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Efraim Racker

Bioenergetics and the Problem of Tumor Growth

An understanding of the mechanism of the generation and control of biological energy may shed light on the problem of tumor growth

The generation of biological energy and the growth of tumors, two subjects which at first sight do not appear to be related, nevertheless have a possible relationship. The key to the mechanism of bioenergy formation is oxidation. Adenosine triphosphate (ATP) formation in cells is completely dependent on oxidative events. This is true for energy generation in mitochondria by oxidative phosphorylation and for the much simpler system of glycolysis which is catalyzed by soluble glucose-degrading enzymes. The two pathways of energy generation have three features in common: (a) the oxidation step takes place first, independent of phosphorylation; (b) the energy of oxidation is conserved in a high-energy intermediate (X∼Y); and (c) a separate coupling device utilizes the energy of X∼Y for the dehydration of adenosine diphosphate (ADP) and inorganic phosphate to generate ATP. In the key step in glycolysis, the oxidation of glyceraldehyde-3-phosphate, the mechanism of energy conservation, and the formation of ATP are known (*1*). As shown in Table 1, the oxidation of the aldehyde leads to the formation of an acyl-enzyme. This nonphosphorylated, high-energy intermediate is cleaved by phosphate to yield an acyl-phosphate (1,3-diphosphoglycerate) which finally donates the phosphoryl group to ADP to yield ATP and phosphoglycerate. In the course of these reactions the oxygen is removed from inorganic phosphate and appears in the carboxyl group of the glycolytic intermediate 3-phosphoglycerate.

In contrast, the mechanism of oxidative phosphorylation is unknown. There are basically two major hypotheses: the chemical and the chemiosmotic (Fig. 1). Some reviewers mention a third hypothesis, the conformational hypothesis which was first proposed by Boyer (*2*). In principle this hypothesis is a useful and interesting variant of the chemical hypothesis, proposing that the energy of oxidation is conserved in a conformational alteration of a protein rather than in a new chemical bond.

The difference between the chemical and chemiosmotic hypotheses is more fundamental. The chemical hypothesis (cf. *1*) is modeled according to the mode of action of glyceraldehyde-3-phosphate dehydrogenase. Its current formulation involves three high-energy intermediates: a nonphosphorylated intermediate of the oxidation chain (A∼X) which arises by oxidation of the substrate ($A_{reduced}$); a second nonphosphorylated intermediate (X∼Y) without a respiratory component; and a third phosphorylated intermediate (X∼P). In the chemiosmotic hypothesis of Mitchell (*3*) the key feature is a translocation of protons from one side of the membrane to the other, which takes place during mitochondrial oxidation of substrates. This translocation gives rise to a proton gradient and a membrane potential which generates the formation of a high-energy intermediate X∼Y as discussed in detail in several reviews (*3–5*). The process of energy conversion from X∼Y to ATP is also formulated somewhat differently in the chemiosmotic hypothesis, the major differences being that it is linked to proton translocation in the direction opposite to that occurring during oxidation, and it does not involve formation of a phosphorylated high-energy intermediate.

The most fundamental difference between the two hypotheses lies, however, in the steps leading to X∼Y formation. Are the proton movements which have been shown to take place during oxidation primary or secondary to the formation of X∼Y? This question is difficult to answer because in both formulations the high-energy intermediate X∼Y is in equilibrium with ion gradients. This means that X∼Y can produce an ion gradient and an ion gradient can generate X∼Y. Indeed there is experimental evidence that either a proton gradient (*6*) or a potassium gradient (*7*) can give rise to ATP formation. The basic question which therefore remains to be answered is whether the proton gradient and formation of a membrane potential are essential for oxidative phosphorylation as proposed by Mitchell (*3*), or whether the proton pump is linked "in parallel" to the phosphorylation process as formulated by the chemical hypothesis. It should be pointed out, how-

Efraim Racker, professor of biochemistry and molecular biology at Cornell University, received his M.D. degree at the University of Vienna in 1938. An early interest in psychiatry led him to studies of the central nervous system and of alterations in the energy metabolism of brains of mice infected with poliomyelitis. Realizing that an understanding of the basic mechanisms is necessary for the intelligent analysis of problems of applied medicine, he began to investigate the fundamental reactions responsible for the generation and control of biological energy. He first studied simple soluble enzymes and is now investigating the complex membrane-bound multienzyme systems of mitochondria. For the past fifteen years he has also maintained an active interest in the control of energy metabolism in tumors and has published several papers on this subject. The present article is based on his Sigma Xi National Lecture, given in 1971. *The original work was supported by a grant from the Cancer Institute of the National Institutes of Health (CA 08964). The author wishes to acknowledge the collaboration of many colleagues in the recent work discussed in this article, particularly Drs. J. Fessenden-Raden, Y. Kagawa, A. Loyter, R. L. Christiansen, W. Arion, C. Burstein, and D. Schneider.* *Address: Wing Hall, Cornell University, Ithaca, N.Y. 14850.*

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ever, that in either case the translocation of protons is important in mitochondrial metabolism because of the role it appears to play in the regulation of electron flow. This phenomenon of respiratory control will be discussed later.

What is the current evidence for and against the chemical and the chemiosmotic hypotheses? As discussed in detail elsewhere (*5*), it is not possible at the present time to make an objective decision. Some experiments seem to be more readily explained by the chemical hypothesis while others support the chemiosmotic hypothesis. Few crucial experiments permit an unambiguous decision. For example, if oxidative phosphorylation could be demonstrated in a soluble system, the chemiosmotic hypothesis would have to be abandoned. Such a system was recently claimed to exist (*8*), but thus far has not been reproducible in several other laboratories. Needless to say, if operative, it would represent a major breakthrough in the field.

The chemiosmotic hypothesis requires not only a membrane but a closed compartment which permits separation of inside and outside, and the formation of a proton gradient and of a membrane potential. It also requires an asymmetric organization of the respiratory chain across the membrane, resulting in proton translocation from one side to the other as illustrated in Figure 2. If it could be unambiguously shown that electron transport takes place on the membrane surface, rather than across the membrane, again the chemiosmotic hypothesis would have to be abandoned. Such findings are not available and in fact experiments on the topography of the respiratory carriers which I shall discuss later reveal an asymmetric organization. In contrast, the chemical hypothesis does not require either an asymmetric organization of the membrane or a vesicular structure. As shown in Figure 2 a linear arrangement of the electron transport chain without associated proton translocation should suffice.

Experimental approaches

If we survey the history of the analysis of biochemical pathways, we can see that a full understanding has come about only after multienzyme systems have been resolved into the individual components. We have therefore set out to separate the individual catalysts of the mitochondrial membrane which participate in the oxidation of substrate from the components of the coupling device which are required for the formation of ATP from X∼Y. Although the coupling system is quite labile, its resolution proved to be easier than the separation of the catalysts of the oxidation chain. The reason for this is interesting in itself. Most of the components of the coupling device are proteins (coupling factors) that are located on the side of the inner mitochondrial membrane which faces the matrix (M-side). After the preparation of submitochondrial particles from mitochondria disrupted by sonic oscillations, this side faces the medium, and the coupling factors which are attached to this surface are readily accessible, as illustrated in Fig. 3. Some of the components of the oxidation chain are, however, more firmly embedded in the phospholipids of the membrane (e.g. cytochrome oxidase). Some are on the M-side (e.g. succinate dehydrogenase), while others (e.g. cytochrome *c* and cytochrome c_1) are on the C-side, which faces the outer membrane in intact mitochondria.

There are two experimental approaches to the problem of resolution and reconstitution. One we refer to as resolution from without. In this procedure, the intact membrane is exposed from the outside to chemicals or enzymes which attack surface components. This method, which has been used mainly for the resolution of coupling factors, has the advantage that it is relatively mild and need not affect the basic structure of the membrane. A second advantage is that because of its mildness, the resolved membranes can be used to assay the resolved components. Reconstitution of functional activity can be achieved by simple addition of the soluble, purified coupling factors to the resolved membrane. A third advantage is that the procedure can give information about the topography of the membrane, since (dependent on the extent of exposure) surface components can be resolved sequentially (*9*). A distinct disadvantage of this approach is that it is limited to surface components.

The second experimental approach involves disintegration of the membrane by detergents and separation of individual components by chemical and physical means. This method suffers from the severe disadvantage that purification of the components must often be carried out without the aid of a good biological assay. Instead, nonfunctional assays which depend, e.g., on the absorption spectrum of the component are being used. This can lead to the isolation of

Table 1. Mechanism of glyceraldehyde-3-phosphate oxidation

1. Aldehyde + DPN + Enzyme ⇌ Acyl-enzyme + $DPNH_2$
2. Acyl enzyme + Phosphate ⇌ Acyl-phosphate + enzyme
3. Acyl phosphate + ADP ⇌ Acid + ATP

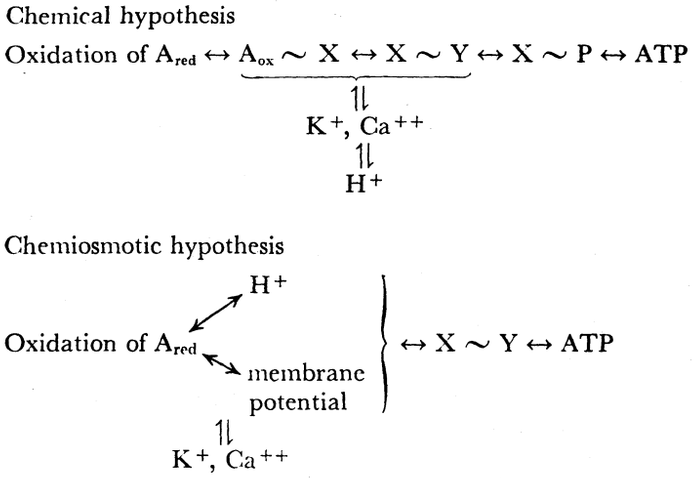

Figure 1. The two hypotheses of oxidative phosphorylation.

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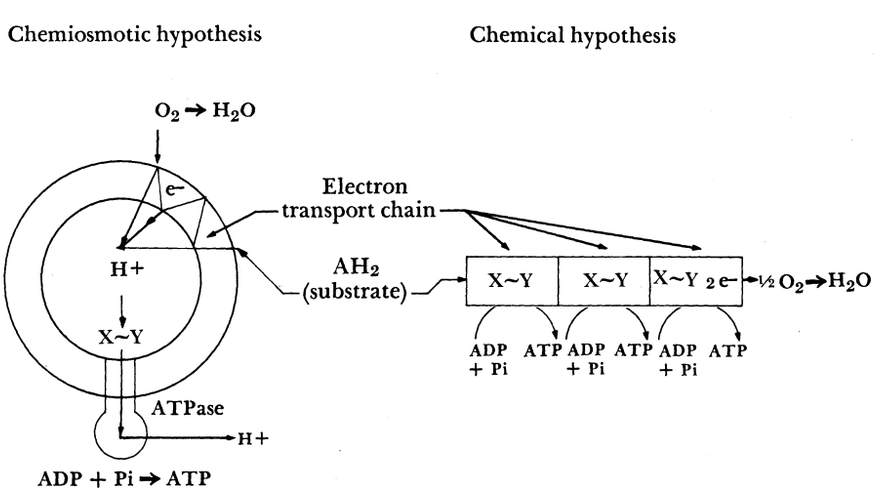

Figure 2. Anatomy of the chemiosmotic and and chemical hypotheses.

damaged components as will be shown.

Resolution from without. Outlined in Table 2 are two of the procedures most extensively used in our laboratory. Also shown in this table are the coupling factors that are required to couple phosphorylation to oxidation. Sonic oscillation of mitochondria at pH 9.2 in the absence of salt yields submitochondrial particles that we call A-particles (alkali-particles). We have made them either in the presence of EDTA (*10*) or in its absence and find little difference. We therefore prefer not to call them EDTA-particles as they have been referred to by others (*11*). The key condition for their preparation is an alkaline pH combined with low ionic strength of the medium. As pointed out previously (*10*) these particles are only partially resolved with respect to coupling factors. In order to resolve the coupling factor more completely, A-particles were treated with Sephadex and then with urea (*12*).

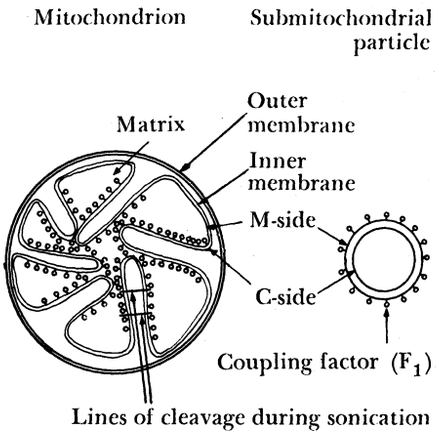

Figure 3. The sidedness of mitochondria and submitochondrial particles.

The resulting ASU-particles catalyzed oxidation but not oxidative phosphorylation.

Addition of coupling factors fully restored oxidative phosphorylation. As shown in Table 3, P:O ratios of 1.6 or higher were obtained with succinate as substrate. With DPNH as substrate, P:O ratios between 2.5 and 3.0 were observed. An alternative and simpler procedure to remove coupling factors is to treat A-particles with silicotungstate (*13*). These STA-particles are even more depleted than ASU-particles. They require more coupling factors and longer periods of incubation for optimal reconstitution. P:O ratios with DPNH were usually not higher than 1.0, but the major value of the STA-particles is that they serve for the assay of F_2 and F_6, while ASU-particles are conveniently used for assay of F_1 and of OSCP (F_3 + F_5) as shown in Table 3.

Four coupling factors have been isolated in a reasonable state of purity. Coupling factor 1 (F_1) is the mitochondrial ATPase (*14*). Added to ASU-particles alone, F_1 does not restore phosphorylation, but in electron micrographs, complete restoration of the inner membrane spheres to the submitochondrial particles can be seen (*12*). The second coupling factor (F_2) has recently been shown to be identical with factor B (*15*). OSCP (*16*) is a highly purified protein which can substitute for F_3 and F_5 (*13*), and F_6 is a highly purified, heat-stable coupling factor (*17*).

We know little about the function of these coupling factors, but one clue is the role they play in conferring oligomycin sensitivity to the ATPase activity of membrane-bound F_1. Membrane fragments which have been exposed to alkali and to silicotungstate do not induce this allotopic property to added F_1. On addition of two mitochondrial proteins (F_{c_1} and F_{c_2}) to these membrane fragments, oligomycin-sensitivity is induced (*18*). F_{c_2}, which is identical with or closely related to F_6, appears to be required for the binding of F_1 to the membrane. F_{c_1}, which can be replaced by OSCP, interacts with F_1 in the membrane in such a manner that ATP hydrolysis does not take place unless phospholipids are added (*19*). It was also shown that F_1 and OSCP are required for respiratory control (*20*) and for proton translocation (*21*).

These findings therefore appear to support Mitchell's formulation of a role of F_1 in proton translocation. The function of OSCP (F_{c_1}) may be to block the water site of membrane-bound F_1 (*19*) and, in the presence of phospholipid vesicles, to assist in the process of proton translocation.

Resolution by disintegration of the membrane. Many components of the respiratory chain have been purified after disintegration of the mitochondrial membrane with detergents and solvents. Assays during purification were dependent either on catalytic oxido-reductions with artificial electron donors and acceptors, or on spectral properties of the reduced or oxidized chromophore attached to the protein. Although some of these procedures have yielded proteins of high purity, there is no assurance that the enzymes have not undergone severe alterations. An excellent case in point is succinate dehydrogenase, which can be purified either in the absence or presence of succinate. However, only the latter preparation recombines with the mitochondrial membrane to reconstitute an active succinate oxidase complex (*22*). Since succinate dehydrogenase is a surface component of the M-side of the inner membrane, the reconstitution of this enzyme after resolution with silicotungstate requires no more than simple addition to depleted submitochondrial particles (*13*).

The problem is much more difficult in the case of respiratory enzymes which are embedded in the membrane or are localized on the C-side of the

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membrane. It was therefore a decisive advance in this area of research when the resolution of respiratory components and their reconstitution to an active succinoxidase complex were achieved (*23*). With this system as an assay, the individual components were further purified and a new component required for the operation of the respiratory chain was discovered (*24*). In spite of numerous efforts, it has not as yet been possible to obtain oxidation coupled to phosphorylation in the reconstituted succinoxidase complex even when coupling factors and oligomycin-sensitive ATPase were included during reconstitution. (While this paper was in press a successful reconstitution of an asymmetric membrane which catalyzes oxidative phosphorylation was achieved [*49*]).

Topography of the inner mitochondrial membrane. Two possibilities were considered to explain this failure. Either we were still missing an unknown component or the reconstitution procedure was faulty, resulting in an assembly of respiratory carriers that could catalyze oxidation but be incapable of energy conservation. The latter possibility is particularly probable if Mitchell's formulation, which requires an asymmetric assembly of the respiratory carriers, is correct. We therefore initiated a systematic analysis of the topography of the inner mitochondrial membrane with respect to both proteins and phospholipids. Since the details of these studies have been reviewed elsewhere (*25*, *26*) a brief summary accompanying Figure 4 should suffice.

The first and clear image is that of asymmetry. Succinate dehydrogenase is located on the M-side of the membrane together with the known coupling factors. Cytochrome *c* and cytochrome c_1 are located on the C-side of the membrane. Cytochrome oxidase is on both sides of the membrane. The location of cytochrome *b* has not been established, but since it is functionally located between succinate dehydrogenase and cytochrome c_1 it may be placed across the membrane as indicated in the figure. Alternatively, in view of our insufficient knowledge of this segment of the respiratory chain, there is room here for another "loop" of the respiratory chain. In any case, it is apparent that the catalysts of the respiratory chain are organized asymmetrically across the membrane, consistent with their proposed function in the translocation of protons and formation of a membrane potential (*3*).

Table 2. Resolution from without

1. Submitochondrial particles
   - Step 1 Mitochondria sonicated at alkaline pH → A-particles
   - Step 2A A-particles treated with Sephadex and urea → ASU-particles
   - Step 2B A-particles treated with silicotungstate → STA-particles
2. Coupling factors
   - F_1 (mitochondrial ATPase)
   - F_2 (factor B)
   - $F_3 + F_5$ (OSCP or F_{c_1})
   - F_6 (F_{c_2})

Table 3. Reconstitution of ASU-particles and STA-particles with coupling factors

ASU-particles	P:O (succinate)	*STA-particles*	P:O (DPNH)
Complete*	1.6	Complete**	1.06
Omit F_1	0.1	Omit F_2	0.16
Omit $F_3 + F_5$	0.15	Omit F_6	0.23

* with F_1, F_2, $F_3 + F_5$

** with F_1, F_2, OSCP, and F_6

Role of phospholipids in oxidative phosphorylation. Our studies on the role of phospholipids in oxidative phosphorylation (*27*, *28*) have considerable bearing on the controversial structure of membranes. Since up to 70 percent of the phospholipids can be hydrolyzed by phospholipase C under conditions of functional integrity of the membrane, and since a similar portion is sensitive to attack by phospholipase A, it is apparent that a large fraction of the phospholipids is on the surface of the membrane. Since the phospholipids are susceptible to enzymes which attack either the hydrophobic or the hydrophilic portion of the molecules, there must be considerable mobility of the phospholipids within the membrane, an assumption born out by spin label studies of Kornberg and McConnell (*29*). On the other hand, there is a core of phospholipids that cannot be digested under functional conditions of the membrane which provides limits to the mobility of phospholipids in the membrane and also provides evidence for the lack of penetration of the lipases into the membrane. Recent experiments in our laboratory have furthermore shown that these core-phospholipids, including cardiolipin, can be digested by phospholipase C from *Bacillus cereus* after disintegration of the membrane with cholate.

A critical advance in the role of phospholipids has been made recently by the chemical separation of hydrophobic membrane proteins from phospholipids (*28*) and reconstitution of a membrane capable of catalyzing $^{32}P_i$-ATP exchange. Again consistent with the chemiosmotic hypothesis is the finding that only after slow assembly and formation of a vesicular membrane is the capacity of energy transduction restored.

Bioenergetics and the cancer problem

Otto Warburg, who first discovered the presence of a high rate of aerobic glycolysis in tumor cells, died two years ago. Few will challenge the statement that Warburg was one of the great biochemists. His experimental approach was monumental and ingenious. Yet Warburg's views in the two vital areas of his research interest, cancer and photosynthesis, are now almost universally dismissed as erroneous or naive. There may be a clue to this curious phenomenon in the personality of this great scientist. Almost twenty years ago Warburg wrote a paper on the mechanisms of action of glyceraldehyde-3-phosphate dehydrogenase (*30*), which was en-

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tirely devoted to an attack on "Racker's *Umweg*" (detour). This is what he called our formulation of the oxidation of glyceraldehyde-3-phosphate via a thiolester intermediate of the enzyme. I remember well how I reacted when I first read this paper—with a mixture of surprise and annoyance, but distinctly flattered that this great scientist should attack me, a young and obscure biochemist.

Warburg's formulation of the mechanism of glyceraldehyde-3-phosphate oxidation was simple and ingenious: a phosphate combines with the aldehyde group which is oxidized to the acyl phosphate. He could not accept an alternative mechanism which he considered unnecessarily complex, and he clearly did not bother to examine the experimental evidence in its favor. Although I chose not to respond to the attack, I became interested in Warburg's mind and in some of his other polemics. An even more extraordinary example of Warburg's train of thought was published in the field of photosynthesis (*31*). An experiment which did not conform to his predictions, but was performed in his own laboratory, failed to persuade him to abandon his hypothesis. Instead, he arrived at the conclusion that the accepted view of the chemical structure of chlorophyll must be erroneous!

It is mainly because of Warburg's tenacity and commitment to his own convictions that there is hardly a biochemist who now takes his broad views of photosynthesis and cancer very seriously, and I have often pointed out to students the danger of committing oneself too rigidly to a hypothesis. Indeed, I try to persuade them that it is more stimulating to work on several hypotheses at the same time even though they may be contradictory. It is not a matter of being right or wrong but of learning to listen to nature.

Warburg's contributions to the cancer field reveal again his brilliant and yet rigid mind. His experiments were decisive and his discoveries impressive. All tumors which he examined showed a characteristic pattern of energy metabolism (*32*). In contrast to most "normal" mammalian cells, tumors exhibited a high aerobic glycolysis. The regulatory inhibition of glucose utilization, known as the Pasteur effect, appeared to be defective. Warburg concluded that cancer is caused by a defect in the respiratory chain which results in a reversion of the cell to the more primitive bioenergetic pathway resembling that of fermenting yeast. Again, experimental evidence that did not fit, such as the presence of a normal respiration chain in some tumors, the presence of aerobic glycolysis in some nontumor tissues, and later data indicative of normal pattern of glycolysis in slow-growing Morris hepatoma (*33*), was either dismissed or ignored. Nevertheless, I shall proceed now with the proposition that what I refer to as the Warburg effect—the presence of high aerobic glycolysis in tumors—deserves more attention in the field of cancer research than it has received in recent years.

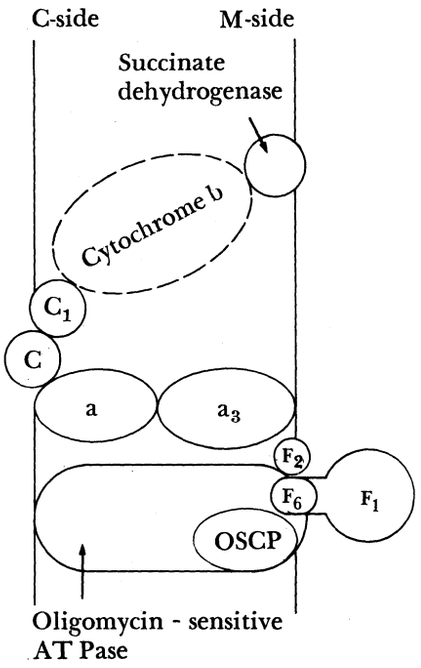

Figure 4. The topography of the inner mitochondrial membrane.

The new hypothesis

My hypothesis of tumor growth centers around the findings of Warburg as a significant feature of cancer pathogenesis. Stated simply, I propose that tumors can be caused by a multitude of different primary lesions, which all have in common the ability to cause a persistent alteration in the intracellular pH (and perhaps also temperature), thereby upsetting the normal regulatory mechanism that prevents uncontrolled growth.

This hypothesis differs from Warburg's in two significant respects. Warburg proposed that the primary lesion is in the respiratory chain followed by the emergence of a fermenting pathway of energy supply. An important feature of the new hypothesis is that it predicts a *variety* of possible primary lesions, which can be induced by viruses or carcinogens or mutations, all of which give rise to a persistent change in pH. Thus aerobic glycolysis is viewed as a secondary phenomenon, most significant because of its frequent occurrence, but neither essential nor sufficient to cause tumors, as will be elaborated. The second difference is that the emphasis is no longer placed on the emergence of a fermenting bioenergetic pathway (although this may be among the contributing factors) but on the disturbances in regulatory mechanisms of biosynthetic processes caused by changes in pH. Moreover, the new hypothesis has the attractive feature that it suggests new experimental approaches and has clear implications for cancer chemotherapy. The following aspects are relevant to the new hypothesis.

The Warburg effect. Production of lactic acid in vitro and in vivo is a very common feature in tumors. Moreover, there appears to be a rough correlation between the degree of malignancy and rate of glycolysis (*34*). In a way, the slow-growing Morris hepatoma supports this correlation.

The Pasteur effect. Before we can evaluate the significance of the Warburg effect, we have to discuss some aspects of regulatory mechanisms of carbohydrate metabolism. Glucose utilization is controlled by the phenomenon known as the Pasteur effect. Stated in simple terms, the operation of oxidative phosphorylation in mitochondria results in an inhibition of glucose utilization. That phosphorylation rather than oxidation is important in this control mechanism is documented by the fact that uncouplers of oxidative phosphorylation, which abolish ATP generation but not respiration, eliminate the inhibition. There is a vast literature on the mechanism of the Pasteur effect in cells. A similar control mechanism has been duplicated in reconstructed cell-free systems (*35*).

From these and other experiments, the following view of the Pasteur effect has emerged (*1*). The enzymes of glycolysis can metabolize glucose only when (a) inorganic phosphate and

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ADP are available for the oxidation of glyceraldehyde-3-phosphate, and (b) the ATP:ADP ratio and the concentration of inorganic phosphate and other allosteric effectors of glycolytic enzymes are suitable for catalytic action. The multiplicity of these allosteric control mechanisms is remarkable (*1*), and I will mention just a few. Phosphofructokinase is inhibited by a high ATP:ADP ratio and stimulated by phosphate. Hexokinase is inhibited by glucose-6-phosphate which accumulates when phosphofructokinase is inhibited by high ATP. Inorganic phosphate counteracts the inhibition by glucose-6-phosphate. Inorganic phosphate also regulates the rate of glyceraldehyde-3-phosphate oxidation which is controlled by the DPN:DPNH ratio. The latter in turn is dependent on the availability of pyruvate or of another hydrogen acceptor. Pyruvate cannot be formed from phosphoenolpyruvate unless ADP is available. We see that ATP, ADP, and inorganic phosphate serve as allosteric effectors and regulators of substrate flux at several steps of glycolysis (Fig. 5).

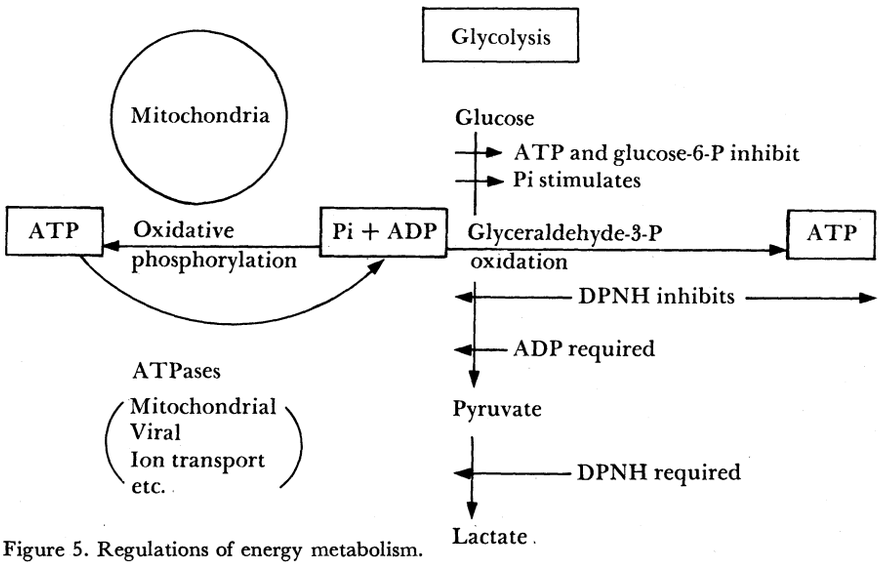

Figure 5. Regulations of energy metabolism.

In the presence of phosphorylating mitochondria and of a glycolytic system, there is an active competition for available inorganic phosphate and ADP. Oxidative phosphorylation can raise the ATP level to concentrations inhibitory for phosphofructokinase and it has a profound influence on the intracellular ATP:ADP:P_i ratio. Some years ago we made an experimental observation that greatly affected our thinking with regard to the importance of inorganic phosphate as a rate-limiting factor in glycolysis of ascites tumor cells (*36*). When we raised the external phosphate concentration to levels which significantly increased intracellular phosphate of the ascites cells, the rate of glycolysis was stimulated severalfold.

Oxidation control and ATPase activity. It is apparent from these considerations as well as from studies of reconstituted glycolysis (*37*) that the rate of ATP hydrolysis that generates phosphate and ADP controls the rate of glycolysis. ATP is hydrolyzed in the course of a large number of biosynthetic processes such as the synthesis of protein, nucleic acids, fats, and carbohydrates. ATP is hydrolyzed during energy-consuming processes of ion transport, muscular contraction, and other work. ATP can also be hydrolyzed by mitochondrial ATPase in the presence of natural uncouplers such as fatty acids. Yet living cells maintain a remarkably high concentration of ATP and are equipped with a powerful ATP-generating machinery which, for the sake of economy, must be effectively controlled.

Indeed, a very simple and ingenious mechanism regulating the flow of bioenergy operates throughout nature. It is based on a tight coupling of oxidation to the phosphorylation process. Oxidation does not proceed unless phosphorylation takes place, i.e. ADP and inorganic phosphate are available. In other words, oxidation is dependent on the utilization of ATP. In the case of mitochondrial oxidative phosphorylation, this ingenious device has been referred to as respiratory control. Since respiration is a term used exclusively for oxidations by molecular oxygen and since the same mechanism is operative in the oxidation steps of glycolysis and photosynthetic phosphorylation, it seems appropriate to introduce the more general term, oxidation control. The mechanism of oxidation control has been analyzed in the well-defined and soluble system of glycolysis and has been shown to include multiple sites of control (*1*). In fact, the complexity of the mechanism of glyceraldehyde-3-phosphate oxidation, which Warburg objected to, may well have evolved to satisfy the need for an effective control mechanism in the oxidation process.

Oxidative phosphorylation in mitochondria and substrate-level oxidative phosphorylation in glycolysis are (in contrast to photophosphorylation) readily reversible processes under physiological conditions. Thus, in the presence of an uncoupler, an agent which allows oxidation to proceed without generation of ATP, the coupling device goes into reverse, resulting in the hydrolysis of ATP. Natural uncouplers exist, e.g. long-chain fatty acids which uncouple oxidative phosphorylation in mitochondria. In the case of glycolysis, there is an enzyme that hydrolyzes 1,3-diphosphoglycerate. However, our knowledge of the action of these natural uncouplers under physiological conditions is very limited. In any case, I want to emphasize here that a breakdown in oxidation control of either mitochondrial or glycolytic oxidations gives rise to an increased hydrolysis of ATP and thereby stimulates aerobic glycolysis.

The role of pH and temperature. The hydrolysis of ATP to ADP and phosphate lowers the pH and increases the intracellular temperature. Relatively little attention has been given to the effect of these variables in allosteric control mechanisms. One example is the inhibition of phosphofructokinase by ATP which is markedly influenced by pH, the control becoming more effective at more acid pH (*38*). In terms of physiological control, this makes sense since it represents a feedback mechanism caused by glycolytic acid production. Even less is known about the effect of pH on biosynthetic processes and their control, particularly in the important areas of protein and nucleic acid biosynthesis, cell division, and growth. Limitations in acquiring

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knowledge in these areas are largely caused by experimental difficulties associated with, e.g., assays of DNA biosynthesis (not repair) in cell-free systems, as well as with the control and measurement of intracellular pH in intact cells. But even experimentally feasible approaches have been neglected. For example, it is surprising how little information has been available until recently on the effect of extracellular pH on the growth rate of cultured mammalian cells. The remarkable effects achieved by maintaining the pH in the cell culture medium on the growth of normal and tumor cells (*39*) are indeed most interesting. Unfortunately, it is not possible at the present time to interpret these data in terms of changes of intracellular pH. Some, if not all, of the observed effects may be due to influences on the surface of the cell membrane. This does not detract from the significance of these findings. I shall return to the important role of surface components in tumor cells.

Mechanisms of acid production. We have proposed as part of our hypothesis that a persistent change in pH (and temperature) alters intracellular conditions in such a manner that control mechanisms of growth and replication are disturbed. A lowering of pH can be caused by increased aerobic glycolysis. Some tumor-inducing viruses have ATPase activity (*40*). This activity of the virus may contribute to the pool of available ADP and phosphate and induce an increased glycolytic rate. An increased acid production could take place, however, completely unrelated to either glycolysis or ATP hydrolysis, e.g. by an increased rate of lipolysis. The observation of a low rate of glycolysis in Morris's hepatoma (*33*) represents a challenge to search for other acid-producing reactions in these tumors.

Stimulation of glycolysis could be caused by a large number of different primary lesions. In addition to the introduction of ATPase by a virus, ATPase activity could be elicited, e.g., by a mutation which leads to an alteration of the protein that controls mitochondrial ATPase (*41*). If this protein is either missing or altered, ATP generated by the mitochondria may be hydrolyzed by mitochondrial ATPase unless it is utilized in other ATP-dependent reactions. ATPase activity could take place if the mitochondrial ATPase which appears to be synthesized on cytoplasmic ribosomes (cf. *42*) is lost into the cytoplasm by a defect of the mysterious transport mechanism, which deposits the enzyme on the inner surface of the inner mitochondrial membrane. Emergence of ATPase activity could take place by degeneration of a phosphotransfer enzyme such as hexokinase (*43*) or argininosuccinate synthetase (*44*), both of which are known to slowly hydrolyze ATP. A defect in an ion-transport system leading to wasteful pumping represents yet another way of increasing ATP hydrolysis. A search for other ATPases in the cell may be fruitful.

However, as mentioned earlier, an increased glycolysis could be induced without increase of ATPase activity by increasing the flow of inorganic phosphate into the cell. We have observed considerable differences in the phosphate-transport properties of various cells (cf. *1*), and further exploration of this transport system seems indicated. A lowering of the natural barrier to phosphate or stimulation of a restrained active phosphate-transport system are among the possibilities that may induce an increased flux of phosphate into the cell due to alteration of the cell membrane. There is a rapidly growing literature on the surface properties of cells and the changes that lead to agglutination by plant extracts and other agglutinins (*45*, *46*). We are considering at present a possible correlation between these surface changes and phosphate transport.

In summarizing, I first want to emphasize one aspect of the new hypothesis that could be easily misunderstood. We propose that in tumor cells persistent pH changes are induced either by glycolysis or by another system, resulting in the release of control mechanisms which govern biosynthesis and division in normal cells. Although induction of a permanent pH change thus becomes an essential common denominator, it should be obvious that by itself it cannot be sufficient for tumor growth. A cell that has lost part of its biosynthetic or mitotic apparatus, or a cell that has a wider pH range of control, could well tolerate changes in pH without becoming a cancer cell. If acid formation takes place in cells with little tolerance for pH changes, or if changes in pH are so extensive as to be incompatible with survival, the result will be cell death rather than tumor growth. In view of all these considerations, we are prepared to find exceptions to the correlation between lactic acid production and the emergence of a malignant tumor.

This new hypothesis, which has built-in excuses on both its loose ends, might not sound so attractive were it not for the fact that it can stimulate numerous interesting experiments and therapeutic approaches. A renewed search for glycolytic inhibitors that are less toxic than iodoacetate or fluoride might well be indicated. A promising candidate is oxamate, an inhibitor of pyruvate reductase (lactate dehydrogenase), which has been explored as an inhibitor of tumor growth (*47*). More fundamental investigations are required to elucidate the specific mechanism of increased glycolysis in various types of tumors. Our approach to the problem of chemotherapy should be different, depending on whether the increased glycolysis is caused by a virus ATPase or by an endogenous mitochondrial ATPase or by a soluble cytoplasmic enzyme. In each case one would hope to design a specific agent directed against the undesirable enzyme. One would certainly have to approach the problem differently when changes in phosphate transport are responsible for an increased rate of glycolysis.

The hypothesis calls for more intensive research on methods to measure intracellular pH and on its effect on biosynthetic and mitotic processes. It should stimulate more systematic studies of the effect of external pH on growth in cell cultures and on the properties of surface components that are influenced by such changes. The hypothesis should also stimulate exploration of regulation of carbohydrate metabolism by agents which appear to influence cell morphology of tumor cells, such as that recently reported for cyclic adenosine monophosphate (*48*).

Considering its potential, I feel that this is a good hypothesis, worthy of a lot of hard work. Following our own good advice, it is not the only hypothesis we entertain. Should this hypothesis cease to feed us with new ideas for experiments, we shall turn to another one. It is as simple as that: we like learning better than dreaming.

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