aeration, compensatory processes in response to heat injury were detected in isolated muscles of rats. Further investigation has shown that in other species of rodents (*M. arvalis*, *Microtus gregalis*, *L. lagurus* and *C. pygmaeus*) similar reactions can be found without additional aeration (Fig. 2a, b, c, d). The amount of oxygen in a solution (0·4–0·5 per cent) seems to be high enough to provide for the compensatory processes. Curves of such a type were found for cells of some marine and terrestrial molluscs (Dzhamusova, 1960; Arronet, 1963).

A sharp increase in the compensatory processes occurs in the muscles of rodents under the influence of oxygen. Excitability persists 10 times longer in the isolated muscles of *L. lagurus* immersed in the aerated Ringer's solution at average temperatures than in the non-aerated one (Fig. 3). These processes depend also upon the physiological state of an organism. In *C. pygmaeus* Pall. the compensatory processes can be found only in spring or summer, i.e. in the period of the most active viability, and are absent in other seasons of the year.

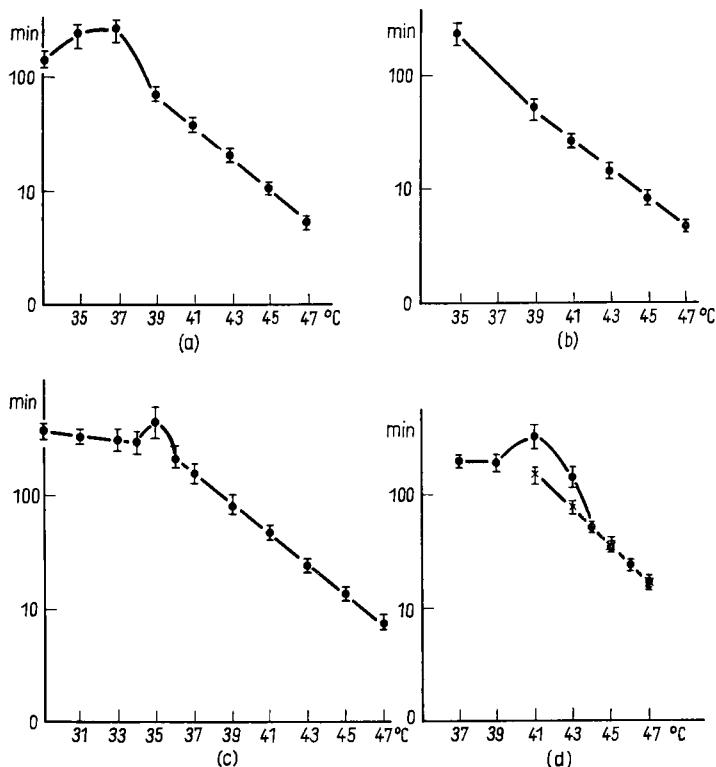


FIG. 2. Thermostability curves of anterior tibialis in (a) *Microtus gregalis* Pall., (b) *Microtus arvalis* Pall., (c) *Lagurus lagurus* Pall., and (d) *Citellus pygmaeus* Pall. Points designate summer; crosses—autumn; other designations are the same as in Fig. 1.

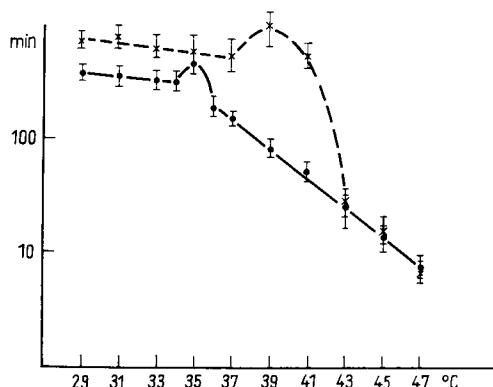


FIG. 3. The thermostability curve of anterior tibialis of the *L. lagurus* in aerated (crosses) and non-aerated (points) Ringer's solution. Designations the same as in Fig. 1.

Thus the investigation of heat-resistance of isolated muscles of rodents has shown that they are capable of partial compensation of heat damage. Active compensatory processes accompanied by intensification of protoplasmic protein synthesis can be brought about only in the presence of oxygen in the medium.

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REACTION TO HEATING OF THE CILIATED CELLS OF SOME GASTROPOD MOLLUSC LARVAE

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CILIATED cells of the trochophore prototroch and the veliger velum of molluscs, as well as those of worms developing in egg clusters are suitable specimens for studying certain questions of cellular resistance physiology. These objects can easily be kept under laboratory conditions and permit a prolonged observation of the cells *in situ* and an estimation of the activity of these cells by their ciliary movement. Embryos are surrounded by envelopes which are impermeable to micro-organisms, but permeable to a number of substances, including vital dyes.

It has been shown on the veligers of the Barents Sea gastropod molluscs, such as *Lacuna divaricata* (Fabricius), *Dendronotus frondosus* Ascanius and *Cuthona* sp., that the process of repression of ciliary activity in velum cells which results from heating in the high temperature zone (32°C and higher) shows a Q_{10} of an order of magnitude of 10, 100 and 1000. The Q_{10} of the repression of velum cells by heat was in three different species of mollusc 68, 1290 and 21,000. Consequently, such cell thermonarcosis is due to heat denaturation of the protoplasmic proteins. Hence, in this temperature range, cells behave as if they were passive protein systems incapable of counteracting heating. This coincides with the reaction of muscle cells (Ushakov and Gasteva, 1953), plant cells (Alexandrov, 1956), ciliated epithelium cells (Zhirmunsky, 1959) and spermatozoans (Svinkin, 1959, 1961) to high temperatures.

It has been demonstrated for ciliated cells of the veliger velum of *L. divaricata* ("young" and "adult" veligers), under lower temperatures, that the cells are able to counteract heat damage in the course of heating. As a result, ciliary movement persists for a longer period (by 6–8 times at $28\text{--}29^{\circ}\text{C}$) than is predicted by the regularity of cell-protein heat denaturation which holds true within the higher temperature region. In this lower temperature zone one can observe injury reparation during heating. Continued heating restores the ciliary motion in part of the cells which have ceased it at the beginning of the action of high temperatures. The same repair of the function in the course of heating was discovered in plant cells (Alexandrov, 1956), for animal cells an

analogous description was made on muscle cells of *Arenicola marina* (Gorodilov, 1961). An injury evoked by higher temperatures can also be repaired but not before heating has ceased. In the case of considerable heat damage of cells, ciliary motion recovers on the 5–6th day after heating. In the high temperature region minimum doses of irreversible cessation of ciliary motion ("lethal dose") determined by heating are about 3·5 times greater than the minimum doses of reversible thermonarcosis ("thermonarcosis dose").

The difference between the lethal and thermonarcosis doses for the same period of heating makes up a reversibility zone. In the high temperature region, these zones of reversibility are equal to 3°; they are considerably diminished within lower temperature limits, for thermonarcosis dosage here approaches the lethal dose.

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CHANGES IN HEAT-RESISTANCE OF ISOLATED TISSUES AS A RESULT OF PRELIMINARY HEATING

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THE increase in the heat-resistance of isolated cells after preliminary heating of frog nerves was described by Thörner (1919, 1922). Jamada (1924) and Zhukov (1935) repeated these experiments with myelinated and unmyelinated nerves and thus confirmed Thörner's evidence.

We studied the effect of preliminary heating on the resistance of isolated muscles of frogs (*Rana temporaria* L. and *Rana ridibunda* Pall.) to high temperatures. Experiments were conducted on paired muscles. Having heated one of the muscles we immersed the pair into Ringer's solution heated to 38 and 42°C (for m. sartorius of *R. temporaria* and iliac peroneus muscles of *R. ridibunda* respectively). The heat-resistance was determined by the time of retention of excitability under a sinusoidal current.

It has been found that a preliminary heating of muscles of *R. temporaria* results in a two-phase change of their thermostability: a short period of heating increased survival time approximately by 58 per cent, while a long exposure led to a decrease. An increase in resistance caused by short-time heating at 34°C was relatively nonspecific: resistance to the injurious effect of alcohol and quinine also increased; heat-treatment did not alter resistance to calcium chloride, potassium chloride, chloral hydrate and hypotonic solutions. The maximal increase in resistance of muscles previously exposed to 34°C was observed after 15 min of heating (Schlachter, 1959).

The effect of preliminary heating of muscle from *R. ridibunda* to 35, 36, 37, 38, 39 and 40°C was studied. Extreme temperatures did not increase the muscular heat-resistance. A preliminary heating at 36–39°C also induced a two-phase change in the heat-resistance of muscles. Heating for 5–10 min evoked an increase in thermostability, while prolonged thermal treating decreased the heat-resistance. The maximal increase in resistance to heating was obtained for all the applied temperatures after a 10-min preliminary action of temperature. Thus after exposure for 10 min of the muscles to temperatures of 36, 37, 38 and 39°C the survival time increased by 17, 21, 32 and 39 per cent respectively (Chernokozheva).

It has been stated that the level of muscular heat-resistance of *R. temporaria* undergoes regular seasonal shifts. It is interesting to follow the increase of resistance after preliminary heating in different seasons of the year. It has been found that in autumn and winter (from August to February) heat-resistance of the muscles increases after a heating for 15 min at 34°C. In spring and summer, when the thermostability alters, this phenomenon is not observed. Thus seasonal changes of the heat-resistance of muscles are observed after the effect of a short period of preliminary heating (Schlachter, 1961).

Heat-resistance increases in March and decreases in May. Apparently these phenomena are also connected with the changes in the intensity of preliminary heating which causes an increase in muscular heat-resistance. Chernokozheva found that 33°C in spring induces the same increase in thermostability of muscles as 34°C in autumn and winter. The maximal rise was also noted after preliminary heating for 15 min (Chernokozheva and Schlachter, 1963). Thus we found a shift in the effect of preliminary heating in causing an increase in heat-resistance in different seasons.

A comparison of our results with the data obtained by Thörner, Jamada and Zhukov permits us to draw the conclusion that the increase of heat-resistance under experimental condition can be observed in different tissues of an organism.

Ushakov (1958) and others have shown that usually the heat-resistance of the whole organism is lower than that of most of its tissues, while an increase in heat-tolerance under our experimental conditions is observed at temperatures which are lethal for the intact organism. Therefore, the increase in cellular heat-resistance induced by a preliminary heating may have an adaptive significance for an animal organism only in case of the local influence of temperature on the organ, but not on the entire organism.

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DISCUSSION

Discussion of B. L. Astaurov's Paper

N. L. FELDMAN: According to your experimental data the increase in the heat-resistance of the silkworm eggs is connected with the transition to the diapause. How can you explain the similar increase of thermostability in case of non-diapause development?

B. L. ASTAUROV: According to Chino, glycogen is not transformed into glycerol and sorbitol in the first short phase of non-diapause development as it is during the diapause development. The mechanism causing the increase of heat-resistance in the first case is different as compared with that in the second case. However, the nature of this phenomenon is not yet clear.

L. K. LOSINA-LOSINSKY: Does the cold-resistance of the silkworm eggs change simultaneously with their thermoresistance?

B. L. ASTAUROV: In the pre-diapause phase of the silkworm egg development the changes of the two characteristics are parallel to each other. In both phases of hardening and hibernation the thermoresistance decreases while the cold-resistance increases.

C. L. PROSSER: What is the basis for your statement regarding protein denaturation? Have you any direct evidence?

B. L. ASTAUROV: We did not perform any special experiments. Our conclusion that we deal with the denaturation of proteins is based on very high values of the temperature coefficient (Q_{10}) of the rate of heat injury. Q_{10} is equal to several thousands.

H. A. PRECHT: It is well known that the diapause is regulated by hormones. May we assume that the changes in cold-resistance which occurred during the diapause are also induced by hormones?

B. L. ASTAUROV: The diapause is regulated not only by hormones. I must say that hormonal mechanisms as a basis for changes in cold-resistance seem hardly possible since the shifts in the cold-resistance occur at the very early period of the embryonal development—at the germ-band stage.

G.I. POLJANSKY: In connection with Prof. Astaurov's considerations I would like to call your attention to the correlation between thermo- and cold-resistance of cells. The data reported indicate that there exists a certain correlation between them. The same was stated for the higher plants in results from the laboratory headed by Prof. Alexandrov. On "hardening" both thermo- and cold-resistance change simultaneously. The correlation is quite opposite for

Protozoa and marine algae: the increase in the thermostability is accompanied with the decrease in the cold resistance. Apparently two different mechanisms constitute the basis of these phenomena. In the first case we can think of certain changes of proteins, while in the second case alterations in some specific sites of metabolism are to be assumed. The correlation between heat- and cold-resistance is a question of a great biological importance.

GENERAL DISCUSSION

C. L. PROSSER

I SHOULD like to start the discussion with a few general statements concerning temperature adaptation. In this symposium we have been using very non-precise terms such as capacity-adaptation, resistance-adaptation, thermostability, heat and cold death with operational meanings. We should attempt to define them in specific molecular terms.

Cold death, for example, may have many causes. We have been told how freezing may kill by dehydration and salt concentration, by disruption of colloidal structure and rupture of membranes, by denaturation which results from unmasking of specific bonds. In addition, cold death at non-freezing temperatures may result from a reduction of energy-yielding reactions below basal levels, or by general increase in cell permeability. Further, cold death in metazoans may result from failure of integrative function of nervous and circulatory systems.

Heat death may result from irreversible denaturation and coagulation of proteins, from reversible denaturation of specific enzymes thus giving reaction rates less than maximal, from changes in the physical state of lipids, from generalized increase in permeability, and indirectly from toxic compounds liberated by heated cells. Precise knowledge of the causes of cold and heat death is necessary to an understanding of thermostability.

Capacity adaptation is a change in rate functions within the normal range of tolerated temperatures. It is normally a compensation which permits relative constancy of activity. Resistance adaptation is a change in survival or tolerance at the limits of the temperature range. The extent to which capacity and resistance adaptation have similar molecular mechanisms is not clear. In general, the resistance limits of intact animals are narrower than those of isolated tissues.

In cold- and heat-adaptation the following kinds of changes have been suggested:

I. *Genetic changes—mutations and chromosomal alterations.* Natural selection of phenotypes which are permitted by the genotypes leads to formation of races and species. This means that nuclear DNA changes randomly and selection acts on the proteins which result. Qualitative changes in primary structure of proteins, formation of isozymes, etc., have a strict genetic basis. High temperature can accelerate mutation rate but cannot direct mutations. Genetic changes are important for determining protein structure which sets temperature limits for denaturation.

II. *Non-genetic or environmentally induced changes.* These occur within the limits of the genotype.

A. Indirect effects. There may be changes due to effects on the following: endocrines, nervous system and behavior, toxins liberated from tissues, morphological changes in homeotherms, diapause, hibernation and estivation, synthesis of protective agent in the cold, such as glycerol.

B. Direct effects on cells, demonstrated for plants, unicellular organisms, and for capacity adaptation in animals.

1. Direct action on DNA can be excluded.

2. Qualitative effects on proteins:

(a) There is probably no direct effect on primary structure of proteins, that is, on sequence of amino acids since this is determined by *m*-RNA and the *m*-RNA is determined by DNA.

(b) Structural changes, as in tertiary structure, are possible but not proved to be directly influenced by temperature. These changes would result in alterations in degree of molecular folding and exposure of reactive groups. Such changes have been seen in purified enzymes such as ribonuclease according to conditions of preparation.

3. Quantitative effects on proteins. There can be stimulation or depression of alternate enzyme pathways, due to quantitative changes in amounts of given enzymes. The control of such quantitative changes can be by induction, repression and inhibition. A protein normally present in very small amounts could be formed in quantity.

4. Changes in fatty acids (degree of saturation) and in proportions of various lipids may occur.

5. Changes in proportions of small molecules:

(a) Coenzymes may be involved in the quantitative alterations of alternate metabolic pathways.

(b) Changes in balance of critical ions; changes in water content and distribution.

6. Changes in membrane permeability may result from some of the above molecular alterations.

H. PRECHT: Two methods can be used when studying the problem of heat-adaptation: the temperature treatment of isolated proteins or that of the organisms themselves. In the latter case proteins and enzymes are separated just before measurement.

Many years ago Fraenkel observed the increase in the thermostability of gelatine when this protein was kept at high temperatures for a long time. The increase in the thermostability was accompanied by the decrease in the molecular weight. Christophersen and Thiel found similar changes in the heat-resistance of pancreatin. However, in many other cases pure enzymes did not show any heat-adaptation. Meves observed some changes in heat-resistance

of proteolytic enzymes of *Helix pomatia* only after the mollusc's adaptation to relatively high temperature was established.

My colleagues will greatly oblige me if they tell me of all the cases they know of cold- and heat-adaptation of proteins.

B. L. ASTAUROV: I would like to give you my consideration concerning studies made by Ushakov and his co-workers on the conservatism of heat-resistance. I highly appreciate the great work my colleagues have performed; however I must say, that some additional investigation is to be undertaken to build up a summarizing theory. The conservatism of heat-resistance is not the exception from other characteristics of the species. This index is known to possess some individual variations which constitute the basis for selection and the formation of races or populations differing in their thermostability occurs. In addition, in the course of the individual development different kinds of changes in cell thermostability can be revealed. These changes directly or indirectly are in correlation with the environment.

Thus, I think that this index possesses all the peculiarities which are also characteristic of any other species criterion.

It is difficult, of course, to suggest a certain adequate method for comparing the stability of indices. In this respect, I believe, one must apply the various statistical methods of estimation and make an attempt to use such a criterion as variation coefficient of indices etc. The comparison of semilogarithmic curves is not a good method because these curves are likely to diminish the quantitative differences of thermostabilities.

I would like to say that up till now there is little evidence as to the ontogenetic variability of thermoresistance as a physiological index. Experimental data are at the very initial stage of accumulation and, therefore, the generalizations of facts available seem rather premature. At present the ontogenetic variability of thermostability means mainly changes related to the sexual cycle. Data in respect to other aspects of the problem under discussion are very scanty. These are some findings on alterations with age in the salmon and on insects which I spoke about during previous sessions of the Symposium. That is why I suppose it is too early to conclude that the thermostability is the result of selection at ontogenetic stages. Directly or indirectly the index of thermostability had rather to be selected at the most effective stages of selection, i.e. at the stage of adult organism or during the embryonic period. I believe that selection can influence this index, maybe not directly, for it can be correlated with some other characteristics, for example, with the thermostability of an organism which I have had the opportunity to speak of. And even in this case it can hardly be concluded that the thermostability of adult forms is the relic of heat-resistance of sexual cells and of early embryonic stages.

A. V. ZHIRMUNSKY: There is a question I would like to ask Professor Astaurov. Do you not think that two aspects of the problem must be dis-

tinguished when we deal with cell thermostability as a species characteristic? Firstly, it is interesting to learn whether cell thermostability can be used as a species index. Choosing as species indices different morphological peculiarities, e.g. the number and position of lamelli in the sea urchin skeleton or the character of shell structure of molluscs, zoologists argue not so much whether the index discussed is "good" or "bad" for a given group of animals as whether it is a species or a genus characteristic. Cell thermostability can be used as a species criterion for the majority of types of poikilotherms and it is nothing but a species index. Like any other characteristic it has some differences and limitations, e.g. fluctuations related to the reproductive cycle or shifts connected with the salinity of the sea water. However, these restrictions can be eliminated under special conditions.

Secondly, and this it seems to me more significant, cell thermostability is a species characteristic. Species divergencies in cell thermostability reflect differences in the structure of proteins of their protoplasmic complexes. This property is fixed genetically and its initial cause is evidently related to the structure of nucleic acids.

Thus, I think we must distinguish the two aspects of this problem and your remarks towards cell thermostability as a species characteristic may be referred to the first part of the problem. Do you agree with me?

B. L. ASTAUROV: I did not object to the role of thermostability as a taxonomic characteristic and I did not want to diminish the significance of this criterion as an important biochemical characteristic which seems to be closely related to the DNA structure. For a certain group of animals this index is probably useful from the taxonomic point of view. However, for some other groups of animals living under changeable environmental conditions this index is not very good. As to the second part of this problem, I agree with you that it deserves a serious investigation but in any case the thermostability should not be opposed to other indices. Thermostability like the latter is genetically conditioned and shows a certain variability based on both genetical and phenotypical variabilities. In this respect cell thermostability does not differ from other species indices and I think that there is no reason to stress specially its conservatism.

B. P. USHAKOV: I thank Professor Astaurov for his critical remarks. There is no doubt that many things we are speaking about will be changed in the future. This is our working hypothesis and we can foresee the difficulties of investigation. You say I place cell thermostability in opposition to all other indices. You are mistaken in this respect because I always stress that this criterion cannot be the only one. We know cases when cell thermostabilities of two species of Tritur coincide but we have never thought they belonged to one and the same species. We do not speak of cell thermostability as of an absolute index. I wrote about it in 1958. We accentuate the coincidence of cell thermostability in individuals of one species. When we passed from studies

of the thermostability of somatic cells to examination of that of sex cells and cells of early embryonal stages we found other experimental support. I speak of Runnström, Moore and Danilevsky who reported on the coincidence in the thermostabilities of embryos of representatives of different populations of one and the same species. In addition I would like to mention your data on the coincidence of the thermostabilities in different races of silkworm eggs.

I do not see any essential difference between cell thermostability and other indices. But I consider this criterion to be very important because the finding of such a universal index, i.e. for a large groups of animals, is a novelty for the species problem. The fact is that the genetic characteristic is throughout the only serious criterion nowadays. However physiological and genetical mechanisms of non-crossing populations are different in different species. For one group of species isolation can be by a different mode of behaviour while for another one it is the different structure of sex organs or chromosomes.

In other words it is supposed that during the process of speciation there arise very significant as well as insignificant (partial changes) physiological differences. The data obtained show that in the process of speciation natural selection causes very profound rearrangements of the organisms which concerns chiefly the bulk of its cells and proteins.

Adaptive changes in cell thermostability were followed during phylogenesis and ontogenesis when the integration of the organism is not yet clearly pronounced. It concerns lower plants and animals in phylogenesis, and early embryonal stages in ontogenesis. As the organism grows its thermostability is being regulated by nervous and humoral mechanisms. At the same time cell thermostability undergoes some indirect alterations (by the way, such a view point accounts for the existence of frequently occurring paradoxical changes in the thermostability. In this case changes of cellular resistance are not of adaptive character. They can be regarded as an accessory effect of adaptive changes of the organism ("Nebeneffekt" according to the terminology of Professor Precht) and are not correlated with the environmental temperature.

V. Y. ALEXANDROV: Professor Prosser proposed for discussion his scheme representing different paths and mechanisms of temperature adaptation of the organism. This scheme is rather complicated, nevertheless it is simple compared with reality. The question of cell resistance, from an ecological point of view in particular, is not exhausted by the resistance of proteins. I say this despite the fact that I took an active part in founding the protein denaturation theory of injury. Therefore, it does become me least of all to diminish the significance of proteins for the determination of the level of resistance in general and that of heat-resistance, in particular. However, considering the cell as the whole living system we must admit that protein thermostability is one of the components which is responsible for the thermostability of the cell.

Now I want to call your attention to one of the figures I demonstrated during my report. In the graph (Fig. 1) one can see the curves of cell thermo-

stability of *Dactylis glomerata*, the cereal growing in Murmansk, Leningrad and Dushambe. Average lowest and highest temperatures proved to be rather different: in Murmansk the lowest temperature is 4°C, the highest 18°C, in Leningrad 11°C, and 18°C, and in Dushambe 13°C, and 31°C, respectively.

I want to stress that in spite of different temperature conditions available for the growth of these plants, the portions of the heat-resistance curves which characterize the protein resistance to the denaturating effect of heating coincided. The break of the curve representing the heat-resistance of plants grown

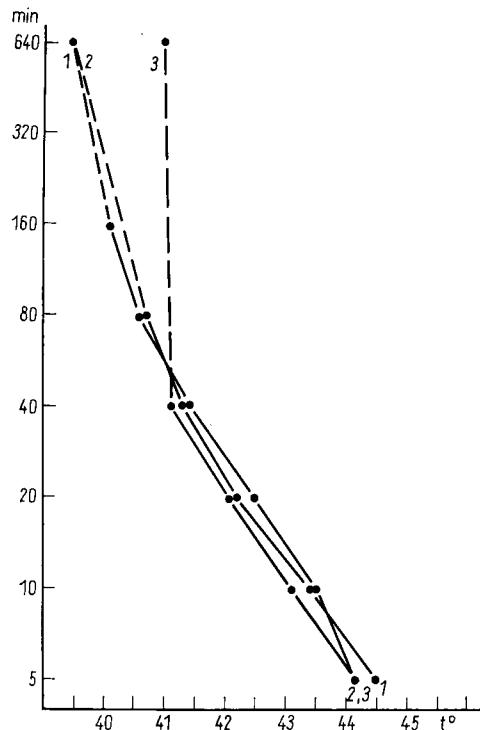


FIG. 1. *Dactylis glomerata* from different regions. Heat-resistance of epidermal cells of the leaf sheaths. 1. Kirovsk (Kolsky peninsula). 2. Leningrad. 3. Dushambe (the Middle Asia). Abscissa—the temperature t° of heating in °C. Ordinate—the duration of retention of protoplasmic streaming (in min), logarithmic scale. (Alexandrov and Feldman, 1958, The Cell and Environmental Temperature.)

at hot temperatures occurs earlier. Hence, within the range of low temperatures the plants show a greater resistance, in spite of similar thermostability of their protoplasm, compared with that of plants from colder regions. The same results were obtained from comparison of heat-resistance of epidermal cells of mulberry-tree leaves growing in Leningrad and Ashkhabad.

But there is no reason to underestimate the significance of protein heat-

resistance. A vast material gathered by Dr. Ushakov's laboratory on animal cells as well as our experimental data on plant cells can testify to the fact.

Protein heat-resistance may be considered from two points of view—genetically conditioned changes of thermostability and changes not connected with a new protein synthesis. The investigation of thermal mutants of Neurospora in which one of enzymes is characterized by a high heat-sensitivity enabled Horowitz and his collaborators to work out in detail a genetic aspect of the question and to point out the very loci which determine the protein resistance of the mutant.

Professor Prosser asked me to acquaint you with the following interesting fact: the cultivation of infusoria at higher temperatures carried out in the laboratory of Prof. Sonneborn changed their antigenic properties rapidly. In this case the rearrangement of the protein structure of infusoria occurred due to the action of temperature. There is no doubt that changes in heat-resistance of the protein are not necessarily related to the changes in its synthesis, i.e. they do not involve the genetic control of the protein synthesis.

I would like to remind you that Dr. Lomagin and Dr. Zavadskaya, from my laboratory, observed an increase in heat-resistance of cells measured after 5–10 sec following a 1-sec heating. In particular, the resistance increased in response to a short-term (5-min) heating causing damage to the cells due to heat denaturation of protoplasmic proteins. This means that the resistance of protoplasmic proteins may change considerably in the process of individual reaction not at the expense of the synthesis of new protein molecules. Here one can suggest a rapid liberation of some cellular substances which increase the resistance of proteins to denaturation.

I think that Dr. Ushakov's proposal that only the thermostability of developmental stages undergoes selection will meet a number of difficulties especially as concerns the vegetable kingdom. It is difficult for me to explain the following facts from the viewpoint just mentioned. We compared heat-resistance of different parts of the plant. The heat-resistance of epidermal cells of the cotton plant leaf proved to be considerably lower than that of the cells inside the seedcase of this plant. The difference was about 6°. However, on hot days, due to a profuse transpiration, the temperature of the leaf was 7–8° lower than that inside the seedcase. We are prone to regard this difference as an adaptive one. But this adaptive mechanism cannot be selected before the very appearance of leaves and seedcases. I think that the difference in the thermostability of tissues of adult organisms also has an adaptive significance and therefore it can serve as material for selection.

Our Symposium is the first conference on cytoecology of temperature relations. The problems we are dealing with are more complicated than we were even inclined to think and I believe it constitutes a good reason for us to proceed with our research. However, the fact is, biological evolution has had much more time for the creation of multiform and perfect mechanisms of adaptation that we, cytoecologists, have at our disposal.

B. P. USHAKOV: I shall dwell mainly on the first part of Professor V. Y. Alexandrov's communication concerning the mechanism of changes in the protein heat-resistance during ontogenesis. I would like to add that changes in the protein heat-resistance occur chiefly due to changes in the state of protein protoplasmic complexes. It seems to me that the example given by Professor H. Precht showing shifts in the molecular weight of gelatin particles suggests such a possibility. On the other hand, our experiments on the changes of cell thermostability during reproduction of frogs under natural conditions and due to hypophyseal injections have shown that the thermal death of muscles was not accompanied by their becoming opaque (no protein coagulation was observed), whereas under non-reproductive conditions the thermal death of muscles is always accompanied by a noticeable increase in opacity. In the blood serum (West and Keye, 1950) it was demonstrated that a hypophyseal hormone changed the thermostability of proteins due to the formation of complexes. This gives us good reason to suggest that in our experiments on muscles we observed a similar phenomenon.

Concluding Remarks

Prof. K. SCHLIEPER

IN CLOSING the session and the Symposium allow me to tell you on behalf of all the invited foreign guests that all of us are very thankful for the scientific exchange of opinions, for the hospitality we have had at the Symposium in Leningrad and at the institutes and laboratories of the Academy of our old and new Russian friends. Scientific results delivered in papers and communications at the Symposium are extremely valuable.

The discussions have given many concepts and stimuli for our future research work.

Many thanks to all of you—Prof. Troshin, Dr. Ushakov, Dr. Zhirmunsky—and to all who spoke and who contributed to the success of the present symposium.

Concluding Remarks

G. I. POLJANSKY

ALLOW me as the chairman of today's sitting and a member of the Organizing Committee of the Symposium to make some concluding remarks.

I want you to know that my concluding speech will be very short, taking into consideration that we have already listened to 43 papers and that if I were to spend even one minute in discussing each of them it would turn out to be a very bad end of the Symposium. There is a good Russian proverb "a fly in the ointment". I do not wish that the end of our Symposium should remind you of this proverb. Nevertheless, I would like you to let me concentrate your thoughts for some minutes.

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It is the first time that a symposium has been held at which scientists from different countries have gathered together to discuss a very important question of general biology—the question of the role of cellular reactions in adaptation of multicellular organisms to environmental temperature. This is one of the problems of the discipline which is intermediate between cytology and ecology and which was called cytoecology in the U.S.S.R.

Foreign specialists from different countries, Bulgaria, Czechoslovakia, France, the German Federal Republic, Japan, India, Poland and the U.S.A., have participated in the work of our Symposium. A number of our Soviet colleagues from different towns and regions of our immense country have come to the Symposium. The total number of foreign guests is 15 and that of the Soviet scientists is 61. But it is not the number of participants that matters so much. I believe, the quality of the event you all have participated in must be the strongest point of the Symposium.

I think I am voicing the opinion of our Organizing Committee when I express our deep satisfaction in the results of the Symposium which can be preliminarily estimated in the following way. I wish to draw your attention to the number of various aspects of the problems of cellular adaptation which were discussed in connexion with the organismal adaptation to high and low environmental temperatures.

Cold- and frost-thermostability of plants also received serious discussion. Mechanisms of hardening and of plant death on freezing, the role of sugars and some carbohydrates in the cell, the role of different biochemical mechanisms with special references to SH groups in increasing the resistance of the organism, all these and some other important questions were reported by Prof. Asahina, Dr. Meryman and Dr. Heber.

In this report, the paper of Prof. Tumanov must also be mentioned in which the results of studying frost-resistance in plants were presented.

A detailed analysis was made towards the problem of cold-resistance of animals to extremely low temperatures by our French colleagues—Dr. Rey and Dr. Simatos. In addition, they presented a film which was of great interest and success with the audience.

Prof. Lozina-Lozinsky reported an interesting observation concerning the possibility of the endurance of extremely low temperature by higher invertebrate Metazoa and of the maintenance of their vital capacity in conditions of the temperature of liquid helium.

In a number of papers some other aspects of cold-resistance of animals were discussed. Dr. Volfenson and her colleagues reported on the relationships between the increased cold-resistance of animals and their ecological conditions (hibernating and non-hibernating rodents).

Data on adaptation of tropical animals to low temperatures reported by our Indian colleague Prof. Rao produced a great impression. He managed to reveal some biochemical mechanisms of the adaptation and their dependence upon the hormonal influence.

An especially large number of papers was devoted to the relationships between cell and organismal thermoresistance both of plants and animals.

Dr. Lange presented a vast and interesting material which allows us to see the dependence of plant thermoresistance upon various factors of both exogenous and endogenous characteristics: developmental stage, age, water content of the organism, seasonal factors etc.

Prof. Alexandrov and his collaborators made a detailed analysis based on studies over many years of mechanisms which determine the level of cell resistance to injurious factors, particularly to temperature. These investigators touched the problem of denaturating action of heating, of hardening, of reparation capacity and its limits in plant cells, and finally the problem of specificity and non-specificity in reference to temperature adaptations.

Specialists in the field of plant physiology adduced their observations on development of physiological processes at high temperatures. In this respect, very interesting data were presented by Dr. Altergott who pictured changes occurring in plant organism under the influence of high temperatures.

Valuable material was presented by Prof. Schlieper who reported on an adaptive significance of fluctuations of thermoresistance of ciliated epithelial cells of various Lamellibranchia. According to Schlieper, these fluctuations are closely related with ecological peculiarities of species examined.

Prof. Precht pictured the diversity of processes connected with temperature adaptations at both cellular and organismal levels of organization. His original method for investigation of simultaneous influences of different temperatures upon different parts of the same organisms (fish) created a great deal of interest among the participants of the Symposium.

Prof. Prosser presented a thorough examination of acclimation and temperature adaptation phenomena carried out mainly on fish. Of particular interest was his analysis of the adaptive changes in nerve functions on acclimation. His diagram reflecting general principles of adaptation and acclimation will make us think much over the question discussed, because it represents a synthesis of the problems which are to be solved and elaborated.

The participants of the present Symposium have had the possibility to listen to some papers and discussions from one of the largest Soviet laboratories dealing with the problem of temperature adaptations of animals—the laboratory of comparative cytology headed by Dr. Ushakov. Data reported by Ushakov and his collaborators—Dr. Zhirmunsky, Kusakina, Dregolskaya, Dzamusova, Pashkova, Andronikov and others—received much attention from the audience. The problems they raised induced lively discussion. This very fact shows that the work carried out at the laboratory is of great interest from a biological viewpoint. Thermoresistance as a species characteristic, cell thermoresistance and its relation to the species ecology, biochemical mechanisms of protein thermoresistance, cyclic changes in thermoresistance,—this is not a complete list of questions which are dealt with at the laboratory

and which seem to constitute a programme of future investigations by Dr. Ushakov and his students.

In connexion with the papers just mentioned is a series of biochemical studies presented at the Symposium. In particular, Prof. Braun and his collaborators reported an interesting phenomenon concerning thermal resistance of proteins (actomyosins) from two close amphibian species. These differences in the increases in the thermostability they revealed are thought to be connected with ecological peculiarities of the species.

Prof. Astaurov presented an interesting paper which allowed us to get acquainted with the work carried out at this laboratory on the age dynamics of thermoresistance of mulberry bombyx (*Bombyx mori*) beginning with the fertilized egg stage of development.

Some questions concerning the role of cellular level of organization in adaptation to various thermal conditions at different steps of phylogenesis, unicellular organisms included, also received attention of the participants.

I have mentioned only a few of the communications reported at the Symposium. However, even this very brief and incomplete survey shows how wide is the range of questions touched upon at the Symposium.

I would like to stress that the field of science which the present Symposium is devoted to and which we call cytoecology, is in a very close connexion with a great number of various biological disciplines. Here, at the Symposium, we can see botanists, zoologists, cytologists, biochemists etc. Thus, a very strongly fortified and strengthened subject, namely the problem of adaptation of organisms, is attacked from the viewpoint of different disciplines. This is not at all astonishing because adaptation is one of the most typical characteristics of living organisms, and the question of establishment and evolution of mechanisms of such adaptation is doubtless one of the central questions of modern biology.

Of course, the importance of the present Symposium does not come only from the papers read or the discussion to which they gave rise. The main significance of the meeting comes, to my mind, in those personal contacts which we have made with our foreign colleagues. Most of them we met for the first time. And we have had the possibility to discuss questions of mutual interest to all of us. I hope the contacts between us will continue and develop further.

It goes without saying, that not all the viewpoints coincide in every particular. We witnessed some rather lively discussions and divergences of opinions. I would like to believe that different approaches for settlement of the problems constitute one of the guarantees of further success in their solution. There is no science when there is no discussion or exchange of opinions.

Before closing the Symposium I would like to express some words of gratitude first of all to the Academy of Sciences of the U.S.S.R. which gave the opportunity of holding the present meeting, and to the Department of

Natural and Mathematical Sciences of UNESCO which rendered great assistance in organizing our Symposium.

On behalf of the Organizing Committee I would like to address some words of warmest gratitude to our foreign colleagues who have come to our town and who took an active and fruitful part in discussion of problems we all were concerned with.

We are very much obliged to our Soviet colleagues who had to cover long distances in coming to Leningrad for taking part in the Symposium. Their participation increased interest and importance of our work a great deal.

In conclusion I wish to express sincere thankfulness to our young research workers, without whose help this Symposium could have hardly been organized. They shouldered almost the whole organizational work of the Symposium. Our interpreters are not at all professional ones—they are also research workers of our institutes. They ensured simultaneous translation.

Before closing the Symposium, I would like to wish a happy journey and good health to our foreign colleagues and friends, our Soviet guests from other towns and all the participants of the Symposium, and to express my confidence that questions which were raised and discussed at the Symposium will serve as a material for further scientific discussion and studies because the problem of adaptation looked at from various aspects of science is, remains and will be one of the central problems of general biology.

Thank you very much for your attention.

INTERMOLECULAR ASPECTS OF THE STRUCTURAL STABILITY OF PROTOPLASM AT THE TEMPERATURE EXTREMES†

J. BĚLEHRÁDEK

Department of Biology, Middlesex Hospital Medical School, University of London

INTERMOLECULAR forces—mainly hydrogen bonds (HB) and the equally important (Waugh, 1954; Salem, 1962) London-van der Waals forces (vW)—are responsible in the first place for the structural stability of protoplasm as depending on the balance between cohesion and thermal agitation, or between molecular order and disorder, prevailing at various temperatures.

Accepting that the structure of liquid systems differs only quantitatively from that of corresponding vitrified systems (Simon, 1930; Christophersen and Precht, 1952; Randall, 1934; Wolkenstein and Ptitsyn, 1959; Zhukov and Levin, 1950) the author has found (1957a) that the vitrification temperature α , practically identical with Tammann's T_g point, can be determined with relative safety by means of Slotte's equation for viscosity (η_v) changes with temperature (t°): $\eta_{v^0} = A/(t^\circ - \alpha)^b$; further (Bělehrádek, in prep.) that in non-associated liquids α (in $^{\circ}\text{K}$) is a simple function of the added mass of atoms in actual intermolecular contact; and that, as the strength of HB can equally be determined, it should be possible to assess the size and probable structure of the glass units, or crystallites (Stewart, 1930; Simon, 1930; Randall, 1934; Miles and Hamamoto, 1962), now believed to exist in the liquids as steadily reforming units of a short thermovariant life-time. Cohesion forces at $t^\circ = \alpha$ are given for a number of liquids in Table 1, and α -values for some solutions of biological interest in Table 2.

Although a direct biological application would be hardly possible at this stage, these results indicate in a general way the importance for protoplasmic cohesion of the HB on the one hand, and of the length of the hydrocarbon chains on the other. The extent of H bonding of water is known to vary with temperature and with the concentration of solutes, especially of ions. The values given concern two extremes, namely a dimer (von Thiele *et al.*, 1957) as if held either by vW only, or by and HB as well.

As a very general idea, based on a consideration of intermolecular forces, living substance can be imagined as essentially a polyphasic liquid system

† The paper is given as an addendum: it was not read because the author, although invited, was unable to attend the Symposium.

characterized by a relatively high degree of structural order, approaching the condition of a polyphasic glass melt, or as a system in the pre-vitreous state. Its various phases, of course, differ considerably in this respect; membranes, for example, are closer to their solidification than are the more hydrated phases which vitrify only at the temperature of overall vitrification of the system (Luyet and Gehenio, 1940; Luyet, 1957; Rey, 1959).

The arrest of cellular reactions by chilling is the consequence of an excess of ordering leaving too little scope for the kinetic energy to remain effective. An increase in structural order seems also to accompany the denaturation and coagulation of proteins, but it can be said that on the whole the specific ordering of the protoplasmic system is destroyed by heat oscillations, and the heat damage on the whole is caused by too much disorder, affecting at least one, the most sensitive, phase. Variations in the damaging temperatures can be caused by any agent able to modify the molecular ordering, whether by a modification, even slight, of the molecular configuration, or by various admixtures, even in traces (Davies and Jones, 1953), acting as steric or electrostatic modifiers of the intermolecular order (Miles and Hamamoto, 1962).

The share of the main constituents in determining the biological temperature limits and their ecological variations can be summarized as follows:

(a) Proteins

A century-long discussion of the role of proteins in the determination of the upper temperature limits of life and their modifications (cf. Bělehrádek, 1935; Precht *et al.*, 1955) has not been concluded. Adaptation to heat or cold of various specific proteins has been frequently postulated, but, to the author's knowledge, never really demonstrated by comparing cognate proteins in a strictly pure state. Definite proof, therefore, seems to be missing that the thermosensitivity of pure proteins would copy, or parallel, that of living cells of any sort. Thus, as far as such differences have been described, they can as well be attributed to the secondary effects of admixtures, such as ions and various concomitant organic substances, mostly metabolites, and also the non-protein moieties of protein complexes, e.g. lipoproteins. The attractive idea that the proteins of the more resistant forms owe their supposed particular stability to the abundance in their molecules of S—S links has apparent support in the fact that many such forms live in ecological media rich in SH₂ and that some of them have a high requirement of the S-containing amino acids (Bhat and Bilimoria, 1955; Bilimoria and Bhat, 1955). But no qualitative differences in the amino-acid composition seem to exist between the thermophilic and mesophilic bacteria (Eurejnova, 1948), and isolated flagella of these two groups are equally unaffected by the SH-disrupting agents, while the flagella of the thermophiles seem to be relatively resistant to HB-disrupting agents (Mallet and Koffler, 1957). Proteins of the thermophilic bacteria also seem to be relatively poor in phosphorus (Sobotka and Luisada, 1957).

Indirect adaptation of a protein to cold is exemplified by the behaviour of actomyosin: the extent of contraction developing in thawing depends on the temperature of thawing, and the curves are different in the frog and rabbit, in accord with their respective usual temperature requirements; however, the curves can be shifted at will in either direction by adding substances such as histamin, guanidin or chloroform (Szent-Györgyi, 1951).

The possibility of a modification in the chemical composition of a protein as a result of individual acclimatization has to be regarded as highly doubtful in view of the template being fixed by the genotype. Only in the lowest plants and protozoa more "plastic" protein synthesis is now being admitted (Alexandrov *et al.*, 1961). Serologically discernible mucoproteins have been found to form in some ciliates at various temperatures, but this effect is genotypical as it is due to different genes being activated at different temperature intervals (Beale, 1954).

(b) Enzymes

Particular metabolic patterns characterizing certain groups of heat-resistant organisms, e.g. the absence of chlorophyll in some thermal algae and prototrophic metabolism in some thermal bacteria very likely are associated with specific enzymatic equipment (Vouk, 1950). The lack in catalase and excess in peroxidase in thermal algae (Harvey, 1924) does not seem to be a general feature in this group (Kubin, 1959). It has been pointed out (Allan, 1950) that no special enzymes need be postulated in such organisms if it is assumed that a rapid heat destruction of enzymes is compensated by an equally rapid enzyme production; this, however, only devolves the problem on the template (Bělehrádek, 1957b). To quote an example of a cold-adapted biochemical pattern, the antarctic "ice-fish" completely lacks haemoglobin, its metabolism is excessively slow and its enzymatic outfit very likely is adapted accordingly, as suggested by some peculiarities in the blood-clotting mechanism (Ruud, 1959).

Still, a definite proof does not appear to have been produced that highly purified or crystalline enzymes would be temperature-adapted, in parallel with the species—in contrast with the adaptive differences in temperature optima and inactivation temperatures frequently occurring in crude enzymes, and in the enzyme activity of tissue extracts and homogenates. Nonetheless, negative (Firdman, 1958) and contrary (Grisolia and Joyce, 1959), findings also have been reported, and the existing comparative tables (e.g. Mansour-Beck, 1954) of enzyme optima and maxima temperatures do not allow us to draw conclusions one way or the other.

It would appear, then, that the thermostability of a given enzyme within the cell is genotypically determined at two levels, concerning (a) the chemical constitution of the enzyme molecule as determined by the template and (b) the normal cytochemical environment of the enzyme molecule, including the

promoting, stabilizing, inhibiting and sensitizing concomitants, and the structure, if any, in which the enzyme molecule is held.

Phenotypical acclimatization of a given enzyme activity has been frequently reported (cf. Precht *et al.*, 1955). It involves a shift of the optimum and maximum (e.g. Morosov, 1939), or, more frequently, a compensatory increase in the rate in cold-adjusted cells (Ushakov and Kusakina, 1960). It is the protoplasmic environment rather than the constitution of the enzyme that can be supposed to allow such changes to take place (Rotini and Gallopin, 1952; Labouesse, 1957).

Although some enzymes in solution lose their activity at the temperatures of liquid gases only (Luyet and Gehenio, 1940; Precht *et al.*, 1955; Rivers, 1927), the threshold temperatures of enzyme action are closer to 0°C and harmonize with the threshold temperatures observed in cellular processes (Table 3). If it is assumed that the arrest of enzyme activity by cold is due to a tight coiling of the enzyme molecule (Kavanau, 1950; Bělehrádek, 1954), the variations of these limits with ionic composition and other factors can be easily understood. The reported spontaneous heat acclimatization of pancreatic amylase in solution (Christophersen and Thiele, 1952) deserves a further study.

(c) DNA

Threshold temperatures at which heterochromatin segments appear in the chromosomes of certain plants on chilling, presumably due to a disturbance in the formation and distribution of DNA, have been claimed to be lower in cold-adapted species (Simon, 1930). On the other hand, the heat-resistance of DNA has been demonstrated to be very precisely determined by the proportion of guanine + cytosine (Marmur, 1960) but shows no correlation with the temperature requirements of the species (in bacteria, Marmur and Doty, 1959).

(d) Lipids

Fats and phospholipids are the only protoplasmic constituents in which the molecular structure and the resulting physico-chemical behaviour depend on the temperature of formation. The presence of unsaturated bonds in the hydrocarbon chain is promoted by low temperature of formation and reflects on the high iodine number and low consistency, connected with a low solidification point, of the respective lipids. The last two properties can be explained as due to an incomplete vW attachment of the chains resulting from the existence of a bend in the middle of the chain where the double bond is situated. Such seems to be the case of the oleic acid where the distant halves of the molecules probably remain without a side-to-side attachment. Consequently, its α point is considerably lower than that of stearic acid (Table 1).

This property of fatty acids and lipids, which agrees with the thermochemical principles, has been confirmed in natural fats of many various animal and vegetable species from diverse conditions of temperature, and also in

variously acclimatized individuals of a species (Henriques and Hansen, 1901; Hilditch, 1940). Protoplasmic lipids have been found to behave similarly (Terroine *et al.*, 1930). Thus the possibility is close at hand that this property of protoplasmic lipids may intervene in the mechanism of cytoecological adaptation and acclimatization (for earlier literature, cf. Bělehrádek, 1930,

TABLE 1. AVERAGE COHESION BETWEEN TWO MOLECULES AT VITRIFICATION POINTS, CALCULATED FROM THE VALUES OF THESE POINTS (IN °K)

Substance	°C	°K	Cohesion (kcal/mole)	Reference
H ₂ O	-46	227	0·9 to 17·9	Thorpe and Rodger
n-Butane, C ₄ H ₁₀	-142 ^a	131 ^a	2·7	I.C.T. ^c
n-Octane, C ₈ H ₁₈	-145	128	5·3	I.C.T.
n-Butanol, C ₄ H ₉ OH	-139 ^b	134 ^b	16·9	Thorpe and Rodger
n-Octanol, C ₈ H ₁₇ OH	-65 ^a	208 ^b	17·3	I.C.T.
Hexadecanol, C ₁₆ H ₃₃ OH	33 ^a	306 ^a	25·1	Firdman
Formic acid, HCOOH	-60	213	17·8	Thorpe and Rodger
n-Butyric acid, C ₃ H ₇ COOH	-94	179	19·0	Thorpe and Rodger
Caprylic acid, C ₇ H ₁₅ COOH	-38	235	19·6	I.C.T.
Stearic acid, C ₁₇ H ₃₅ COOH	1	274	23·0	I.C.T.
Oleic acid, C ₁₇ H ₃₃ COOH	-92	181	15·2	Kavanau

^a α -value calculated by the author from the data under reference.

^b Above 43°C the (apparent) value of α is -92°C (Thorpe and Rodger).

^c I.C.T.—International Critical Tables.

1935). The composition of natural fats, of course, is primarily genotypical (Bonner, 1950) but obviously plastic enough to allow individual fluctuations, sometimes surprisingly wide. Be it stressed that the consistency of fats does not depend on the proportion of unsaturated carbons only and that the melting points are not simply proportional to the iodine numbers, because the latter do not reveal the effect of the lower saturated fatty acids. In general, protoplasmic lipids are less consistent than the corresponding reserve lipids (André, 1925).

The functional stability of lipid-containing protoplasmic structures, such as the surface membrane, various protoplasmic membrane formation, especially the mitochondria, is easily upset at extreme temperatures; the role of lipids as "spacers" in certain enzyme systems (Picken, 1960) and as concomitants indispensable to some enzymes (Crane and Ehrlich, 1960) undoubtedly also is temperature-conditioned. It is known that the contractile system of the muscle does not react to ADP in absence of its lipid constituent (Szent-Györgyi, 1951); it is likely that the conspicuous shift of the peak on the tension-temperature curve occurring in response to acclimatization of a frog muscle (Varga, 1952) can be explained by a modification of the concomitant lipid rather than by that of the molecule of myosin or actin.

The association of the two moieties in protoplasmic lipoproteins is mostly

weak (Chargaff, 1944) and is easily disrupted by heat and other agents; salts acting at low temperatures (Lovelock, 1953) cause their denaturation. The impulse for the disruption may originate in the protein part (Ushakov, 1958) as a result of a change in coiling or of ionic interaction, but a simple effect of thermic vibrations loosening the vW attachment is also possible.

The effect of the lipid may consist in strengthening the thermolabile configuration of the protein by means of the rigid hydrocarbon chain. The "cloud temperature" of serum albumin can be raised by as much as 20°C on addition of fatty acids, and the effect on the whole increases with the length of their molecule (Boyer *et al.*, 1946); it has been suggested (Giese, 1947) that this effect may be indirectly indicative of the mechanism of the structural lipid action.

(e) Water

Water content, primarily a genotypic feature, is subjected to variations caused, among other factors, by acclimatization. In general, cold promotes hydration (see Beale, 1954; Christophersen and Precht, 1952; Precht *et al.*, 1955) which may involve an increase in distances between ions and molecules, and increased hydration of the hydrophilic constituents, with consequences to their stability (Christophersen and Precht, 1952; 1953). In forms capable of anabiosis and in many organisms undergoing acclimatization in nature, increased stability is generally associated with a reduction of water content, and an increase in osmotic concentration and hydrophilic power. An essential factor seems to be a relatively firm coat of H-bonded water molecules around the protein (Bogen, 1948; Christophersen and Precht, 1953; Levitt, 1951) which does not rotate in dielectric relaxation. On the contrary, many ordinary cells and tissues show reduced resistance to temperature extremes when partially dehydrated, e.g. the frog heart shows a reversible drop of temperature optimum on perfusion with a liquid rendered hypertonic by glucose or mannitol (Bělehrádek, unpublished). The drastic dehydration accompanying extracellular ice formation can be harmless to specially hardened or periodically freezing plants and animals (Luyet and Gehenio, 1940; Levitt, 1951; Scholander *et al.*, 1953) but is otherwise usually harmful. In view of the generally less injurious effect of an osmotic dehydration, the withdrawal of water in freezing need not be the sole damaging agent, and thermochemical effects have to be admitted, in analogy with the damage produced by cold even without ice formation (cf. Bělehrádek, 1935, 1957b). The possibility of a harmless intracellular freezing is now admitted in some instances (Kalabuchov, 1934).

Liquid water—apart from being a mixture of two crystallographic variants at a thermovariant equilibrium (Bernal and Fowler, 1933)—also is characterized by a thermovariant distribution of the vW and HB forces, their equilibrium being affected by ions and many molecular substances (Robinson and Stokes, 1959). Such alterations in the structural order are reflected by the value of α found in various solutions (Table 2). Like some compounds with an —OH

TABLE 2. VALUES OF α CALCULATED FOR VARIOUS SOLUTIONS AND SOME BIOLOGICAL SYSTEMS

System		°C	Reference
NaCl	1 mol	—46	I.C.T. ^a
KCl	1 mol	—40	
LiCl	1 mol	—50	
KBr	1 mol	—46	
NaBr	1 mol	—45	
Glucose		—42	Powell
Sucrose	20%	—44	Bingham and Jackson
Sucrose	40%	—40	
Sucrose	70%	—15	
Gelatin	0·25%	—43	Loeb
Gelatin	0·5%	—40	
Gelatin	1·0%	—35	
Gelatin	2·0%	—20	
Gelatin	4·0%	—15	
Na caseinate	9·4%	—25	
Peptone plasma (dog)		—40	Snyder and Todd
Cytoplasm, eggs of <i>Nereis</i> virens		—5	Pantin
Cytoplasm, hypocotyl cells of <i>Phaseolus multiflorus</i>		—40	Weber and Weber

^a I.C.T.—*International Critical Table*.

group, water shows two different values of α , the lower one observed experimentally as the T_g point when amorphous ice is produced from steam (Pryde and Jones, 1952), the higher one, calculated from the temperature-viscosity relation (Table 1). Although not confirmed experimentally, the technical obstacles to experimentation in the relevant temperature zone being almost insurmountable (Pryde and Jones, 1952), the higher α value recurs as the starting point in all aqueous solutions of varied concentration, coinciding smoothly with the value for zero concentration. As the value of η_t in protoplasmic viscosity, and the zero temperature points calculated or observed for various cellular processes lie above —46°C, the conclusion seems justified that water engaged in life phenomena is the structural variant of a supercooled liquid rather than that of a supercooled steam. The same seems to apply to enzyme reactions (Table 3).†

† From the applicability of the same equation it should not be inferred that the viscosity governs the rates; the views previously expressed are no more tenable in the original simple form (Beale, 1954; Bělehrádek, 1930, 1935). The protection against the action of freezing by glycerol and other substances (Root, 1932; Polge, 1957; Rey, 1959) rich in OH groups seems to consist in preventing or delaying the structural changes in water and its intermolecular relations. The detailed mechanism is a complex one (Rey, 1959) and is not yet clear.

TABLE 3. LOWEST TEMPERATURES OF REPORTED OBSERVABLE ACTIVITIES IN VARIOUS ORGANISMS AND ENZYMES, AND α -VALUE OF ENZYMATICAL ACTIVITY, COMPUTED FROM THE TEMPERATURE-VELOCITY RELATION

	$t^{\circ}\text{C}$	Reference
Vibrio phosphorescens, luminescence	-6 ^b	Root
Actinomyces sp., active existence	-7 to -10	Haines
Bacteria from frozen fruit, growth	-9	Luyet and Gehenio
Winter wheat, respiration	-6 to -7	Walter
<i>Helix pomatia</i> , heart beat	-7	Fischer
Various insects, O_2 uptake	-15	Kalabuchov
Chironomid larvae, O_2 uptake	-26	Scholander <i>et al.</i>
Trypsin	-15	
Lipase	-24.5	
Invertase	-18	
Ptyalin	-18	
Trypsin	-15 ^a	
Yeast invertase, $pH = 3.2$	-30 ^a	
Yeast invertase, $pH = 5.6$	-20 ^a	
Malt invertase	-60 ^a	
Urease (crystalline)	-30 ^a	
Phosphatase (cat, bone)	-15 ^a	

^a α -values computed from available data.

^b Observed, and identical with the computed α -value (Root, 1932).

(f) Salts

Potassium is known to accumulate in some tissues of warm-adapted plant species (Bachrach, 1943) and has also been reported to facilitate acclimatization to higher temperatures in certain micro-organisms (Bachrach, 1944). Excess of K^+ in the perfusion of snail's heart slightly elevates the optimum temperature, but produces a narrowing of the biokinetic zone because it raises the lower temperature limit considerably more (Bachrach, 1944). But some earlier detailed work showed that K^+ promotes heat injury in various animal and vegetable cells (cf. Bělehrádek, 1935). It also lowers the resistance to freezing of sea urchin ova (Asahina, 1962). Similar facts are known on the role of other ions, but more research would be needed for a fruitful comparison.

(g) Pluralistic Hypothesis

None of these (a-e) substances and, indeed, no other single product of protoplasmic disintegration, can explain the mechanism of damage by, and the resistance to, the extreme temperatures. Several protoplasmic constituents in their interaction have to be considered in any attempt at a satisfactory hypothetical interpretation of the cytopathological phenomena caused

by extreme temperatures and of the corresponding cytoecological regulations. Single factors, of course, have first to be looked for and studied extensively in order to determine their place in the relative cytochemical and cytophysical interactions, not to promote one of them rather dogmatically to be the sole responsible agent. This pluralistic view is supported by observational facts covering the whole range of supra-optimal and subzero temperatures, by the composite character of the time-temperature curves for the regions revealing usually one or more breaks (irrespective of the formula chosen, cf. Bělehrádek, 1935), and by the existence of several types of the adaptive or regulatory changes (Precht, 1955). While it never has been claimed that e.g. lipids would be the only factor in heat-stability and its variations, an opposition of the "lipoid theory" and the "protein theory" would be artificial and formalistic.

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