

Determinants of the Specificity of Behavioral Effects of Drugs*

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With 9 Figures

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I. Introduction

Pharmacology has always been characterized by close relations with other biological sciences; it has freely borrowed from other sciences, and it has generously contributed to them. The diverse effects of drugs and ways of studying them have caused a continuous increase in the number of specialized branches of pharmacology. Historically this tendency to encompass aspects of other disciplines has been important for pharmacology. The present review will be concerned with behavioral pharmacology, a relatively new branch of pharmacology concerned with the effects of drugs on behavior. Over the past ten years, interest in this field has been stimulated by discoveries of new drugs which are useful clinically in modifying human behavior. The goal of behavioral pharmacology is to provide by means of experimental studies a coherent account of the action of these drugs. The purpose of this review is to discuss characteristics of behavioral pharmacology as a basic scientific discipline.

Behavioral pharmacology must be concerned with both drugs and behavior. There are many problems that are common to all branches of pharmacology. For example, the quantitative effect of a drug usually depends upon the dose that is administered; a large enough dose of any drug would be sure to have behavioral effects. The behavioral pharmacologist must determine characteristic relations between drug doses and behavioral effects. Only when such dose-effect relations have been clearly established is it possible to compare the effects of several drugs or to determine how the effects of a given drug are specific.

In studying the actions of drugs on behavior, the behavioral pharmacologist uses techniques and concepts that have come from experimental psychology. In the last quarter century, experimental psychologists have developed objective and quantitative techniques appropriate for conducting basic pharmacological studies on behavior. Because the subject matter of behavior is new to pharmacology, the behavioral pharmacologist necessarily uses procedures and technical terms that may seem strange to other pharmacologists. The strangeness of the concepts may have diminished the interest of many pharmacologists in behavioral pharmacology and given the impression that behavioral pharmacology is somehow remote from other branches of pharmacology. Although new concepts emerge when pharmacological principles are applied to new subject matters, accepted pharmacological criteria should always be used in evaluating the actions of drugs. A critical survey of the literature in behavioral pharmacology indicates that it is neglect of basic pharmacological principles more than any inherent difficulty in studying behavioral effects of drugs which leads to seemingly inconsistent results and contradictory interpretations of results.

An outstanding feature of behavioral pharmacology is that the behavioral effects of drugs are largely determined by the environmental circumstances. Such dependence is not qualitatively unique; the effects of a drug on any

biological system depend upon the state of the system. For example, the effect of epinephrine on blood pressure depends in part on the height of the blood pressure when the drug is administered. The effects of past circumstances are, however, much greater in behavioral pharmacology. The profound effects of environmental factors have not been fully recognized because physiological and pharmacological experiments on intact animals usually attempt to hold environmental determinants constant and thereby obscure their importance. For behavioral pharmacology, however, the study of the nature of the environmental determinants of behavior is essential.

In behavioral pharmacology, as in other interdisciplinary fields, many serious difficulties in interpretation of results develop because results come from two different types of experiments: 1) those in which a behavioral procedure is used as a tool for analyzing effects of drugs; and 2) those in which a drug is used as a tool for analyzing behavior. Under ideal conditions, type 1 experiments would use thoroughly understood behavioral procedures to provide information about how drugs are acting; type 2 experiments would use thoroughly understood drugs to provide information about behavioral processes. In the brief history of behavioral pharmacology, it has been necessary to conduct both types of experiment simultaneously. Type 1 experiments have used arbitrary behavioral procedures, usually selected for their simplicity, to sort drugs into different classes. On the other hand, type 2 experiments have used representative drugs from different classes to learn more about other behavioral procedures which can subsequently be used in type 1 experiments. This complementary use of the different types of experiment has been essential for progress in behavioral pharmacology. It is apparent that behavioral pharmacology is advancing in an irregular fashion. Concepts are being continually modified by results from the complementary types of experiments just referred to.

A main difficulty has been the ready acceptance of seemingly plausible interpretations that went far beyond the experimental findings. For example, it was found that phenothiazine tranquilizers, such as chlorpromazine¹, decreased escape or avoidance responding (COURVOISIER *et al.*, 1953; COOK and WEIDLEY, 1957; VERHAVE *et al.*, 1958); that is, responding under procedures in which responses eliminated or postponed electric shock. Many writers interpreted these results as indicating that chlorpromazine specifically attenuated the effects of noxious stimuli on behavior, through a reduction of the fear or anxiety which they assumed to underlie such behavior. Subsequently, it was found that chlorpromazine did not attenuate the suppression of behavior by electric shock (GELLER *et al.*, 1962). Rather than questioning the original interpretation, many concluded only that the suppression procedure was a poor procedure for measuring effects of drugs on fear and anxiety. But minor tranquilizers, such as meprobamate, although relatively ineffective in decreasing

¹ Chemical names of drugs are given in the Appendix.

avoidance responding, can attenuate the suppression of behavior by electric shock (GELLER and SEIFTER, 1960). Assumptions about the nature of tranquilization in terms of fear and anxiety have led to inconsistencies that are irresolvable.

Another example of a plausible assumption that has been readily accepted is that amphetamine decreases responding maintained by food presentation through an anorexic effect. Yet the same dose of amphetamine also decreases comparable responding maintained by termination or postponement of electric shock. The suggestion of an anorexic action of amphetamine was reasonable, but it has been uncritically accepted on the basis of limited experimental evidence and without the demonstration of a specific effect on behavior maintained by food presentation.

A primary purpose of this review is to provide a framework that will enable other pharmacologists to evaluate the results and significance of experiments in behavioral pharmacology. The next section (II) deals with behavioral procedures and concepts, emphasizing the characteristics of behavior that are most relevant to an understanding of how drugs affect behavior. The subsequent sections will describe some of the general characteristics of behavioral pharmacology, and will present some of the general principles which are emerging from studies in behavioral pharmacology. These sections will describe some representative experimental results obtained with a number of different classes of drugs and will interpret these results in the light of the general concepts presented.

II. Characteristics of behavior

The scientific study of behavior poses many difficulties. One difficulty results, paradoxically, from our familiarity with numerous isolated facts about the behavior of ourselves, other people, and animals. The interpretations customarily given to these facts lead to preconceived opinions, which frequently interfere with the unbiased study of behavior. Moreover, behavior is essentially dynamic in the sense that behavioral processes reflect changes in the interactions between an individual and its environment which take place *in time*. Even the simplest relationships may not be readily apparent to casual observation. Finally, a pattern of behavior is the result of many interrelated factors, including environmental circumstances that have long since ceased to exist, thus posing special problems for identification and study.

A. Elicited responses

Studies in which behavior is to be used as a tool for investigating drugs often attempt to minimize the complexities of behavior by dealing with unconditioned responses. Two general classes of unconditioned responses are elicited or reflex responses and spontaneous or emitted responses. A reflex response characteristically occurs regularly and promptly when a specific elici-

ting stimulus is presented. Examples of reflex responses are salivation elicited by food placed in a dog's mouth and paw withdrawal elicited by an electric shock delivered to a dog's paw. Elicited responses can be objectively quantified in terms of the time between onset of stimulus and occurrence of response (latency), the stimulus intensity required to elicit the response (threshold), or the magnitude of the response. An essential characteristic of a reflex response is its close relationship with its eliciting stimulus; the reflex response depends on parameters such as the duration, intensity, and frequency of presentation of the eliciting stimulus. Reflex responses have been widely used in the assessment of the analgesic effects of drugs such as morphine. Reflex responses are also frequently used to assess the specificity of a drug effect on behavior; for example, it is useful to know that a drug will not affect reflex responses at dose levels that do affect other types of behavior. Reflex responses are an important part of behavior, but it should be recognized that all reflex behavior constitutes only a small fraction of the total behavior of any animal.

B. Pavlovian conditioning

The control of behavior can be extended by conditioning procedures. In Pavlovian conditioning, a neutral stimulus (one that does not initially elicit the response under study) is presented prior to an eliciting stimulus. After a few paired presentations of the two stimuli, the neutral stimulus itself may be followed by a response. For example, if a tone is sounded just before food is placed in a dog's mouth, the tone itself will come to be followed by salivation. The originally eliciting stimulus is called the unconditioned stimulus; the neutral stimulus is called the conditioned stimulus; and the response to the neutral stimulus is called the conditioned response. Conditioned responses can be measured in terms of their latency, magnitude, or frequency of occurrence. The rate of acquisition of a conditioned response depends on many factors, such as intensities of the stimuli and temporal relations between the stimuli. If the conditioned stimulus is repeatedly presented without the unconditioned stimulus, the conditioned response ceases to occur; this process is called experimental extinction. Typically, extinction is slower than conditioning. Although Pavlovian conditioning extends the range of stimuli controlling responses, it is limited to responses for which there is an initial eliciting stimulus.

C. Emitted responses

It is impossible to identify an eliciting stimulus for much of the behavior of an individual that can be predicted and controlled. To say that behavior occurs in the absence of an identifiable eliciting stimulus does not imply that the behavior is not determined, but simply that it does not have the functional properties of reflexly elicited behavior. For example, a food-deprived rat given access to a supply of small food pellets will eat for a period of time and then

cease to eat. If the rate of ingesting pellets is recorded, a simple and reproducible curve of eating is obtained, which describes the ingestion of food under these conditions (SKINNER, 1930, 1938). Although the rate of eating following food deprivation is a reproducible temporal process, it is not possible to analyze this behavior simply in terms of eliciting stimuli. Because the presentation of food is often immediately followed by ingestion, it may seem that the ingestion of food is elicited by the presence of food itself (presumably its sight and smell). But the rate of eating declines in time; the presence of food does not continue to have the same preemptiveness. Thus, it is necessary to invoke some other factor, such as deprivation (hunger drive), operating in conjunction with the sight and smell of food. The deprivation of food critically determines rate of eating and also changes other classes of responses in a reproducible way, but it is not an eliciting stimulus in the sense in which the term is used in reflex physiology.

The occurrence of emitted behavior generally bears a temporal relation to the deprivation and presentation of particular environmental conditions, whether or not the deprivation produces any conspicuous physiological change. For example, if a rat is confined in a small space and then given access to a revolving wheel, it will run for some time and then gradually cease to run. As in the example of the rat eating, a record in time of the running behavior will reveal that the running has a characteristic temporal pattern. After deprivation of access to the wheel, the availability of the wheel is closely followed by the running of the rat, yet the running is not controlled by the presence of the wheel as an eliciting stimulus.

It is customary in psychology to refer to any movement as a "response." Thus, spontaneously emitted "bits of behavior" are called responses even though they are not elicited by an identifiable stimulus. In studying the occurrence of emitted responses in time, it is clearly desirable that particular instances be easily identified, reproducible, and functionally significant. The exact criterion for specifying functionally significant emitted responses will be taken up in the next section.

One approach to the study of emitted responses is to try to observe all of an animal's responses during a specified period of time. Because it is difficult to quantify such data objectively, investigators often develop rating scales and elaborate scoring techniques, but detailed ratings have the disadvantage that independent replication of results is difficult (DEWS and MORSE, 1961). Such observational procedures sometimes provide clues to variables that are influencing behavior; they can also be very useful in an initial assessment of the effects of a drug.

Motor activity can be studied objectively and quantitatively with photocell chambers or "jiggle" cages which automatically record the level of such activity. Measurements of motor activity have been widely used in assessing the

effects of drugs. Levels of motor activity depend on factors such as time of day, degree of food or water deprivation, and number of exposures to the testing chamber. Problems with studying such behavior are that the variables influencing it are poorly understood, difficult to control, and of limited generality.

D. Operant conditioning²

Future behavior is mainly determined by the consequences of past behavior. In operant conditioning, the occurrence of a particular type of event (a reinforcer) immediately following a response increases the subsequent frequency of occurrence of responses of the same kind. To return to the earlier example, a food-deprived rat begins eating when food is presented not because the food elicits eating, but because this behavior, or similar behavior, has occurred in the past. The principles of operant conditioning can be demonstrated with the same rat in an apparatus containing a food dispenser and a lever projecting from the wall. Depression of the lever closes a switch, which operates the food dispenser. Any response of the rat which depresses the lever will be followed by the presentation of food. Under such circumstances a lever-pressing response is said to be "reinforced," and the likelihood that a similar response will occur again is increased. The consequence defines the property with respect to which responses are called similar; the word "operant" is used by SKINNER (1937, 1938) to describe this functionally identifiable class.

If only depressions of the lever exceeding a certain force are followed by food presentation, weaker responses diminish, and stronger responses become more frequent and can be selected through further differential reinforcement. In the example, food is the reinforcer and presenting food when the response is emitted is the reinforcement. The operant is defined by the property upon which reinforcement is contingent — the depression of the lever with a certain minimal force. The change in frequency of the operant is the process of operant conditioning. Under appropriate conditions, the effect of a single presentation of a reinforcer can be demonstrated as an immediate increase in frequency of an operant response (SKINNER, 1938). The effects of reinforcement persist in time; if the response is no longer followed by a reinforcer, its frequency of occurrence will only slowly decline (experimental extinction).

Operant conditioning is important in that complex human behavior develops as a result of past experience. Operant conditioning is also a phenomenon of great generality. Although some response classes are more readily modified by reinforcers than others, the range of behaviors that can be controlled by operant conditioning is vast. In restricted experimental situations in which an arbitrary response is intermittently followed by an effective reinforcer, the frequency of occurrence of that response is a useful and significant dependent

² This brief account of operant conditioning is based on some of the writings of B. F. SKINNER (1937, 1938, 1953a, b, 1965, 1966).

variable which is a function of many subtle experimental conditions. In practice, each of the response classes most commonly selected for experimental study meets four criteria: it is so easily identified that repeated instances can be reliably counted; it is easily recorded with automatic equipment; its occurrence requires little time; and it is readily repeatable. The kinds of responses which meet these criteria usually operate a small electrical switch (key); for example, a key for the pigeon is a translucent circular plastic disc mounted vertically, and a key for the rat or monkey is a horizontally projecting lever.

Although Pavlovian and operant conditioning procedures have certain common characteristics, there are basic differences between them, which should be noted. Pavlovian conditioning is concerned with responses elicited by stimuli (for example, salivation), while operant conditioning is concerned with the rate of occurrence of emitted responses (for example, pressing a key). In Pavlovian conditioning, the temporal sequence of conditioned stimulus followed by unconditioned eliciting stimulus occurs independently of the animal's behavior. On the other hand, the presentation of the reinforcer in operant conditioning is necessarily related to behavior; that is, the reinforcer follows the emission of a response. Some ways in which discriminative stimuli modify responding under operant conditioning will be described in Section II G. The present review is primarily concerned with the effects of drugs on operant behavior, and to provide a framework for this discussion, the following sections will describe in more detail characteristics of operant behavior.

E. Reinforcers

Reinforcement is a central concept in operant conditioning. An event is identified as a reinforcer when it follows a response and there is a subsequent increase in the occurrence of similar responses; the presentation of a reinforcer following such responses is called reinforcement. Although it is usually arranged that a specified response actually produces a reinforcer, other responses that happen to precede a reinforcer may subsequently occur more frequently (adventitious reinforcement) (SKINNER, 1948). Some events function as reinforcers when they are presented (for example, food); others function as reinforcers when they are terminated (for example, electric shock). (An event which can sustain behavior that precedes its *presentation* is often called a positive reinforcer; an event which can sustain behavior that precedes its *termination* is often called a negative reinforcer.) Events that have been used as reinforcers in experiments in behavioral pharmacology include food, water, electric shock, temperature changes, loud noise, and intracranial stimulation.

Reinforcement and punishment are defined in analogous ways. The decreased occurrence of responses similar to one that immediately preceded some event identifies that event as a punisher; the presentation of a punisher following such responses is called punishment. Punishment is sometimes defined

as the presentation of a negative reinforcer. This formal definition of punishment emphasizes the usual contrasting effects of presenting and terminating a particular negative reinforcer, but there is not always a reciprocal behavioral effect (KELLEHER and MORSE, 1964).

There is, as yet, no coherent theoretical concept that explains why certain events are reinforcers. Nevertheless, many researchers still feel that reinforcers and punishers act by changing motivational states or "drives." For example, from the observation that the effectiveness of food as a reinforcer is partially dependent upon the extent to which the animal is deprived of food, it is inferred that the animal has a hunger drive that is reduced by the presentation of food, and that this drive reduction underlies reinforcement. Similarly, it is assumed that electric shock or other noxious stimulation engenders a drive of pain, fear, or anxiety that accounts for its properties as a punisher or as a reinforcer. These motivational interpretations of behavior are largely tautological, but have had a strong influence on behavioral pharmacology because many drugs are thought to be clinically useful in specifically reducing hunger or anxiety. Experimental studies on the effects of drugs on behavior maintained by a variety of reinforcers, to be discussed in Section III C, have made such seemingly simple motivational interpretations of drug effects untenable.

F. Schedules of reinforcement

The most interesting characteristics of operant behavior are revealed when reinforcers are delivered intermittently. Because the effects of reinforcers persist in time, it is only when reinforcers are delivered intermittently that their full effects can be seen. Under intermittent reinforcement the parameters of the sequential and temporal relations between responses and reinforcers are called the schedule of reinforcement. Schedules of reinforcement engender reproducible temporal patterns of responding which can give more information on the dynamic properties of reinforcers than does the defining criterion of a mere increase in frequency of responding. The schedule under which a reinforcer is delivered can be a more important determinant of behavior than the type of reinforcer (KELLEHER and MORSE, 1964; MORSE and KELLEHER, 1966).

Although there is a vast number of types of schedules of reinforcement, many characteristics of schedule-controlled behavior can be illustrated with two representative schedules. The fundamental classification of schedules distinguishes between schedules that reinforce a response on the basis of number of responses (ratio) and on the basis of time (interval). [For comprehensive accounts of schedules of reinforcement, see SKINNER (1938), FERSTER and SKINNER (1957), and MORSE (1966).] In fixed-ratio schedules, the reinforcer follows the occurrence of a constant number of responses. In fixed-interval schedules, the reinforcer follows the first response occurring after a constant minimum interval of time.

Fixed-ratio schedules of reinforcement of the easily repeated responses that are usually studied characteristically engender high rates of responding (for example, 3 responses per sec) whenever the individual is responding. The exact pattern of responding depends upon the parameter value of the schedule. When only a few responses are required (for example, 10), a high rate of responding is sustained between presentations of the reinforcer. When the schedule parameter is changed so that many responses are required (for example, 300), the latency of the initial response in each ratio is long and variable; however, once responding has begun it continues at a sustained high rate. The development of responding on a large fixed ratio usually requires the prior development of responding by reinforcement; if the individual were abruptly exposed to the large response requirement, it might never emit 300 responses.

Fixed-interval schedules of reinforcement engender a characteristic pattern of responding. An initial period of no responding (pausing) is followed by acceleration of responding to a final rate that is sustained until reinforcement. This characteristic pattern of responding occurs over a wide range of parameter values (30 sec to more than 24 hr) and is relatively independent of the individual's previous schedule performance. Under most conditions, only one-quarter of the total number of responses in each fixed interval have been emitted when about 60 % of the minimum time of the interval has elapsed (quarter-life).

There are many reasons why schedules of reinforcement are relevant to behavioral pharmacology. The most significant of these is the critical dependence of the effects of drugs upon steady-state behavior, which in turn is dependent upon the maintenance schedule. The action of a drug seems to depend more upon the schedule-controlled pattern of responding than upon any other environmental influence. The reasons for this generalization will be discussed in the following sections.

Initially schedules of reinforcement were used in studying drugs because of other advantages. Schedule-controlled behavior appears to be a phenomenon of great generality (cf. DEWS, 1958a). First, patterns of responding under schedules of reinforcement have been found to be highly reproducible under diverse conditions. For example, characteristic fixed-interval performances have been engendered in many species (Fig. 1) including man (HOLLAND, 1957)². In comparative pharmacology, as in comparative psychology, it is convenient to start with phenomena that are common to diverse species before looking for differences. It is usually of interest to know whether an effect of a drug is

² In cumulative response records, each response moves the recording pen a constant distance upward while the paper moves horizontally at a constant speed. This produces a smooth record when the paper moves slowly and each upward step is small. A horizontal line indicates no responding, and positive slopes are directly related to rate of responding. For example, in the upper frame of Fig. 1 a slope of 45° indicates a rate of 0.5 response per sec.

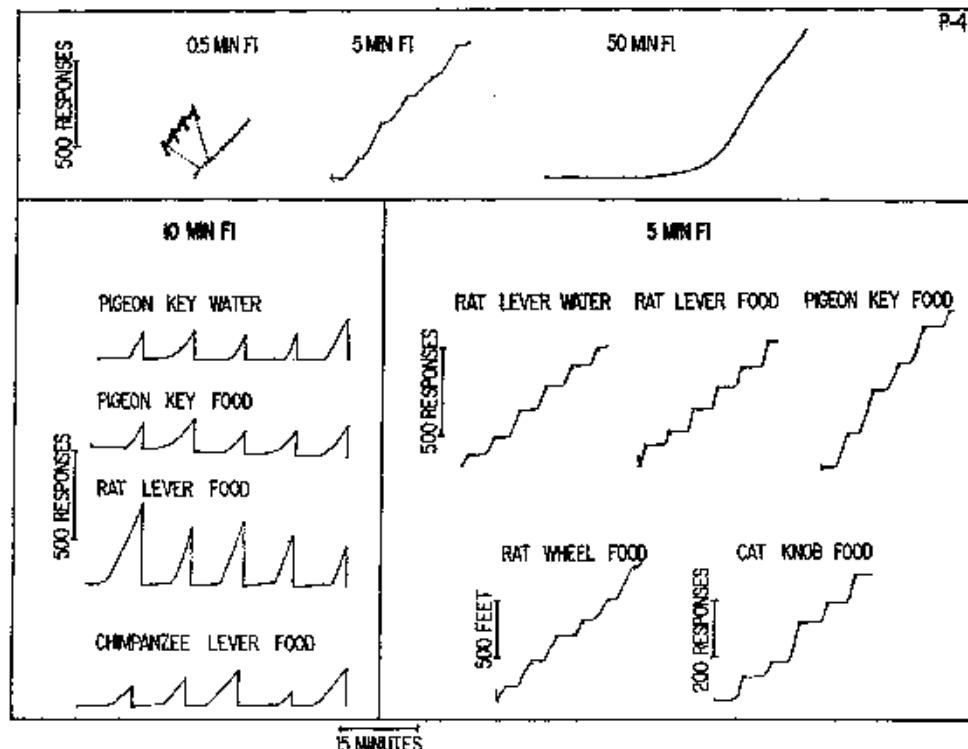


Fig. 1. Generality of characteristic fixed-interval performance (no responding, then acceleration to a maintained steady rate of responding). Ordinate: cumulative number of responses; abscissa: time. A fixed-interval schedule of presentation of food or water was in operation in all examples shown in this figure. Upper frame: individual pigeon (P-4) pecking plastic key (food). Three different durations of the fixed interval are shown; the general pattern persists despite the 100-fold change in the schedule parameter. Food presentations, ending each fixed interval, are marked by short diagonal strokes on the cumulative record. Lower left frame: performances under a 10-min fixed-interval schedule. Food or water presentations, ending each fixed interval, are marked by the resetting of the recording pen to the baseline. Lower right frame: performances under a 5-min fixed-interval schedule. The species, the type of switch recording the response, and the reinforcer presented are indicated above the records. The pigeon pecks a plastic key with its beak; the rat and chimpanzee press a horizontal lever with their paws; the cat depresses a rounded knob with its paw. The rat turns the wheel by running; only a turn of 180° is reinforced, but the cumulative distance the wheel turns is recorded directly. Modified from DEWS (1958a).

limited to a particular species or whether it is a more general phenomenon. Second, comparable schedules of reinforcement can be used to engender comparable patterns of responding with a variety of different reinforcers. For example, in the rat or pigeon fixed-interval performance can be maintained with food or water (Fig. 1); in the monkey fixed-interval performance can be maintained by presentation of food or by termination of a stimulus correlated with occasional electric shocks (Fig. 2). The schedule-controlled performance provides a common frame of reference for comparing the effects of drugs under diverse reinforcers and upon the motivational variables underlying the effectiveness of reinforcers. A drug that similarly affected performances controlled by food, water, and electric shock could not be having specific effects on motivational variables. Finally, the same reinforcer can be used with different

schedules of reinforcement to engender different patterns of responding. Schedules of reinforcement make it possible to produce a wide range of patterns and rates of responding occurring in a given sequence, and to determine the extent to which the effect of a drug depends upon the ongoing behavior under control conditions.

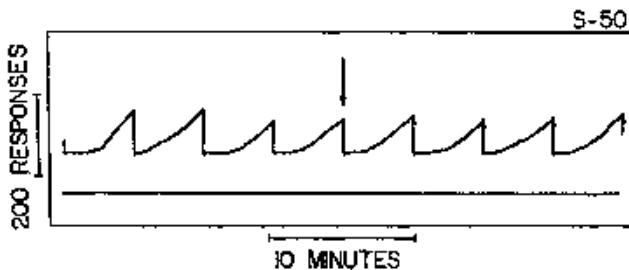


Fig. 2. Characteristic fixed-interval performance in the squirrel monkey under a multiple schedule of stimulus-shock termination and food presentation (Monkey S-50). Each type of schedule is correlated with a distinctive visual stimulus. The schedule of stimulus-shock termination was in effect during the four record segments to the left of the arrow. In the presence of a white light, electric shocks were scheduled to occur at 3-sec intervals starting after 5 min; the first response after 5 min terminated the stimulus-shock complex for 1 min. No shocks were delivered in the record segment shown, as indicated by the absence of diagonal strokes on the event record. The schedule of food presentation was in effect during the four record segments to the right of the arrow. In the presence of a red light, the first response after 5 min was followed by food presentation and terminated the light for 1 min. Food presentations are indicated by short diagonal strokes on the cumulative record. The recording pen reset to the baseline at the end of each fixed interval. The recorder did not run during the minute of darkness following each fixed interval. The arrow indicates the change from the schedule of stimulus-shock termination to the schedule of food presentation.

G. Discriminative stimuli

In the above description of operant conditioning, there was no consideration of any stimulus that preceded the response. In the example described in Section II D, the lever-pressing response was not elicited by a stimulus in the reflex sense in which, for example, paw withdrawal is elicited by electric shock; that is, emitted operant behavior is maintained by consequences rather than elicited by prior stimuli. Nevertheless, operant behavior can be brought under the control of environmental stimuli; indeed, such control is probably inevitable following operant conditioning. If the lever-pressing response is reinforced only in the presence of a light, eventually lever-pressing occurs predominantly when the light is present. The stimulus (the light) is the condition under which a response (lever-pressing) is followed by the reinforcer (food presentation). A stimulus, such as the light, that is correlated with a schedule of reinforcement is called a discriminative stimulus. In the limited case in which each response following the presentation of a stimulus is reinforced, a discriminative stimulus may be like an eliciting stimulus in that the response occurs immediately after the stimulus is presented. However, the development of control by a discriminative stimulus is different, particularly under intermittent schedules of reinforcement.

Several different kinds of schedule-controlled behavior can coexist in an individual, each schedule of reinforcement being correlated uniquely with a different exteroceptive discriminative stimulus; such a compound schedule is called a multiple schedule (FERSTER and SKINNER, 1957). The discriminative stimuli and their corresponding schedules may be presented in a regular or an irregular sequence. With multiple schedules it is possible to sample several different kinds of behavior in an individual within a short period of time. The component schedules constituting the multiple schedule can be identical but controlled by different types of reinforcers, or they can be different but controlled by a single type of reinforcer. As control procedures, multiple schedules have the advantage that many variables that might change the performance of an individual over time are controlled. Further, multiple schedules can be used to study the effects of drugs on the discriminative control of behavior by different stimuli. More significantly, different drugs selectively affect different schedule performances within the same session, sometimes in opposite ways.

III. Characteristics of behavioral pharmacology

A. Dose-effect relations

It is an important characteristic of behavioral pharmacology that most drugs modify behavior over only a relatively narrow range of doses, and that few drugs produce a single graded effect. The characteristics of dose-effect curves depend in part upon the level of complexity of the preparation being studied. In a simple preparation, such as an isolated piece of smooth muscle, increasing doses of a drug can produce graded increases in contraction up to a maximum. In more complex preparations, drugs produce bidirectional changes that depend upon interacting effects of a number of variables. For example, the magnitude and direction of the effect of a drug on the blood pressure of an anesthetized cat will depend on the type of anesthetic agent used, homeostatic reflexes, and initial level of the blood pressure; in unanesthetized intact animals, the interaction of variables in both the internal and external environment further complicates how a drug affects the blood pressure. Thus, the effect of a drug on a complex preparation is a composite. Integrated behavior comprises many interrelated components which are simultaneously affected by most drugs that influence behavior. Whereas a drug can have a single graded effect on a simple system over a wide range of doses, its separate effects on different components of a complex system produce a more complex net result.

The effects of a drug that decreases behavior must be interpreted cautiously since a large enough dose of any drug will abolish responding. In characterizing drugs that mainly decrease most patterns of responding, it is useful to compare dose-effect curves obtained with several different patterns. For example, chlorpromazine decreases responding in many commonly-used laboratory mammals.

Under different circumstances or different schedules, however, dose-effect curves for responding are changed, thus indicating that chlorpromazine does have selective effects on different performances (for example, COOK and WEINSTEIN, 1957).

A powerful pharmacological technique for characterizing the actions of a drug is to test the effects of the drug in the presence of antagonistic drugs. The antagonism is best studied over a wide range of doses of both agonist and antagonist. In behavioral pharmacology, however, the range of doses over which the antagonist exerts only selective antagonism without other effects is usually limited. VAILLANT (1964a) has suggested that evidence for specificity of antagonism can be sought by studying the effects of agonist and antagonist on different schedule-controlled patterns of responding that are selectively affected by many drugs. This can be considered analogous to general pharmacological studies which seek evidence for specific antagonisms by determining whether the antagonism can be demonstrated for a variety of effects on a variety of tissues. For example, VAILLANT (1964a) found that physostigmine or nicotine produced complete suppression of responding in pigeons under a multiple fixed-interval fixed-ratio schedule of food presentation. An appropriate dose of atropine could completely restore the two different complex patterns of responding suppressed by physostigmine, but atropine did not antagonize the effects of nicotine. That atropine restored both patterns of responding suppressed by physostigmine, but not nicotine, is evidence for the specificity of the antagonism.

Drug-produced increases in behavior are particularly interesting because they are unlikely to be caused by nonspecific effects of a drug. Most drugs that have specific behavioral effects enhance the output of some responses while decreasing the output of others. The full spectrum of behavioral actions of a drug is often disregarded because it has seemed convenient to classify drugs that affect behavior as stimulants or depressants. Investigators often express surprise over decreases in responding caused by drugs that can produce convulsions and increases in responding caused by drugs that can produce sedation or even general anesthesia. Although such effects are frequently called paradoxical, they can be reliably obtained under appropriate conditions. In autonomic pharmacology, sympathomimetic amines used to be classified as having excitatory or inhibitory actions on autonomic effector organs. Study of the effects of several sympathomimetics on a variety of organs led to the distinction between two types of receptors (α and β) based on specific pharmacological properties rather than on an assumed excitatory-inhibitory classification. In behavioral pharmacology, the misleading classification of drugs as stimulants and depressants should be abandoned. Only by taking account of the full spectrum of behavioral actions of each drug will it be possible to develop a satisfactory classificatory system.

The need for investigating a range of doses before characterizing the behavioral action of a drug cannot be overemphasized. Equally important is the need to understand the determinants of ongoing behavior when the drug is given. Unfortunately, many investigators reporting on the behavioral actions of drugs study only single doses and often tend to use complex behavioral procedures which have not been previously studied. Finally, many investigations on the behavioral effects of drugs are conducted with powerful drugs at ineffective or minimally effective doses. Because minimally effective doses usually have variable effects, the comparison of single doses of a drug in different situations or comparisons among single doses of different drugs can be completely misleading.

B. The importance of environmental circumstances

A primary concern in behavioral pharmacology is how the behavioral effects of drugs are modified by the past and present environmental determinants of behavior. The earliest work in behavioral pharmacology simply established that most drugs have selective actions on behavior in different situations, and that the prediction of the behavioral effect of a drug required knowledge about the conditions under which the drug was acting and the determinants of behavior in that situation. As noted previously, attempts to hold environmental determinants constant in physiological and pharmacological experiments have obscured the profound effects of environmental factors in modifying the actions of drugs.

For example, the toxic effects of amphetamine depend upon environmental circumstances. When amphetamine was administered to mice alone in their cages, the LD₅₀ was 114 mg/kg; when amphetamine was administered to mice caged in groups of ten, the LD₅₀ was reduced to 15 mg/kg (CHANCE, 1946). The toxic effects of amphetamine could also be enhanced by increasing room temperature, increasing ambient noise level, decreasing cage size, or administering electric shocks (GUNN and GURD, 1940; CHANCE, 1947; HÖHN and LASAGNA, 1960; WEISS *et al.*, 1961). Under conditions in which environmental circumstances were well controlled, the toxicity of amphetamine could be reliably determined. Yet changing only the environmental circumstances could produce a tenfold enhancement in the lethality of amphetamine.

With dependent variables that are less gross than life or death, environmental variables can be even more influential in modifying the magnitude or even type of effect of a drug. For example, HILL *et al.* (1957a) found that the effects of morphine and pentobarbital on simple reaction times in man were completely altered by changing experimental conditions. Three groups of subjects, all former morphine addicts, received morphine (the "incentive") for

their participation in the study⁴. The low-incentive group received a fixed amount of morphine at least one week before the experiment; the standard-incentive group received a fixed amount of morphine at the end of the experiment; the high-incentive group received an amount of morphine at the end of the experiment that depended upon how fast they reacted.

In control sessions, subjects in the low-incentive group reacted slowest; subjects in the high-incentive group reacted fastest. When an intramuscular injection of morphine sulfate (15 mg) was given 50 min before a session, the reactions of subjects in the standard and high-incentive groups were the same as in control sessions, but subjects in the low-incentive group reacted significantly faster than in control sessions. When an intramuscular injection of pentobarbital sodium (250 mg) was given 50 min before a session, the reactions of subjects in the standard group did not differ from those in control sessions, but subjects in the low-incentive group reacted significantly slower than in control sessions, while subjects in the high-incentive group reacted significantly faster than in control sessions. Whether pentobarbital produced slower reactions, no effect, or faster reactions depended upon the experimental conditions. What HILL *et al.* (1957a) refer to as different incentive conditions includes not only different amounts of morphine, but also different schedules of presentation of morphine and different control reaction times. Such selective modifications of behavior by drugs have been found in studies with a variety of species. A primary concern in behavioral pharmacology is to go beyond the demonstrations of differences to clarify the nature of the interrelations between the effects of drugs and the environmental determinants of behavior.

C. Interrelations between effects of drugs and behavior maintained by reinforcers

The primary determinant of the frequency of occurrence of operant behavior is reinforcement. As noted in Section II E, psychologists have been much concerned with explaining why certain events are reinforcers. Most psychological theories have assumed that there are basic motivational drives underlying behaviors maintained by different types of reinforcers. It is assumed that behavior maintained by food presentation depends upon a hunger drive, which is directly related to the duration of food deprivation. Similarly it is assumed that behavior maintained by termination of a noxious stimulus depends upon a drive of fear or anxiety, which is directly related to the intensity of the noxious stimulus. Theories of behavior and clinical accounts of how drugs are affecting behavior have emphasized the dependence of behavior on

⁴All subjects were volunteers who were serving prison sentences for violations of the Harrison Narcotic Act. They were selected according to various criteria designed to ensure that their chances for rehabilitation would not be jeopardized by participation in the study.

assumed drive states that underlie and support the behavior. Thus, there has been a widespread belief that any specific effects of drugs on behavior necessarily reflect the differential effects of the drugs on drive states.

The notion that drugs mainly modify the effectiveness of a particular reinforcer has two clear implications. First, it implies that behaviors maintained by the same reinforcer should be similarly affected by the drug. Second, it implies that behaviors maintained by a variety of reinforcers should be differentially affected by the drug. Results from many experimental studies are relevant to each of these implications.

1. Effects of drugs on different schedule performances maintained by one reinforcer

DEWS (1955a) studied the effects of intramuscular doses of pentobarbital (0.25 to 5.6 mg) on the behavior of four food-deprived pigeons under different schedules of food presentation. For two birds, food was presented following every 50th response (50-response fixed-ratio schedule); for the other two birds, food was presented following the first response after 15 min (15-min fixed-interval schedule). When dose-effect curves had been established, the schedules were reversed for the two groups of pigeons and the experiment was repeated. The average control rates of responding were about 1.7 responses per sec under the fixed-ratio schedule and about 0.4 response per sec under the fixed-interval schedule. Fixed-ratio rates of responding were increased at doses of 0.5 to 4.0 mg, but markedly decreased at higher doses. Fixed-interval rates of responding were increased at doses of 0.25 and 0.5 mg, but progressively decreased as the dose was increased from 1 to 4 mg. Over a more than twofold range of doses, pentobarbital increased rates of responding in pigeons under a fixed-ratio schedule but markedly decreased rates of responding in the same pigeons under a fixed-interval schedule. Further, the effect of a particular dose of pentobarbital could be changed from a decrease in responding to an increase in responding by varying only the type of schedule of food-presentation (DEWS, 1955a). Although only one type of reinforcer, food presentation, was used in these studies, the effects of pentobarbital differed according to the schedule-maintained patterns of behavior.

Changes in parameter values of schedules of reinforcement also produce characteristic changes in the action of drugs. Under fixed-ratio schedules, for example, performance at many parameter values depends more critically upon the subject's past performance and history of reinforcement than does performance under many other schedules. As the response requirement is increased to higher and higher values, a point is reached where there will be long periods of time during which the subject does not respond. Such pauses, characteristically occurring after reinforcement, are a complex product of interrelated

factors; for example, the number requirement, the amount of reinforcer, and the conditions of deprivation.

Fig. 3 shows the effect of amobarbital under two parameter values of fixed-ratio (FR) reinforcement in the pigeon (MORSE, 1962). The conditions of the experiments were identical except that food was presented following every 33rd response under one condition (FR 33) and following every 330th response under the other condition (FR 330). Under non-drug conditions the average rate of responding was almost twice as high under FR 33 as under FR 330, and the frequency of reinforcement on FR 33 was almost 20 times as high. In contrast to the control values, the two dose-effect curves were superficially similar.

At the lowest dose the rate was nearly the same under the two conditions. On a percentage basis, however, the increase in responding was negligible under FR 33 and substantial under FR 330. The 10 mg dose produced an even more marked divergence. Relative to their respective control values, the response output under FR 33 was decreased by 38 per cent and the response output under FR 330 was increased by 84 per cent. The rate of responding after 10 mg amobarbital was less when the bird had to make 33 responses than when it had to make 330 responses per reinforcement. The higher doses of

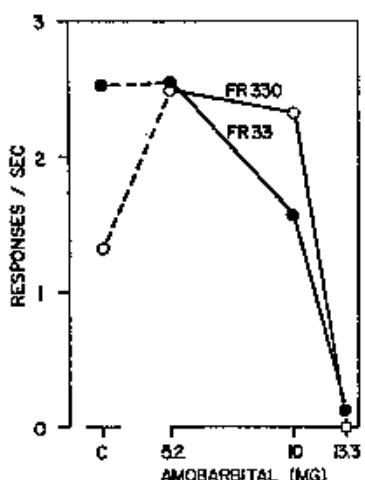


Fig. 3. Dose-effect curves for amobarbital at two parameter values of fixed-ratio (FR) reinforcement. Each drug point indicates the rate of responding in a 10-min session starting 15 min after drug injection. Note that after 10 mg of amobarbital the curve for FR 33 is depressed below its control value, and the curve for FR 330 is elevated above its control value and also above the FR 33 curve. Modified from MORSE (1962); courtesy of Lea and Febiger, Philadelphia, Pa., U.S.A.

amobarbital decreased responding more under the schedule in which control rates were high than under the schedule in which control rates were low; that is, the dose-effect curves crossed. Similar crossing dose-effect curves were also obtained with amobarbital under different parameter values of two other schedules of food presentation (MORSE, 1962). Differences in the shapes and slopes of dose-effect curves under different conditions suggest that the net behavioral effect of the drug is a composite of interacting components.

Systematic studies have shown that the effects of certain drugs are selectively modified by the type and the parameter values of schedules (DEWS, 1960; MORSE, 1962). It is inferred from such results that performance under a schedule represents a dynamic balancing of multiple influences and that drugs shift this balance. The nature of the shift will depend upon the interactions determining the balance, which will change at different parameter values.

Thus, the effects of drugs on behavior have revealed the importance of interactions that were previously unemphasized. That the effect of drugs on behavior changes at different parameter values of a single schedule emphasizes both the intrinsic complexity of even seemingly simple, homogeneous behavioral performances, and the power of drugs to help analyze this complexity.

A particularly useful technique for comparing the effects of drugs on different patterns of behavior maintained by one type of reinforcer is the multiple schedule comprising alternating components. As noted previously, under a multiple schedule different patterns of behavior can be studied in an individual in a single experimental session. The dependence of the effects of pentobarbital

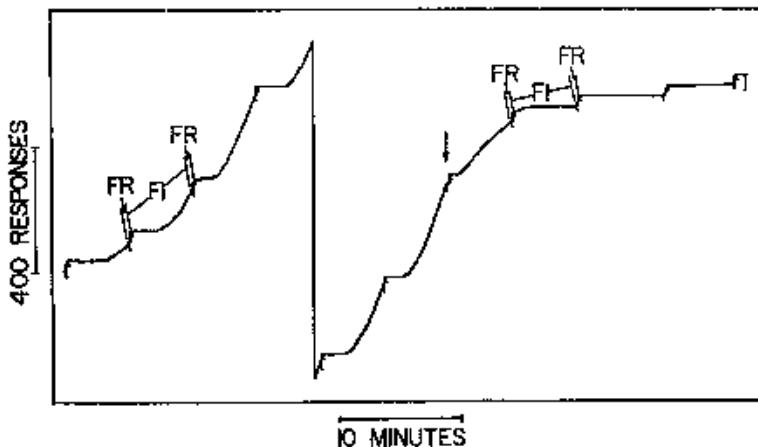


Fig. 4. Selective effect of pentobarbital on multiple schedule performance in the pigeon. An FR 30 schedule of food presentation alternated with an FI 5 schedule of food presentation. Each type of schedule was correlated with a distinctive visual stimulus. Food presentations, marked by short diagonal strokes, were followed immediately by the change in schedule. The variation in the number of responses in fixed-interval components is normal. Pentobarbital sodium (3 mg) was administered intramuscularly at the arrow (body weight, 430 g). The drug markedly decreased responding in individual FI 5 components. Modified from MORSE (1962); courtesy of Lea and Febiger, Philadelphia, Pa., U.S.A.

on different schedule-controlled patterns of responding (DEWS, 1955a) is also obtained when these schedules are combined under a multiple schedule in which the response patterns alternate over short periods of time (MORSE and HERRNSTEIN, 1956; MORSE, 1962). Fig. 4 shows performance of a food-deprived pigeon under a multiple fixed-ratio fixed-interval schedule. Under the fixed-ratio (FR) component, responding occurred at a high steady rate. Under the fixed-interval (FI) component, an initial pause was followed by acceleration of responding to a steady rate that was sustained until reinforcement. After an intramuscular injection of 3 mg pentobarbital sodium (at the arrow), responding was markedly reduced in the fixed-interval component but not in the fixed-ratio component.

An example of a prolonged differential effect of a drug on responding under different schedules was noted by SMITH (1964). The effects of chronically ad-

ministered reserpine were studied on the behavior of pigeons under a multiple schedule comprising 33-response fixed-ratio and 5-min fixed-interval components. Reserpine (0.1 mg/kg, intramuscularly) was administered 16 to 18 hours before each daily experimental session. During the first 7 days of treatment with reserpine, rates of responding in fixed-interval components decreased to about 30 per cent of pre-drug rates, but rates of responding in fixed-ratio components were little changed.

In studies with multiple schedules, the same individual can be studied with the same reinforcer presented according to different schedules sequentially during the same session. Thus, the marked differential effects of the drugs on these repeatedly alternating patterns of responding cannot reasonably be attributed to changes related to the reinforcer. It has been found consistently that different patterns of responding maintained by the same reinforcer are selectively affected by drugs. Although all the experiments presented in this section involved the food-deprived pigeon as the subject and food presentation as the reinforcer, the results with drugs have been consistently confirmed in experiments using other species and other types of reinforcers. Several of these experiments will be discussed in the section which follows.

2. Effects of drugs on performances maintained by different reinforcers

Many different reinforcers have been used to maintain behavior in experiments concerned with the effects of drugs on behavior. We will first consider studies using reinforcers that involve the presentation of a stimulus. Examples of such reinforcers are food, water, heat, and intracranial stimulation. Under appropriate conditions these reinforcers function similarly, but the conditions required for their suitability as reinforcers are different. Deprivation is necessary with food or water; exposure to a cold environment is necessary with heat; and the proper placement of the electrodes is necessary with intracranial stimulation. In the few studies that have compared directly the effects of drugs on behaviors maintained by different reinforcers, the results obtained with extensively studied drugs such as chlorpromazine and amphetamine have been consistent.

Meaningful comparisons among the studies of different types of reinforcers are difficult when different species or important differences in procedure are involved. Thus, it is convenient to consider first experiments studying the behavioral effects of drugs on one species (the rat) responding under one schedule (each response is followed by the reinforcer). Consistently the results indicate that as the dose of chlorpromazine is increased, rate of responding decreases in a graded fashion with the following reinforcers: food (WEISSMAN, 1959), intracranial stimulation (OLDS and TRAVIS, 1960), and heat (WEISS and LATIES, 1963). Appropriate doses of amphetamine increase rates of responding

with the following reinforcers: food (STONE *et al.*, 1958; WEISSMAN, 1959), intracranial stimulation (STEIN, 1964), and heat (WEISS and LATIES, 1963). Higher doses of amphetamine decrease rate of responding (WEISSMAN, 1959; WEISS and LATIES, 1963). On the other hand, amphetamine decreases the intake of food in a free-eating situation (MILLER, 1956; EPSTEIN, 1959; SIEGEL and STERLING, 1959) and the intake of water in a free-drinking situation (ANDERSSON and LARSSON, 1956; EPSTEIN, 1959).

The experiments in which WEISS and LATIES (1963) used heat as a reinforcer are particularly significant for the present discussion because they studied the effects of several drugs on skin and body temperature, as well as on frequency of responding maintained by heat presentation. The experiments were conducted with individual shaved rats in a small chamber in a refrigerated room; whenever the rat pressed a lever within the chamber, it received 2 sec of infrared heat from a lamp above the chamber. Thermocouples were used to record skin temperature and rectal temperature. In experiments monitoring rectal temperature in the cold (2° C) without heat reinforcement, chlorpromazine (2 to 4 mg/kg, intraperitoneally) enhanced the fall in temperature, but amphetamine had little effect on temperature at a dose of 2 mg/kg (intraperitoneally). These same doses markedly affected rates of responding in experiments in which each response turned on the heat lamp. Chlorpromazine decreased rates of responding even though it enhanced the rate at which temperature fell in the cold, and amphetamine increased rates of responding. In other experiments in which the ambient temperature was higher (10° C), the increase in rate of heat presentation after amphetamine caused the skin temperature to rise significantly. Noting that these effects are similar to those obtained when food or water is used to maintain behavior, WEISS and LATIES (1963) concluded that "the behavioral properties of these drugs are largely independent of the reinforcer that maintains the behavior, or, put another way, of the motivational state that supports it".

There have been many studies of the effects of chlorpromazine or amphetamine on responding maintained by the termination of a stimulus associated with a noxious stimulus or by postponement of the onset of a noxious stimulus. The results, like those with presentation of food or heat, have consistently indicated that chlorpromazine decreases rates of responding, while amphetamine increases rates of responding at lower doses and then decreases them at higher doses; for examples, see reviews by HERZ (1960), DEWS and MORSE (1961), and GOLLUB and BRADY (1965). Despite the many different properties among reinforcers, the behavioral effects of chlorpromazine and amphetamine appear to be independent of the type of reinforcer that is maintaining the behavior under study. Rather, what seems to be important is the schedule under which the reinforcer is delivered.

3. Direct comparisons of the effects of drugs on performances maintained by different reinforcers

Behavioral pharmacologists have long been interested in determining whether drugs have selective and specific effects on behavior controlled by noxious stimuli. Many investigators have compared the effects of drugs on behavior maintained by presentation of food with their effects on behavior maintained by termination (or postponement) of electric shock. Much of the interest in such comparisons derives from motivational interpretations of the clinical uses of drugs. For example, chlorpromazine and reserpine are thought to be useful in alleviating fear and anxiety. If electric shocks control behavior by engendering a generalized state of fear or anxiety, chlorpromazine or reserpine might specifically affect this behavior by modifying these underlying motivational states.

Some investigators have reported that chlorpromazine (WEISSMAN, 1959) and reserpine (WENZEL, 1959) have more marked effects on behavior maintained by electric shock than on behavior maintained by the presentation of food. In the study by WENZEL (1959), for example, cats were presented with food when they pressed one lever in the presence of one stimulus, and avoided an electric shock when they pressed a second lever in the presence of a second stimulus. Reserpine (35 µg/kg) was administered, and then performance was repeatedly tested over the next 124 hr. Reserpine increased response latencies more in the presence of the stimulus paired with shock than in the presence of the stimulus paired with food. It was concluded that reserpine acted specifically upon the behavior maintained by avoidance of electric shock.

Other investigators have reported that chlorpromazine has more marked effects on behavior maintained by presentation of food or intracranial stimulation than on behavior maintained by avoidance of electric shock (FERSTER and SKINNER, 1957; OLDS, 1959; COOK and KELLEHER, 1961, 1962). In the study by COOK and KELLEHER (1961, 1962), for example, squirrel monkeys pressed a lever under a schedule in which each press postponed the onset of a brief electric shock for 30 sec, and concurrently every 100th press was followed by food presentation. Just after food presentation, the rate of responding was about 0.1 response per sec for several minutes, and then the rate abruptly increased to more than 1.0 response per sec until food was presented again. Control experiments showed that the low rate was controlled by electric-shock postponement while the high rate was controlled by food presentation. Chlorpromazine, and other phenothiazines, eliminated the high rate of responding (controlled by food) at doses lower than those that affected low rates of responding (controlled by electric shock).

Other studies comparing behaviors maintained by food and by electric shock found no difference in sensitivity to reserpine (WEISKRANTZ and WILSON, 1955; MILLER, 1956; SIDMAN, 1956; RIOPELLE and PFEIFFER, 1958) or to chlor-

promazine (WALLER and WALLER, 1962; COOK and CATANIA, 1964; KELLEHER and MORSE, 1964). In the study by WALLER and WALLER (1962), for example, dogs responded by nuzzling a lever under a multiple schedule comprising two alternating components. Responses in one component were followed by food presentation at variable intervals of time averaging once per min. Responses in the other component postponed a brief electric shock for 20 sec. Steady rates of about 0.8 response per sec were maintained in both components. Chlorpromazine decreased rates of responding under both schedules at the same dose level.

How may these seemingly contradictory results be reconciled? We have already noted that even with the same reinforcer, different schedule-controlled patterns of behavior in an individual can be differentially affected by drugs. In WENZEL's (1959) study of reserpine, the average control latencies in the presence of the stimulus paired with shock were longer than latencies in the presence of the stimulus paired with food. Although the schedules were formally similar, the patterns of responding engendered by these schedules were different (cf. DEWS and MORSE, 1961). In the studies of COOK and KELLEHER (1961, 1962) in the squirrel monkey, the schedules of shock avoidance and food presentation were different, and the patterns of responding controlled by each schedule differed markedly. On the other hand, in the study of WALLER and WALLER (1962), in the dog, the patterns and rates of responding controlled by each type of reinforcer were similar. These findings indicate that one could select pairs of schedules that would seem to show that a drug specifically affected behavior maintained under either type of reinforcer. The result obtained would depend on the type and the parameter of the schedule controlling the patterns of responding.

The difficulties involved in comparing behavioral effects of drugs on performances maintained by different reinforcers are formidable. If different reinforcers are presented according to different schedules, the effects of the drug will be largely determined by the schedule-controlled patterns of responding. For comparing the effects of drugs on behaviors maintained by different reinforcers, it is useful to start with similar schedules of reinforcement; however, there is still no *a priori* basis for equating such parameters as amount of food and intensity of electric shock. It is unreasonable to presume that any one arbitrarily chosen schedule of food presentation is comparable to any one arbitrarily chosen schedule of electric-shock termination.

The most satisfactory way to attack these many problems is to obtain, as nearly as possible, identical patterns of responding maintained by different reinforcers and then to establish dose-effect relations for drugs on these patterns. Functional relations between drugs and behavior maintained by different schedules with each reinforcer can then be compared. Such a program obviously requires a prodigious amount of experimental work, but there is no

alternative at present. Two recent studies have reported procedures for establishing comparable patterns of responding with formally comparable schedules of food presentation and electric shock termination. One study established comparable patterns of responding in squirrel monkeys under fixed-interval schedules with different reinforcers (COOK and CATANIA, 1964). In a group of food-deprived monkeys, the first key-pressing response after 10 min was followed by food presentation. In another group of monkeys, a pulsating electric shock of low intensity was continuously delivered; the first response after 10 min terminated the shock. Response patterns characteristic of fixed-interval schedules were maintained under both food presentation and shock termination. Chlorpromazine and imipramine decreased rates of responding with both reinforcers, while selected doses of amphetamine, meprobamate, and chlordiazepoxide increased rates of responding with both reinforcers. These results support the view that the behavioral effects of the drug depend mainly upon schedule-controlled patterns of responding.

The other study directly compared the importance of type of reinforcer and schedule of reinforcement as determinants of the behavioral effects of drugs (KELLEHER and MORSE, 1964). One group of monkeys was food-deprived and responded under a multiple fixed-ratio fixed-interval schedule of food presentation. A small rectangular window in front of the monkey could be transilluminated by a pattern of horizontal lines, a red light, or a white light. In the presence of the pattern of horizontal lines, responding had no programmed consequences and food was never delivered. In the presence of the red light, a 30-response fixed-ratio schedule was in effect. In the presence of the white light, a 10-min fixed-interval schedule was in effect. A second group of monkeys responded under a multiple fixed-ratio fixed-interval schedule of termination of stimuli correlated with occasional electric shocks. Again, in the presence of the pattern of horizontal lines, responding had no programmed consequences and shocks were never delivered. In the presence of the red light, shocks were scheduled to occur every 30 sec; the 30th response terminated the red light and produced the pattern of horizontal lines. In the presence of the white light, shocks were scheduled to occur at 1-sec intervals starting after 10 min; the first response after 10 min terminated the white light and produced the pattern of horizontal lines.

Although the performances were maintained by different reinforcers, these two multiple schedules maintained similar patterns of responding. Representative performances of two monkeys are shown in Fig. 5. Performance under the fixed-ratio component of each multiple schedule was characterized by a sustained high rate of about 2.3 responses per sec. Performance under the fixed-interval component of each multiple schedule was characterized by a pause (period of no responding) followed by acceleration of responding to a steady rate; the average rate in the interval was about 0.6 response per sec. The

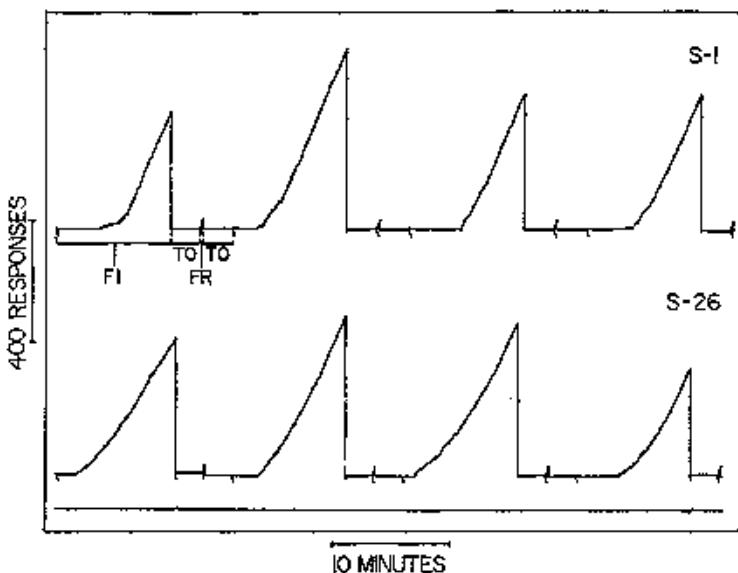


Fig. 5. Generality of characteristic multiple fixed-interval fixed-ratio schedule performance in the squirrel monkey. The performances were maintained by food presentation (upper record, Monkey S-1) and by stimulus-shock termination (lower record, Monkey S-26). The sequence of visual stimuli and corresponding schedules was the same in the upper and lower records. At the beginning of the records, the FI 10-min schedule was in effect in the presence of a white light. At the termination of the FI component the recording pen reset to the baseline. Following reinforcement, a pattern of horizontal lines was present for 2.5 min; during this time-out period (TO), responses had no programmed consequences. The next short diagonal stroke on the cumulative record indicates that the FR 30 component was in effect in the presence of a red light. Again the cumulative recording pen reset to the baseline at reinforcement, and was followed by the 2.5-min TO component. This cycle was repeated throughout each session. At the bottom of the record for Monkey S-26, the short diagonal strokes on the event line indicate electric shock presentations. The variation in the number of responses in fixed-interval components is normal. Note the similarities of the patterns of responding under these multiple FI FR food and shock schedules. Modified from KELLEHER and MORSE (1964); courtesy of Federation of American Societies for Experimental Biology, Bethesda, Md., U.S.A.

effects of *d*-amphetamine and chlorpromazine on rates of responding under each component of each multiple schedule are summarized in Fig. 6. *d*-Amphetamine increased rates of responding under both fixed-interval schedules, except at the highest dose level, but decreased rates of responding under both fixed-ratio schedules. Chlorpromazine produced graded decreases in rates of responding under both fixed-interval and both fixed-ratio schedules. The rates of responding under the fixed-ratio schedules were relatively less affected at the lower doses. COOK and KELLEHER (1962) showed a similar selective effect of chlorpromazine on the fixed-interval and fixed-ratio components of a multiple schedule of food presentation in the squirrel monkey. These results are also consistent with the findings of WALLER and WALLER (1962) that comparable patterns of responding maintained by food presentation or by postponement of electric shocks in the dog were affected similarly by chlorpromazine. The behavioral effects of both amphetamine and chlorpromazine depended more upon the schedule than upon the reinforcer.

Many theories of behavior assume that deviations from a normal physiological steady state are the basic motivational drives underlying the effectiveness of most reinforcers. It once seemed probable that the behavioral effects of drugs would be explained in terms of their effects on motivational drives. But the results described in detail in this section strongly support the conclusion that the behavioral effects of drugs are largely independent of the type of reinforcer maintaining the behavior — and consequently, independent of

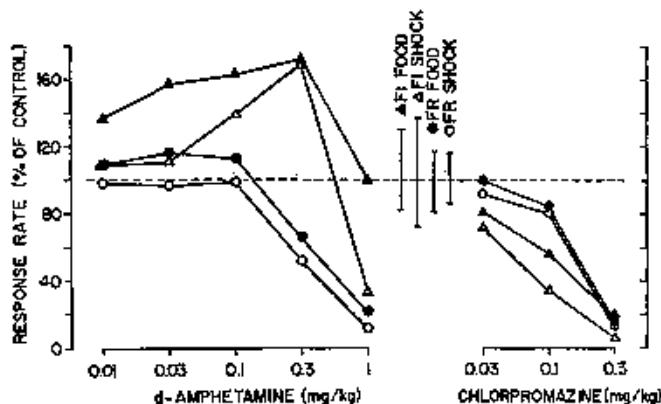


Fig. 6. Effects of *d*-amphetamine sulfate and chlorpromazine hydrochloride on rates of responding under multiple FI FR food and shock schedules. Three monkeys were studied on each multiple schedule. Each drug was given intramuscularly immediately before the beginning of a $2\frac{1}{2}$ -hr session. At least duplicate observations were made in each monkey at each dose level; thus, each point is based on at least six observations. Summary dose-effect curves for the four component schedules were obtained by computing the means of the percentage changes in average response rates from control to drug sessions. The dashed line at 100% indicates the mean control level for each component. The vertical lines in the middle of the figure indicate the ranges of control observations expressed as a percentage of the mean control value. Note the general similarity of the pairs of dose-effect curves for fixed-interval and for fixed-ratio components. From KELLEHER and MORSE (1964); courtesy of Federation of American Societies for Experimental Biology, Bethesda, Md., U.S.A.

assumed underlying motivations. Selective effects of drugs on particular reinforcers may yet be found, and it is possible that such effects may have occurred in some of the studies just described. Nevertheless, these studies show clearly that any dependence of drug effects upon type of reinforcer is relatively small compared to the critical dependence of drug effects upon schedules of reinforcement.

Interpretations of the effects of drugs on behavior have too often assumed an effect of motivational variables on the basis of insufficient evidence. For example, since it is known that amphetamine decreases eating in some situations, any amphetamine-produced decrease in output of behavior maintained by food presentation has been attributed to an anorexic effect of the drug. It has even been proposed that one cannot study behavioral effects of amphetamine independent of its anorexic effects unless one uses reinforcers such as intracranial stimulation or electric shock. Yet the effects of drugs on comparable

performances maintained by food presentation and electric shock termination are similar.

These results have important implications for both the study of behavior and the study of behavioral pharmacology. Historically the fundamental determinants of behavior have been considered to be motivational variables. But motivational interpretations of drug effects on behavior have failed to increase our understanding of how drugs affect behavior. If motivational concepts are to become useful for analyzing either behavior or the effects of drugs on behavior, they must be reformulated to encompass the effects of schedules of reinforcement.

Only recently has the power of schedules in engendering different rates and patterns of behavior been recognized. As behavior is engendered by a reinforcer, the schedule of reinforcement used to maintain the behavior determines the interaction between patterns of responding and the reinforcer; for example, the marked differences between responding under 30-response fixed-ratio and 5-min fixed-interval schedules of food presentation have been described. The sustained high rate of responding under a fixed-ratio schedule might be described as more highly motivated than the lower rate under a fixed-interval schedule. There is a growing body of evidence suggesting that a schedule-controlled pattern of responding is itself a fundamental determinant of behavior; that is, the schedule conditions engender what was formerly thought of as motivation (FALK, 1961; DEWS, 1963; MORSE and KELLEHER, 1966; MORSE, 1966). The current challenge in behavioral pharmacology is to develop an understanding of the ways in which drugs act upon schedule-controlled behavior.

IV. Ongoing behavior as a specific determinant of drug action

The previous section described situations in which the behavioral effects of drugs depended upon patterns of behavior determined by the schedule of reinforcement and the parameter value of the schedule. How does a schedule of reinforcement influence the behavioral effects of drugs? A schedule is, of course, only a specification of the conditions relating stimuli, responses, and reinforcers. In the analysis of behavior it is important to determine how these controlling relations interact with behavior to engender particular patterns; but drugs affect the behavior of the individual rather than affecting controlling relations. Some of the results already presented suggested that what is important is the schedule-produced pattern of responding rather than the schedule itself. In the study by WALLER and WALLER (1962), for example, comparable patterns of responding engendered by different types of schedules were shown to be similarly affected by drugs. In behavioral pharmacology then, the primary interest is in the behavior engendered by the schedule rather than the intrinsic properties of schedules. Thus, it is more precise to say that effects of drugs

depend upon schedule-controlled patterns of responding than to say that they depend upon the schedule of reinforcement. The only justification for the latter statement is that particular schedules do, in fact, usually engender a characteristic behavior pattern. The present section will analyze in detail the role of ongoing behavior as a specific determinant of the effects of drugs.

In 1958, DEWS (1958b) suggested that the frequency of occurrence of the behavior under study was an important determinant of the behavioral effects of amphetamines. The suggestion that the pre-drug rate of responding is a critical determinant of the effects of drugs has wide applicability for predicting the effects of drugs. Its importance has been enhanced in recent years as it has become clearer that traditional interpretations are inadequate. Since DEWS's interpretation was originally developed to account for the effects of the amphetamines, we will first consider evidence that the ongoing rate of responding is a determinant of the effects of amphetamines (rate-dependent effects). We will then attempt to extend the interpretation of rate-dependency to the effects of other classes of drugs.

A. The dependence of effects of amphetamines on average rate of responding under different schedules

DEWS (1958b) studied the effects of methamphetamine on behaviors maintained under four different schedules of food presentation in the pigeon. Two of these schedules, a 1-min variable-interval and a 50-response fixed-ratio, maintained relatively high rates of responding (more than one response per sec). Performances under both schedules were similarly affected by methamphetamine. Relatively low doses had little effect on rates of responding, while higher doses caused progressive decreases in rates of responding. The other two schedules, a 15-min fixed-interval and a modified 900-response fixed-ratio, maintained low average rates of responding (0.1—0.2 response per sec), and under both schedules there were periods of time without responding. Again, performances under both schedules were similarly affected by methamphetamine. In this case, relatively low doses caused marked increases in rate of responding while higher doses decreased rates of responding. Under all four schedules, the dose that decreased rates of responding was the same (an absolute dose of 1 mg/bird, intramuscularly). DEWS concluded that amphetamines tended to increase responding occurring at low rates, but would only decrease responding occurring at high rates.

"The results can probably be best stated in terms of interresponse times; i.e., the times elapsing between consecutive responses (or from the appearance of the key light to a response). When a control performance contains long interresponse times, administration of low effective doses of methamphetamine tends to reduce their number and length. Higher doses (1 mg and greater), in

addition to the foregoing, also tend to increase the length of short interresponse times." (DEWS, 1958b).

The results of many studies using a variety of different procedures and reinforcers in the pigeon, rat, and monkey are consistent with DEWS's interpretation. When food, intracranial stimulation, or heat is presented following every lever-pressing response in the rat, the relatively low rates of responding that develop (0.1 response per sec or less) are markedly increased by amphetamines (STONE *et al.*, 1958; WEISSMAN, 1959; WEISS and LATIES, 1963; STEIN, 1964). Food or water presentation can maintain low average rates of responding at appropriate parameter values under fixed-interval schedules, variable-interval schedules, reinforcement only of long interresponse times, and delayed presentation of the reinforcer following a response; also response rates eventually become low when food or water presentations are discontinued (extinction). In all of these cases it has been found that amphetamines can markedly increase rates of responding (ZIEVE, 1937; SKINNER and HERON, 1937; WENTINK, 1938; SIDMAN, 1955, 1956; BRADY, 1956; MORSE and HERRNSTEIN, 1956; KELLEHER *et al.*, 1961; SCHUSTER and ZIMMERMAN, 1961; COOK and KELLEHER, 1962; SEGAL, 1962; ZIMMERMAN and SCHUSTER, 1962; POSCHEL, 1963). On the other hand, under variable-interval or fixed-ratio schedules that maintain high rates of responding, amphetamines in small doses have little effect, while larger doses decrease rates of responding (OWEN, 1960; HEARST, 1961; KELLEHER *et al.*, 1961).

The rate-dependent effects of amphetamines have also been shown with multiple schedules of food presentation comprising components that maintain high and low rates of responding (COOK and KELLEHER, 1962; SMITH, 1964; RUTLEDGE and KELLEHER, 1965; CLARK and STEELE, 1966). For example, CLARK and STEELE studied the effects of *d*-amphetamine on the behavior of rats under a multiple schedule with three components presented in the following order: a 4-min period in which no responses were reinforced; a 4-min fixed-interval schedule; and three food presentations under a 25-response fixed-ratio schedule. Saline or *d*-amphetamine was given intramuscularly 5 min before the start of the 4-hr session. Mean rates of responding following saline administration were relatively low during the period of no reinforcement (0.01 response per sec) and the fixed-interval schedule (0.19 response per sec), but higher during the fixed-ratio schedule (0.84 response per sec). Increasing doses of *d*-amphetamine (0.5 to 4.0 mg/kg) increased the rate of responding under the two components with relatively low rates of responding, but decreased the rate of responding under the fixed-ratio component. Thus, DEWS's hypothesis about the rate-dependent effects of amphetamine applies even when different patterns of behavior are observed over brief periods of time in the same individual.

Amphetamines also increase low rates of responding maintained under schedules using noxious stimuli. Several studies of responding maintained by

the postponement of electric shocks have shown that amphetamines markedly increase such avoidance responding (SIDMAN, 1956; VERHAVE, 1958; TEITELBAUM and DERKS, 1958; HEISE and BOFF, 1962). Amphetamine increased responding in the rat when responses terminated an intense noise (HARRISON and ABELSON, 1959); and it increased responding in the squirrel monkey under a 10-min fixed-interval schedule of termination of a continuously-pulsating electric shock (COOK and CATANIA, 1964). Although amphetamine decreases drinking in a free situation, it markedly increases drinking when each drinking response postpones an electric shock. TEITELBAUM and DERKS (1958) have reported an experiment in which each lick at a water bottle was recorded as a response that postponed the onset of a brief electric shock. In control sessions, licking responses occurred at a relatively low rate; a rat ingested about 5 ml of water in one hr and received many electric shocks. Amphetamine (5 mg/kg, subcutaneously) markedly increased the rate of licking; a rat ingested 10—15 ml of water in one hr and received few electric shocks. We have previously discussed the effects of *d*-amphetamine on the behavior of the squirrel monkey under a multiple schedule of termination of a stimulus associated with occasional electric shocks. Appropriate doses of amphetamine increased the lower rate of responding under the fixed-interval component while decreasing the higher rate under the fixed-ratio component (KELLEHER and MORSE, 1964).

B. The dependence of effects of amphetamines on patterned responding

The results described so far indicate that the interpretation offered by DEWS is applicable to different rates of responding maintained under a number of different conditions. Previously we have considered only average rates of responding, but many schedules engender patterns of responding with different rates. Recent findings indicate that the net effect of amphetamines on average rate of responding under a schedule can be analyzed in terms of effects on rates of responding in different temporal periods of the schedule.

Responding under a fixed-interval schedule is characterized by an initial pause followed by increasing responding during the interval. In a study of the effects of drugs on the behavior of the pigeon under a multiple fixed-ratio fixed-interval schedule of food presentation, SMITH (1964) compared the effects of *d*-amphetamine (0.01 to 10 mg/kg, intramuscularly) on behavior during the first and last minutes of the 5-min fixed-interval component. He found that *d*-amphetamine markedly increased the low rates of responding characteristic of the first minute and decreased the high rates of responding characteristic of the fifth minute. The dose of 3 mg/kg, which produced the maximal increase in average rates of responding, significantly increased the rate of responding in the first minute and lowered the rate of responding in the fifth minute. The dose of 10 mg/kg, which decreased average rates of responding, produced a

greater increase in rates in the first minute than did 3 mg/kg, but also produced a more marked decrease in rates in the last minute. The change in average rate of responding produced by *d*-amphetamine was the net result of its rate-increasing and its rate-decreasing effects.

VERHAVE (1961) reported an interesting interaction between the effects of methamphetamine and momentary changes in rates of responding. Rats responded under a schedule in which each response postponed a brief electric shock for 30 sec. When the rate of responding fell too low and a shock was delivered, a momentary high rate of responding followed. He found that methamphetamine tended to reduce the momentary high rates and to increase the more prevalent low rate, resulting in a steady intermediate rate of responding.

Results supporting the interpretation of the effects of the amphetamines in terms of rate dependencies have been described in some detail because this interpretation appears to have great generality. The direction of change in responding after amphetamines depends upon both dose and control rate of responding. Evidence from the experiments discussed indicates that rates of responding of about one response or more per sec are only decreased by increasing doses of amphetamines; lower rates of responding are increased to a maximum and then decreased by increasing doses of amphetamines. Some evidence suggests that when the pre-drug rates of responding are very low (about 0.1 response or less per sec), the peak of the dose-effect curve occurs at higher doses than when pre-drug rates are intermediate (about 0.4 response per sec). For example, in the study by CLARK and STEELE (1966) in the rat, after *d*-amphetamine the peak of the dose-response curve for rates of responding during the period of no reinforcement was to the right of the peak for average fixed-interval rates of responding. Also, in SMITH's (1964) study in the pigeon, rates of responding in the first minute of the 5-min fixed-interval schedule were maximally increased by the highest dose of *d*-amphetamine tested (10 mg/kg, intramuscularly).

There is a graded relation between the increase in low rates of responding and the decrease in high rates of responding after amphetamines. The available

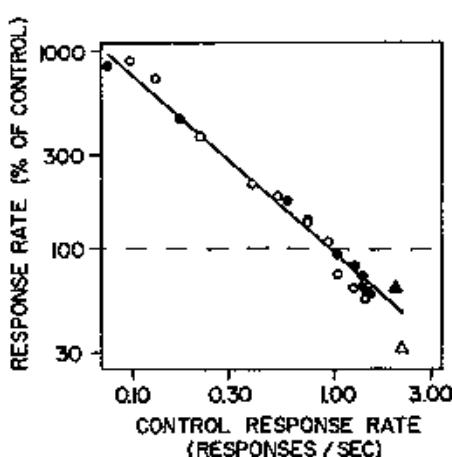


Fig. 7. Dependence of effect of *d*-amphetamine on pre-drug rate of responding in a squirrel monkey. Abscissa: average rate of responding in successive minutes of a 10-min fixed-interval schedule (circles) and under a 30-response fixed-ratio schedule (triangles); ordinate: percentage change in rate of responding after 0.3 mg/kg *d*-amphetamine, intramuscularly. Open and filled symbols indicate data from two different sessions. The line through the points was fitted by visual inspection. Based on data of a single monkey used in computing the averaged data under FI and FR shock termination in Fig. 6

evidence suggests that the change in rate of responding after an appropriate dose of an amphetamine is related to the pre-drug rate of responding in the way that is shown in Fig. 7. The proportional increase in rate of responding is an inverse function of the control rate; above a control rate of about one response per sec, the proportional decrease in rate of responding is directly related to the control rate. The two sets of data points in Fig. 7 are derived from the rates during complete sessions after *d*-amphetamine (0.3 mg/kg, intramuscularly) and the corresponding rates during the previous control sessions for a monkey under a multiple fixed-interval fixed-ratio schedule of stimulus-shock termination. Rates of responding were separately recorded during the fixed-ratio component and during successive minutes of the 10-min fixed-interval component. This same functional relation has been found in several different species under conditions in which different pre-drug rates of responding were engendered by different schedules of reinforcement or by sampling different temporal periods of a single schedule. This model of amphetamine action suggests that observed increases and decreases in responding do not reflect qualitatively different processes.

C. Exceptions to the rate-dependent effects of amphetamines

Although the effects of amphetamines are determined by control rates of responding under a wide variety of experimental conditions, exceptions to this general statement must be considered. First, responding that is maintained at a relatively low level under punishment does not increase following administration of amphetamine. The effects of drugs on punished responding will be discussed in detail in Section V of this review. Second, the rate-dependent effects of amphetamines depend not only upon the actual control rate but also upon the tendencies for different rates of responding to occur.

Under conditions in which responding has not previously occurred or has no programmed consequences, amphetamine may have little tendency to enhance very low rates of responding. For example, VERHAVE (1958) in untrained rats recorded lever-pressing responses during 12 daily 1-hr sessions in which responses had no programmed consequences. The mean rates of responding in consecutive sessions decreased from 15.7 responses per hour in the first session to 0.8 response per hour in the seventh session; three of the six rats did not respond at all in the seventh session. Following the administration of methamphetamine (2 mg/kg, subcutaneously) before the eighth session, the mean rate of responding remained 0.8 response per hour, and four of the six rats did not respond. In the eleventh session, the mean rate of responding was 0.8 response per hour, and five of the six rats did not respond. Methamphetamine (4 mg/kg, subcutaneously) was administered before the twelfth session, and none of the rats responded. In contrast, when responses postponed electric

shock, methamphetamine markedly increased responding; this effect was obtained even in the early stages of training when little responding occurred.

When responding has been decreased to very low levels by nonreinforcement, factors other than control rate must be considered. Given equally low rates, amphetamines have less tendency to enhance responding in individuals that have no history of responding than in individuals that have responded; amphetamines have less tendency to enhance responding when there are no consequences maintaining responding than when there are. For example, DEWS (1955b) studied the effects of methamphetamine on well-established discriminative performances in the pigeon and found that methamphetamine in doses of 0.1 to 3 mg, intramuscularly, did not increase rates of responding in the presence of a visual stimulus in which food was never presented, and key-pecking responses had no programmed consequences.

In contrast to DEWS's (1955b) finding, CLARK and STEELE (1966) found that *d*-amphetamine did increase low rates of responding during a stimulus condition in which food was never presented. Although the conditions of an experiment may specify that food presentation does not occur in the presence of a stimulus, there may still be adventitious consequences that maintain responding in its presence. When food presentation occurs in the presence of a discriminative stimulus, the onset of that stimulus may itself act as a reinforcer to maintain responding that had preceded it. Thus, the appearance of such a stimulus could adventitiously reinforce responses occurring during a previous period in which food was never presented. In the experiment of CLARK and STEELE (1966), the control cumulative response records indicate that responses often occurred in the period just before the onset of the white light associated with the fixed-interval schedule of food presentation. During the training phase of DEWS's experiment, however, each response occurring during the period in which food was never presented prolonged that period. Although it is impossible to say whether the differences in training procedure could account for the different effects of amphetamines in the two studies, the seeming inconsistencies might have depended on the differences in past schedule conditions during the periods in which food was never presented. Careful examination of procedures and results is necessary to determine when responding is a result of past schedule conditions. Further studies of this problem are needed.

D. Rate-dependent effects of the barbiturates

The effects of the barbiturates also seem to be dependent upon the pre-drug rates of responding. As we have already noted, the dose of barbiturates required to decrease rates of responding depends upon both the type of reinforcement schedule and the schedule parameter that is used. At doses lower than those that decrease responding, barbiturates generally tend to increase rates of responding engendered by various schedules of reinforcement (DEWS, 1955a,

b, 1956, 1958b, 1964; SIDMAN, 1956; MORSE and HERRNSTEIN, 1956; MORSE, 1962; WEISS and LATIES, 1964; REID *et al.*, 1964; RUTLEDGE and KELLEHER, 1965). This rate-increasing effect appears to be similar to that described for the amphetamines, except that low doses of barbiturates have been found to further increase already high rates of responding (DEWS, 1955a; KELLEHER *et al.*, 1961; WALLER and MORSE, 1963).

WALLER and MORSE (1963) studied the effects of pentobarbital on the key-pecking response of the pigeon under a 30-response fixed-ratio schedule of food presentation. They selected birds having pre-drug rates of responding that ranged from 0.10 to 3.18 responses per sec; 10 of the 13 birds studied had rates higher than 1.50 responses per sec. The mean control rate of responding of 2.02 responses per sec was increased to 2.67 responses per sec following the intramuscular administration of 2 mg of pentobarbital per bird. The magnitude of the proportionate increase in rate of responding caused by pentobarbital was inversely related to the pre-drug rates of responding of the individual birds. For example, control rates of responding of 0.15, 2.21, and 3.72 responses per sec were increased respectively to rates of 1.94 (129%), 3.83 (173%), and 4.53 (122%). Thus, the failure of the amphetamines to increase relatively high rates of responding (more than 1.0 response per sec) is not due to a physical limitation on rates of responding.

The effects of amobarbital on diverse rates of responding in individual pigeons have been described by DEWS (1964). Each bird responded under a 500-sec fixed-interval schedule of food presentation. This schedule was repeatedly interrupted by the presentation of an additional white light, which was never present when a response was reinforced. Each 500-sec interval was divided into 10 segments of 50 sec each; the additional light was on only during the 1st, 3rd, 5th, 7th, and 9th 50-sec segments of the interval. The mean rate of responding in the presence of the light (0.01 response per sec) was much lower than in its absence (0.63 response per sec). Since the presence of this light suppressed responding, DEWS (1964) notes that it could be considered an inhibitory stimulus according to the common usage of that term. Amobarbital was administered intramuscularly in doses ranging from 6 to 60 mg/kg; doses of 20 or 33 mg/kg caused consistent increases in rates of responding. The relative increase in rate was very much greater in the presence of the additional light than in its absence. For example, after 33 mg/kg the rate in the presence of the light increased to 28 times the control level, while the rate in the absence of the light increased to only 1.87 times the control level. This result indicates that the rate-dependent effects of amobarbital, like those of amphetamine, occur even when different patterns of behavior are observed over brief periods of time.

Because DEWS measured rates of responding in each consecutive 25 sec of the 500-sec fixed-interval schedule, the rate-dependent effects of amobarbital could be examined directly. Under control conditions, rates of responding

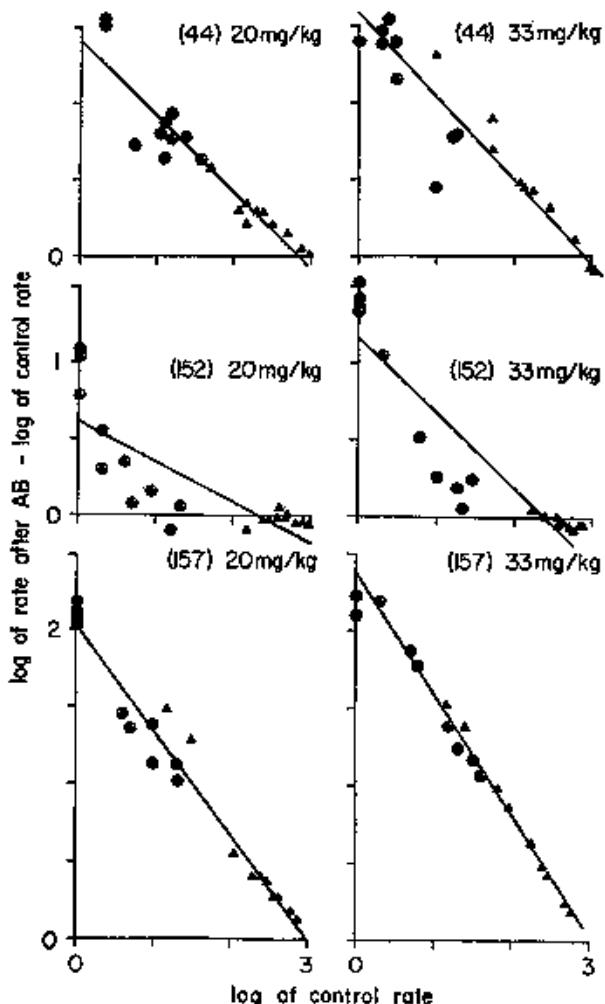


Fig. 8. Dependence of effect of amobarbital on pre-drug rate of key-pecking in the pigeon. Abscissa: log of rate as responses per session (total of 500 sec, since each 25-sec period occurred once in each of 20 cycles). Ordinate: change in log rate following amobarbital, intramuscularly. Since the 500-sec interval was divided for recording purposes into 20 periods of 25 sec, there are 20 points on each graph, 10 representing periods when the light was present (circled triangles) and 10 representing periods when the light was not present (triangles). The line through the points was calculated by least squares from all points. On the $X = 0$ ordinate are plotted all points where the mean rate of responding was 1 or less responses per 500 sec; this arbitrary assignment makes possible the logarithmic plot. Exclusion of these indeterminant points would not appreciably affect the regression line. From Dews (1964)

ranged from near zero at the beginning of the interval to more than 1 response per sec in some 25-sec segments near the end of the interval. For each of the three birds, Dews plotted the relative increase in rate of responding caused by appropriate doses of amobarbital in each 25-sec segment of the fixed-interval schedule against the control rate of responding in each segment. The results obtained at both doses that increased rates of responding are shown in Fig. 8. A linear regression line was calculated by the method of least squares for all of the points in each graph. The magnitude of the increase in rate of responding

after amobarbital was inversely related to the control rate of responding. Further, since the points representing periods in which the additional light was present are distributed around the regression line in the same way as the points representing periods in which it was absent, the effect of the drug was more dependent upon the control rate of responding than upon whether the "inhibitory" light was present. The relative increases in rates of responding following appropriate doses of barbiturates have generally been found to be inversely related to control rate of responding. Although this same general relationship was also shown for amphetamines, doses of barbiturates that increased low rates of responding seemed to have less tendency than doses of amphetamines to decrease high rates of responding (see Fig. 7).

This difference between the rate-dependent effects of the amphetamines and barbiturates has been shown in other studies (DEWS, 1955a; KELLEHER *et al.*, 1961). For example, under multiple fixed-interval fixed-ratio schedules the rate of responding under the fixed-interval component increased to a maximum and then decreased following increasing doses of either *d*-amphetamine (SMITH, 1964; KELLEHER and MORSE, 1964) or pentobarbital (RUTLEDGE and KELLEHER, 1965). Under the same multiple schedules, the rate of responding under the fixed-ratio component decreased with increasing doses of *d*-amphetamine, but was not suppressed by pentobarbital except in doses that decreased responding under the fixed-interval component (see also Fig. 4). Thus, the rate-increasing and rate-decreasing effects of barbiturates appear to depend not only upon the control rate of responding but also upon the type and the parameter value of the schedule (MORSE, 1962). Over a wide range of doses and under different schedule conditions, amphetamines have the same kind of rate-dependent effects whereas barbiturates do not.

E. Effects of combinations of amphetamines and barbiturates

Several studies have investigated the behavioral effects of combinations of amphetamines and barbiturates. STEINBERG *et al.* (1961) and RUSHTON and STEINBERG (1963) found that combinations of amphetamine and amobarbital in selected doses caused a greater increase in locomotor activity of rats than the sum of the maximal increase of either drug administered alone. The increase in activity caused by the drug combination was not obtained with rats that had previously been exposed to the apparatus (RUSHTON *et al.*, 1963).

WEISS and LATIES (1964) studied, in the dog, the effects of amphetamine and pentobarbital on the response of pressing a key with the nose under a schedule in which food was presented whenever the cumulative duration of key-pressing responses exceeded a specified minimum time period. The average frequency of pressing responses and the average duration of pressing responses were recorded. Combinations of amphetamine and pentobarbital increased the frequency of pressing responses and decreased the average duration of

responses more than either drug alone. In this study, as in the studies with rat locomotor activity, the pre-drug rates of responding were relatively low. STEINBERG *et al.* (1961) and RUSHTON *et al.* (1963) had suggested that low levels of locomotor activity were caused by fear and anxiety associated with introduction to a novel environment and that amphetamine-barbiturate combinations might have increased activity by alleviating fear and anxiety. This interpretation would not account for the results of WEISS and LATIES (1964) in the dog. It seems more likely that the results simply reflect a mutual enhancement of the tendency of both drugs to increase initially low rates of responding.

RUTLEDGE and KELLEHER (1965) studied the effects of methamphetamine and pentobarbital on the pecking response of the pigeon under a multiple schedule of food presentation with 5-min fixed-interval and 34-response fixed-ratio components. The effects of combinations of the drugs depended upon the schedule of reinforcement. Under the fixed-interval schedule, which maintained a low average rate of responding, combinations of methamphetamine and pentobarbital increased rates of responding more than the sum of the maximal increases caused by either drug alone. Under the fixed-ratio schedule, which maintained a high average rate of responding, the rate-decreasing effects of methamphetamine were antagonized by pentobarbital. Thus, combinations of methamphetamine and pentobarbital can either antagonize or enhance the effects of the individual drugs on patterns of responding. The type of interaction that occurs appears to depend upon the schedule-controlled pre-drug patterns of responding.

F. Rate-dependent effects of other drugs

The results of many studies suggest that the effects of drugs other than amphetamines or barbiturates are at least partially dependent upon pre-drug rates of responding. In addition to his study of *d*-amphetamine, described in Section IV B, SMITH (1964) studied the effects of cocaine, pipradrol, and imipramine on the pecking response of the pigeon under a multiple schedule of food presentation comprising a fixed-ratio component and a fixed-interval component. Although these drugs also increased low rates of responding and decreased high rates of responding, their effects differed quantitatively from those of amphetamine. [In contrast to its effects in many species, imipramine consistently increases rates of key-pecking in the pigeon under several schedule conditions (DEWS, 1962; COOK and KELLEHER, 1962; VAILLANT, 1964b).] Intramuscular doses of cocaine (1—10 mg/kg) and imipramine (0.3—10 mg/kg), which increased average rates of responding in the fixed-interval component, decreased rates of responding in the fixed-ratio component; doses of pipradrol (0.3—10 mg/kg), which increased average fixed-interval rates, did not significantly decrease fixed-ratio rates of responding. Neither cocaine nor pipradrol

was as effective as *d*-amphetamine in increasing rates of responding in the first minute of the fixed-interval; however, both cocaine and pipradrol had only slight rate-decreasing effects in the last minute of the fixed-interval. The sum of these effects on rates of responding in different parts of the fixed-interval schedule was that cocaine and pipradrol caused larger increases in average fixed-interval rates of responding than did *d*-amphetamine. Imipramine was comparable to *d*-amphetamine in decreasing rates during the last minute of the fixed-interval, but caused much larger increases than *d*-amphetamine on rates during the first minute (cf. DEWS, 1962); thus, imipramine caused larger increases in average fixed-interval rates of responding than *d*-amphetamine.

Abundant evidence indicates that the minor tranquilizing drugs, meprobamate and chlordiazepoxide, also increase low rates of responding. Under appropriate parameter values of several schedules of food presentation, such as fixed-interval schedules, variable-interval schedules, and reinforcement only of long interresponse times, it has been found that rates of responding are increased by meprobamate (KELLEHER *et al.*, 1961) and chlordiazepoxide (RICHELLE, 1962, 1963; RICHELLE *et al.*, 1962; RICHELLE and DJAHANGUIR, 1964). Meprobamate can increase responding when each response produces intracranial stimulation (OLDS, 1959; OLDS and TRAVIS, 1960). Doses of meprobamate that increased lower rates of responding under several different schedules did not affect high rates of responding (3.5 responses per sec) under a fixed-ratio schedule (KELLEHER *et al.*, 1961). COOK and KELLEHER (1962) and COOK and CATANIA (1964) studied the effects of meprobamate and chlordiazepoxide on the behavior of squirrel monkeys under a multiple fixed-interval fixed-ratio schedule of food presentation. In both studies, it was found that both meprobamate and chlordiazepoxide increased average fixed-interval rates of responding. Meprobamate had little effect on fixed-ratio rates, but COOK and CATANIA (1964) found that chlordiazepoxide produced graded decreases in fixed-ratio rates of responding at doses that increased fixed-interval rates.

In Section III C 3 we described experiments by COOK and CATANIA (1964) in which meprobamate (400 mg/kg, orally) and chlordiazepoxide (10 mg/kg, orally) produced comparable increases in rates of responding under fixed-interval schedules of food presentation and fixed-interval schedules of termination of electric shock. Under the fixed-interval schedule of termination, they found that the effects of both meprobamate and chlordiazepoxide depended upon the mean control rate of responding, which in turn depended upon the intensity of the electric shock. The magnitude of the increase in relative rate of responding was inversely related to the control rate of responding for both drugs, except for the highest control rates, which were slightly decreased by both drugs. The rate-dependent effects of meprobamate and chlordiazepoxide appear to be similar to those of the amphetamines and barbiturates but have not been as thoroughly studied. Meprobamate and chlordiazepoxide are more

like amphetamines than barbiturates in causing decreases in high rates of responding, but more like barbiturates than amphetamines in increasing responding that has been suppressed by punishment.

V. Effects of drugs on punished behavior

In recent years the suppression of behavior by punishment has been a topic of both behavioral and pharmacological interest. Punishment is defined in the same way as reinforcement, but the behavioral change is in the opposite direction (see Section II E). A punisher is an event that decreases the subsequent occurrence of responses that preceded the event; presentation of a punisher following a response is punishment. The punisher most commonly used in experimental studies is electric shock. Most techniques for studying the suppression of responding by punishment require a measurable level of ongoing responding, which is usually maintained by a schedule of reinforcement. [For a discussion of recent experiments on punishment, see AZRIN (1960) and AZRIN and HOLZ (1966).]

The effects of drugs on punished responding have been actively studied since GELLER and his co-workers, as well as a number of other investigators, reported that drugs such as meprobamate, chlordiazepoxide, barbiturates, and other minor tranquilizers can be distinguished from chlorpromazine and other major tranquilizers on the basis of their effects on punished responding.

The use of electric shock to suppress responding has always been popular in behavioral pharmacology. The consistency of the pharmacological results obtained using punishment procedures is especially interesting when contrasted with the inconsistent results obtained in the many studies on the suppression of responding under the widely used procedure developed originally by ESTES and SKINNER (1941).

A. Effects of drugs on responding under the Estes-Skinner procedure

ESTES and SKINNER found that ongoing responding maintained under a fixed-interval schedule of food presentation in the rat was suppressed in the presence of an auditory stimulus (pre-shock stimulus) which regularly preceded an unavoidable electric shock. They considered the suppression to be caused by an emotional state of anxiety, which interfered with responding. The magnitude of the suppression was assumed to be a quantitative measure of the anxiety state. Subsequent studies have shown that a pre-shock stimulus can suppress responding of various other species under various schedules of reinforcement (for example, BRADY, 1955; AZRIN, 1956; VALENSTEIN, 1959), though the degree of suppression depends on the experimental conditions (STEIN *et al.*, 1958; CARLTON and DIDAMO, 1960). The general procedure of superimposing

a stimulus terminating with a noxious stimulus on a baseline of ongoing behavior is called the Estes-Skinner procedure.

The Estes-Skinner procedure initially attracted interest in pharmacology because the suppression was said to be due to anxiety. There is no generally accepted definition of "anxiety." Clinically, anxiety is usually characterized on the basis of the verbal reports and demeanor of patients. For many psychologists, anxiety is a drive — something that will sustain avoidance behavior. Other psychologists characterize anxiety as an emotional state which affects a number of different behavioral activities simultaneously and nonspecifically. The suppression of responding under the Estes-Skinner procedure conforms to this last usage of the term "anxiety." It is still not possible to use the term interchangeably in its different meanings, and attempts to do so distract from the careful comparison of specific phenomena which is the only sound basis for generalization.

Many studies have been conducted to determine whether drugs, particularly tranquilizing drugs, would increase responding that had been suppressed under the Estes-Skinner procedure (for example, BRADY, 1956; HUNT, 1956, 1957; GATTI, 1957; HILL *et al.*, 1957b; VALENSTEIN, 1959; BOREN, 1964; YAMAHIRO *et al.*, 1961; KINNARD *et al.*, 1962; LAUENER, 1963; RAY, 1964). Reports that reserpine increased responding in the presence of a pre-shock stimulus (BRADY, 1956; RAY, 1964) were not confirmed in different experiments (YAMAHIRO *et al.*, 1961; KINNARD *et al.*, 1962). Similarly, a report that morphine increased responding in the presence of a pre-shock stimulus (HILL *et al.*, 1957b) was not corroborated under other conditions (LAUENER, 1963). Although it has been generally reported that chlorpromazine, meprobamate, and amphetamine do not increase responding that has been suppressed in the presence of a pre-shock stimulus (BRADY, 1956; GATTI, 1957; BOREN, 1961; KINNARD *et al.*, 1963; RAY, 1964), one recent study does indicate that meprobamate, as well as some barbiturates and chlordiazepoxide, can increase responding under such conditions (LAUENER, 1963). The lack of generality in these results has been disappointing to investigators who assumed both that suppressed responding in the presence of a pre-shock stimulus is an animal analogue of anxiety and that tranquilizing drugs have specific effects on anxiety. The different findings undoubtedly resulted from the use of different schedules, shock intensities, and other parameter values. It is now understood that these procedural differences are more important determinants of the action of a drug than the supposed common component of anxiety.

Many variables inherent in the Estes-Skinner procedure are poorly understood. For example, STEIN *et al.* (1958) found in the rat that the degree of suppression of responding in the presence of a pre-shock stimulus depends upon the relative duration of the presence or absence of the pre-shock stimulus; the suppression was greatest when the pre-shock stimulus was present for relatively

short periods of time. The degree of suppression also depends upon the schedule of reinforcement that is used to maintain responding (BRADY, 1955). The rate of responding in the presence of a pre-shock stimulus can also be determined by the experimental history of the individual; for example, if the individual has previously responded to postpone electric shocks, responding during the presentation of the pre-shock stimulus is enhanced rather than suppressed (SIDMAN *et al.*, 1957; HERRNSTEIN and SIDMAN, 1958; KELLEHER *et al.*, 1963; WALLER and WALLER, 1963). When it is considered that the Estes-Skinner procedure engenders such a variety of rates and patterns of responding, the diverse effects of drugs are not surprising.

The suppression of behavior under the Estes-Skinner procedure is probably the result of interacting behavioral processes. It has usually been interpreted as a disruption of ongoing operant behavior by conditioned responses that have been established by Pavlovian conditioning (ESTES and SKINNER, 1941; HUNT and BRADY, 1955). According to this interpretation, a noxious stimulus (usually an electric shock) elicits both skeletal muscle responses and autonomic responses that interfere with ongoing operant responses. When a distinctive stimulus has preceded the shock repeatedly, it is assumed that this pre-shock stimulus alone becomes a conditioned stimulus and elicits the responses which interfere with ongoing operant responses (conditioned suppression).

The Estes-Skinner procedure can also be viewed as a punishment procedure in which the relations between responses and the noxious stimulus do not follow a prearranged schedule. The temporal relation between the pre-shock stimulus and the noxious stimulus is fixed, but because shocks are presented independently of the operant responses, temporal relations between responses and electric shocks will depend on the pattern of responding (adventitious punishment). AZRIN (1956) directly compared adventitious punishment under the Estes-Skinner procedure with immediate punishment. He found a greater degree of suppression when shocks immediately followed responses (immediate punishment) than when shocks followed responses after unspecified delays (adventitious punishment).

In any experiment using the Estes-Skinner procedure, both conditioned suppression and adventitious punishment may be involved to varying degrees. Further, after responding has been suppressed by the Estes-Skinner procedure, it may remain suppressed for other reasons even when electric shock is discontinued. The suppression of responding in the presence of a pre-shock stimulus usually results in a decrease in the frequency of reinforcement in that stimulus condition. As MORSE and SKINNER (1957) have shown, responding may be suppressed in the presence of a stimulus adventitiously correlated with a decreased frequency of reinforcement. It has been impossible to evaluate the relative contributions of various factors in most pharmacological studies using the Estes-Skinner procedure; the analysis of behavioral processes requires in-

formation on functional relations under different parameter values, which has been lacking. Nevertheless, because of the consistent effects of certain drugs on responding suppressed by punishment (discussed in Section V B, C), the results of pharmacological experiments can offer presumptive evidence on the role of adventitious punishment under the Estes-Skinner procedure.

B. Effects of drugs on responding under immediate punishment procedures

In contrast to the varied results obtained with the Estes-Skinner procedure, pharmacological results with immediate punishment procedures have been highly consistent (see COOK and KELLEHER, 1963). For example, with different types of ongoing behavior (unconditioned and conditioned), different species (pigeons, rats, cats, monkeys), and different schedule parameters and shock intensities, the following drugs consistently increase responding that has been suppressed by immediate punishment: pentobarbital, amobarbital, phenobarbital, meprobamate, chlordiazepoxide (JACOBSEN, 1957; NAESS and RASMUSSEN, 1958; GELLER, 1962; GELLER and SEIFTER, 1960, 1962; GELLER *et al.*, 1962, 1963; MORSE, 1964; KELLEHER and MORSE, 1964). A study by NAESS and RASMUSSEN (1958) illustrates the punishment of behavior not explicitly conditioned. Drinking in the water-deprived rat was suppressed by the delivery of an electric shock each time that the rat licked the water tube. When the rats had been given amobarbital or meprobamate, they drank despite the repeated delivery of electric shocks.

Most studies of punishment have been concerned with the effects of drugs on the suppression of ongoing responding maintained under a schedule of reinforcement. Several studies by GELLER and his co-workers used a procedure, in the food-deprived rat, in which lever-pressing responses were maintained under a 2-min variable-interval schedule of food presentation in the absence of a tone, while each response occurring in the presence of a tone was followed by the presentation of both food and electric shock. The degree of suppression of responding in the presence of the tone was directly related to the intensity of the electric shock. Some shock intensities almost completely suppressed responding in the presence of the tone; lower shock intensities were less suppressing. Even at the higher shock intensities, meprobamate, chlordiazepoxide, phenobarbital, pentobarbital, hedonal, emylcamate, and urethane increased responding that had been suppressed in the presence of the tone (GELLER and SEIFTER, 1960, 1962; GELLER *et al.*, 1962). Similar results were obtained in the monkey with meprobamate and chlordiazepoxide (COOK and CATANIA, 1964). On the other hand, phenothiazine tranquilizers of varying degrees of potency (promazine, chlorpromazine, and trifluoperazine) did not increase responding that had been suppressed by punishment. Indeed, these drugs further decreased the intermediate rates of responding in the presence of the tone at

lower shock intensities (GELLER *et al.*, 1962). Similar decreases were produced by morphine (GELLER *et al.*, 1963) and by *d*-amphetamine (GELLER and SEIFTER, 1960).

C. Interpretations of the effects of drugs on suppressed responding

The consistency of the effects of drugs on responding under diverse immediate punishment procedures suggests that these effects may be specifically related to the process of punishment. It is useful to compare the effects of drugs on suppressed responding under the Estes-Skinner and under immediate punishment procedures. If the Estes-Skinner procedure involved only adventitious punishment, the effects of drugs on responding under these two procedures should be qualitatively similar. But only in the study by LAUENER (1963) were several drugs shown to affect responding suppressed under the Estes-Skinner procedure in the same way as responding suppressed under immediate punishment procedures. LAUENER's experimental parameters differed in two ways from those commonly used under the Estes-Skinner procedure. First, the duration of the compound auditory and visual pre-shock stimulus was variable and relatively short (15 sec to 1 min). Second, LAUENER used a 5-sec fixed-interval schedule of water presentation, which engendered a relatively steady rate of responding (0.17 response per sec). Thus, in LAUENER's study responses occurred during the pre-shock stimulus in fairly close temporal proximity to presentations of the electric shock. The pharmacological results support the view that the degree of suppression maintained in LAUENER's study was primarily due to adventitious punishment. In contrast, in many experiments with the Estes-Skinner procedure, the suppression may have been partly determined by the generalized effects of the presentation of an electric shock. This analysis suggests that the generalized suppression obtained with the Estes-Skinner procedure, usually attributed to incompatible autonomic and skeletal muscle responses, is not reliably attenuated by any drug that has been studied. On the other hand, responding suppressed by punishment can be markedly increased by drugs such as pentobarbital, meprobamate, and chlordiazepoxide. Although both the Estes-Skinner procedure and the immediate punishment procedure involve suppression of behavior controlled by scheduled presentations of electric shock, the pharmacological results suggest that different behavioral processes are usually involved in these two types of suppression (KELLEHER and MORSE, 1964).

Motivational interpretations have assumed that behavior maintained by the termination of noxious stimuli and behavior suppressed by the presentation of noxious stimuli both reflect common components of fear and anxiety. This assumption has a plausible generality because it can account for both increases and decreases in behavior with a unitary motivational concept. For example, SOLOMON (1964) suggests that "what holds for punishment and its action

on behavior should hold also for escape and avoidance training, and vice versa". This assumption is not supported by the results of pharmacological studies. Some drugs, such as chlorpromazine, which are particularly effective in decreasing responding maintained by the termination of a noxious stimulus, are ineffective in increasing responding suppressed by immediate punishment. Other drugs, such as meprobamate, which are relatively ineffective in decreasing responding maintained by the termination of a noxious stimulus, are particularly effective in increasing responding suppressed by immediate punishment. Despite the cogency of psychological theories, drugs which affect punished behavior do not necessarily affect escape or avoidance behavior, and vice versa. This pharmacological separation of behavior maintained by noxious stimuli and behavior suppressed by noxious stimuli is an important instance of the use of drugs as tools for analyzing behavioral processes.

The question remains as to why some drugs increase responding that has been suppressed by punishment. We do not have as yet a satisfactory answer to this question, but some possibilities have been excluded. Drugs which attenuate suppression caused by punishment do not act by raising the threshold response to electric shock. None of the drugs that are effective in attenuating suppression is regarded as an effective analgesic drug, and morphine, a classic analgesic drug, does not increase responding suppressed by immediate punishment (GELLER *et al.*, 1963; KELLEHER and MORSE, 1964).

It may seem surprising at first that morphine has no significant tendency to increase responding suppressed by immediate punishment. In addition to its analgesic action, morphine is considered to alleviate fear and anxiety, but the analysis of the effects of drugs in terms of their alleged actions on motivational states has not made it possible to predict their effects on behavior. As we have already noted, major tranquilizers, such as chlorpromazine and trifluoperazine, do not increase responding suppressed by punishment, so there is no reason to expect morphine to do so through its tranquilizing effects.

Two important considerations help in clarifying the lack of relation between the analgesic properties of morphine and its failure to increase responding suppressed by punishment. First, in the absence of noxious stimuli, morphine decreases rates of responding maintained under various schedules of reinforcement at dose levels below those required to show analgesic effects (EDWARDS, 1961). Thus, the effects of morphine should not be expected to be confined simply to an analgesic action. Second, when punishment is discontinued, an immediate increase in the level of responding previously suppressed by punishment does not regularly occur. Inasmuch as the complete removal of electric shocks does not increase responding that has been suppressed, it is unreasonable to expect morphine to do so through its analgesic action. Using a punishment schedule in which each response was followed by an electric shock, AZRIN (1960) found in the pigeon, that the suppression of schedule-controlled key-pecking

continued after the intensity of the shock was greatly decreased; the rate of punished responding gradually increased to a new level over several daily sessions. If the electric shock has been intense enough to produce an almost complete suppression of responding, or if it has been presented intermittently, then even the abrupt omission of electric shocks will not immediately increase responding that has been suppressed by immediate punishment (AZRIN, 1960; AZRIN *et al.*, 1963; KELLEHER and MORSE, 1964). Although systematic experiments on omission of electric shock under the Estes-Skinner procedure have not been reported, BRADY (1956) found that suppression during the pre-shock stimulus was maintained when more than half of the scheduled shocks were omitted.

KELLEHER and MORSE (1964) studied the effects of morphine on punished responding in the pigeon under a multiple schedule in which punishment and nonpunishment components alternated. A fixed-ratio schedule of food presentation was in effect in both components. Rates of 2 to 4 responses per sec prevailed in nonpunishment components, but responding seldom occurred in punishment components. Morphine was given in intramuscular doses of 1, 3, and 10 mg/kg; none of these doses increased responding in the punishment component. After 3 mg/kg of morphine, rate of responding in the nonpunishment component was slightly decreased; after 10 mg/kg, almost all responding ceased, although the pigeon would still eat when food was presented. In one experiment, 1 mg/kg of morphine was given before a session which began with scheduled electric shocks omitted from punishment components. Even when electric shocks were omitted, the rate of responding after morphine was lower in punishment components than in nonpunishment components. Further, when scheduled shocks were reinstated later in the same session, responding was immediately suppressed in the punishment component. Morphine neither renders electric shocks ineffective nor prevents the redevelopment of suppression. It is clear that, unlike pentobarbital or meprobamate, morphine fails to increase responding suppressed by punishment. The morphine experiments and the behavioral experiments on the omission of scheduled electric shocks indicate that drugs do not attenuate the suppression caused by punishment through an analgesic action.

It has been shown that drugs do not increase responding suppressed by punishment through disruption of control by discriminative stimuli. The effects of drugs on simultaneously punished and reinforced responding have usually been studied with multiple schedules under which responding is punished in the presence of one stimulus (punishment component), but not in the presence of another stimulus (nonpunishment component). Such multiple schedules permit the simultaneous observation of the effects of drugs on schedule-controlled responding that is punished and schedule-controlled responding that is not punished in individual animals. Multiple schedules may, however, produce interactions between punished and nonpunished responding, and it has been

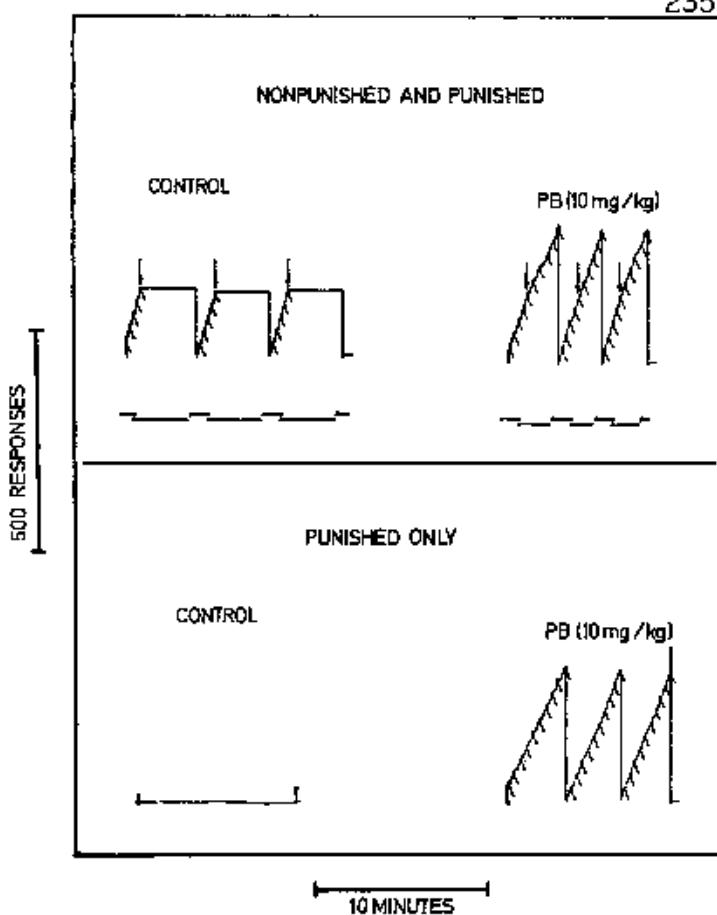


Fig. 9. Effects of pentobarbital (PB) on key-pecking suppressed by punishment. Each frame shows a complete control session followed by a complete drug session for pigeon number 235. The drug was given intramuscularly 15 min before the beginning of drug sessions. Upper frame: in nonpunishment components (event record displaced upward) a 30-response fixed-ratio schedule of food presentation was in effect in the presence of an orange light. The termination of each nonpunishment component is indicated by a small arrow. In punishment components (event record displaced downward), a 30-response fixed-ratio schedule was in effect in the presence of a white light; each of the first 10 responses of each ratio produced a 35-msec electric shock of 6 mA, 60 cycles AC, delivered through gold wire electrodes that were implanted around the pubis bones of the bird. The termination of each punishment component is indicated by the resetting of the pen to the bottom of the record. Lower frame: the punishment procedure was in effect throughout each session in the presence of a white light. Note that under both procedures pentobarbital attenuates the suppression of responding by punishment. From KELLEHER and MORSE (1964); courtesy of Federation of American Societies for Experimental Biology, Bethesda, Md., U.S.A.

suggested that such interactions could account for the drug-induced increases in responding in punishment components. For example, a drug might change the control exerted by the stimuli correlated with each component. In two studies (MORSE, 1964; KELLEHER and MORSE, 1964) this possibility was excluded by experiments in which the punishment procedure alone was in effect throughout each session; it was found that amobarbital and pentobarbital still produced marked increases in rates of punished responding (see Fig. 9).

Effects of drugs on punished responding do not reflect mere nonspecific enhancement or diminution of levels of responding. For example, GELLER and SEIFTER (1960, 1962) report many instances in which meprobamate, phenobarbital, and pentobarbital increased responding during the punishment component and decreased responding during the nonpunishment component of a multiple schedule. It could also be argued that an underlying tendency of a drug such as chlorpromazine to increase responding suppressed by punishment might be masked by its general action in decreasing responding under many conditions. Yet there are species, such as the dog and the pigeon, in which chlorpromazine has little tendency to decrease responding over a wide range of doses and can even increase rates of responding (DEWS, 1958a, c; WALLER, 1961; FERSTER *et al.*, 1962; KELLEHER *et al.*, 1962). In one study in the pigeon (MORSE, 1964), a key-pecking response was well-maintained under a variable-interval schedule over a 30-fold range of doses of chlorpromazine in nonpunishment components, but no increases in rates of responding occurred in punishment components. In another study, KELLEHER and MORSE (1964) studied the effects of chlorpromazine on the key-pecking of pigeons under a 30-response fixed-ratio schedule in which nonpunishment and punishment components alternated. Chlorpromazine (10 and 30 mg/kg, intramuscularly) slightly increased the already high rate of responding in the nonpunishment component (2 to 4 responses per sec), but did not affect the near zero rate of responding in the punishment component. Thus, chlorpromazine does not increase responding that has been suppressed by punishment even under conditions in which it can increase rates of responding.

Some experimental evidence suggests that drugs that increase punished responding are acting selectively on behavior in punishment components, but it has not been established that these effects are specific to the behavioral processes of punishment. As we have indicated previously, the tendency of many drugs to enhance rate of responding depends upon the control level of responding. The dramatic effects of certain drugs in increasing responding suppressed by punishment may occur simply because control rates of responding are relatively low in punishment components. Many studies have provided data that are relevant to this question.

The effects of some drugs on responding suppressed by punishment cannot be interpreted simply in terms of rate-dependent effects. In Section IV A, B, we summarized results indicating that amphetamines have a general tendency to increase low rates of responding. Nevertheless, amphetamines do not increase low rates of responding engendered by the Estes-Skinner procedure (BRADY, 1956; LAUENER, 1963) or by immediate punishment procedures (GELLER and SEIFTER, 1960).

Indeed, the already low rates of responding engendered under these procedures are often further decreased by amphetamine while responding in the

absence of the pre-shock stimulus or the punishment component is increased (GELLER and SEIFTER, 1960). Similarly, chlorpromazine and imipramine, which increase low rates of responding maintained by food presentation in the pigeon, failed to increase responding that had been suppressed by punishment (MORSE, 1964; KELLEHER and MORSE, 1964). The results with amphetamine, chlorpromazine, and imipramine show that some drugs that can increase low rates of responding under appropriate experimental conditions fail to increase responding that has been suppressed by immediate punishment or adventitious punishment (Estes-Skinner procedure).

All the drugs that increased punished responding have, however, been found to increase low rates of responding engendered in other ways. Phenobarbital, pentobarbital, amobarbital, meprobamate, and chlordiazepoxide characteristically increase responding under schedules of reinforcement that engender low rates of responding. COOK and CATANIA (1964) attempted to determine in the squirrel monkey whether the actions of meprobamate and chlordiazepoxide in increasing responding suppressed by punishment could be separated from the rate-dependent effects of these drugs. They studied a multiple schedule in which the schedule parameter had been selected to produce the same average rate of lever-pressing responses in the punishment and nonpunishment components. Responses in the punishment component were maintained under both a 2-min variable-interval schedule of food presentation and an independent 2-min variable-interval schedule of presentation of electric shock; responses in the nonpunishment component were maintained under a more intermittent 6-min variable-interval schedule of food presentation. Although the average control rates of responding in each component were the same, COOK and CATANIA (1964) found that increasing doses of meprobamate or chlordiazepoxide had more marked rate-enhancing effects in the punishment component than in the nonpunishment component. Their finding suggests that these drugs may have specific effects on the behavioral processes involved in punishment that cannot be explained simply in terms of rate-dependence. There is a clear need for further investigation of the relations between the rate-dependent effects of drugs and their effects on responding suppressed by punishment.

VI. Conclusions

The effects of drugs on behavior depend on the pattern of behavior maintained, but are generally independent of the events maintaining the behavior. Different patterns of responding maintained by the same reinforcer are affected differently by drugs. For example, under a multiple fixed-ratio fixed-interval schedule of food presentation, an appropriate dose of an amphetamine will decrease the rate of responding under the fixed-ratio component, while increasing the rate of responding under the fixed-interval component. Similar patterns of responding maintained by different reinforcers are affected similarly

by drugs. For example, the effects of amphetamines on performance under a multiple fixed-ratio fixed-interval schedule are obtained with responding maintained by termination of a stimulus associated with scheduled electric shocks, as well as responding maintained by food presentation. Seemingly qualitative differences in the effects of drugs on performances maintained by different reinforcers disappeared when control rates and patterns of responding were made comparable.

Exceptions to the general independence of effects of drugs on events maintaining behavior have been found when responding was suppressed by punishment. Morphine, the phenothiazine tranquilizers, and amphetamines tend only to further decrease responding suppressed by punishment; in contrast, many barbiturates, meprobamate, and chlordiazepoxide can markedly increase responding suppressed by punishment. These drug-induced increases in punished responding are not the result of an analgesic action, a generalized enhancement of levels of responding, or a disruption of control by discriminative stimuli. Although definitive experiments are still lacking, the consistency with which drugs affect punished responding under diverse conditions suggests that these effects may be specifically related to the process of punishment.

Rate of responding is an especially important aspect of patterns of responding. The way in which many drugs modify responding is quantitatively related to the level of responding before the drug was given; in fact, the rate of responding is a principal determinant of the behavioral effects of drugs. Early work in behavioral pharmacology showed that the effects of drugs on behavior depended upon past and present environmental conditions. It is now clear that differences in the effects of drugs on responding under different schedules of reinforcement are dependent upon the rates of responding engendered by the schedules. Predictions can often be made simply from knowing the absolute rate of responding. Thus, a rate of 3 responses per sec is a performance that would be likely to be decreased by an appropriate dose of amphetamine, while a rate of 0.3 response per sec would be likely to be increased by the same dose. Many of the seemingly qualitative differences in the effects of the drug on different performances result from a quantitative difference in pre-drug rates of responding.

Quantitative information about the effects of drugs on schedule performances is of interest and value mainly to those working directly in the field of behavioral pharmacology; yet the importance of the actual behavior that is occurring should be recognized by anyone concerned with the effects of drugs. For example, the view that the actions of drugs on behavior can be best predicted from knowledge about the ongoing behavior is compatible with clinical observations that the effectiveness of major and minor tranquilizers depends more upon "target symptoms" than upon psychiatric nosology.

In evaluating the results of pharmacological experiments on behavior one should look for quantitative relations between doses of the drug and numerical values of the behavior. Where such information is not presented, it is unlikely that meaningful conclusions can be drawn. One does not need to be an expert on matters dealing with behavior to apply this rule in evaluating experimental results, and the need to apply it cannot be overemphasized. For example, there has been much recent interest in the possibility that drugs affect learning. The numerous current reports showing that certain drugs can enhance performance in specific situations are reminiscent of previous reports of the effects of tranquilizers on responding controlled by noxious stimuli. In order to establish that a drug has a fundamental effect on the rate at which behavior is modified by environmental conditions, at the very least it is necessary to determine quantitatively how the drug effect is changed under important interventions. Such factors as the physical characteristics of the response, the effectiveness of the reinforcer, and the temporal delay between the response and the reinforcer influence the acquisition (or loss) of any new pattern of behavior. The advantage of describing results in fundamental quantitative terms is that they take on an applicability beyond the situation in which they were observed.

The purpose of this review has been to describe basic characteristics of behavioral pharmacology in such a way that interested physiologists, pharmacologists, and psychologists without specialist knowledge in behavioral pharmacology could understand the significance of experimental findings in this field. Because previously held beliefs have been tested experimentally and found wanting, they are disappearing and being replaced by new ones. Many of the earlier interpretations of the behavioral effects of drugs seemed easy to understand because they were easily, perhaps too easily, translated to and from colloquial language. Behavioral pharmacology is becoming a more technical and quantitative subject. The concepts may be less easily understood by those not directly involved, but they provide a more general account of the behavioral effects of drugs.

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Appendix

Amobarbital: 5-Ethyl-5-isoamylbarbituric acid.

Amphetamine: *dL*- α -Methylphenethylamine.

Atropine: *dL*-Hyoscyamine.

Chlordiazepoxide: 7-Chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepine-4-oxide.

Chlorpromazine: 2-Chloro-10-(β -dimethylaminopropyl)-phenothiazine.

Emylcamate: 1-Ethyl-4-methylpropyl carbamate.

- Hedonal: Methylpropylcarbinol carbamate.
- Imipramine: 5-(3-Dimethylaminopropyl)-10,11-dihydro-5H-dibenzazepine.
- Meprobamate: 2-Methyl-2-propyl-1,3-propanediol dicarbamate.
- Methamphetamine: *d*-N, α -dimethylphenethylamine.
- Nicotine: 4-Methyl-2-(3-pyridil) pyrrolidine.
- Pentobarbital: 5-Ethyl-5-(1-methylbutyl)-barbituric acid.
- Phenobarbital: 5-Ethyl-5-phenylbarbituric acid.
- Pipradrol: α,α -Diphenyl-2-piperidinemethanol.
- Promazine: 10-(3-Dimethylaminopropyl) phenothiazine.
- Reserpine: 3,4,5-Trimethoxybenzoyl methyl reserpate.
- Trifluoperazine: 2-Trifluoromethyl-10-[3'-(1-methyl-4-piperazinyl)-propyl] phenothiazine.
- Urethane: Ethyl carbamate.

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Adipose Tissue Dynamics and Regulation, Revisited*

BERNARD JEANRENAUD **

With 13 Figures

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I. Adipose tissue as a central site of lipid metabolism

1. Introduction

Previous reviews (13, 214, 215, 472, 499) have stressed the central importance of adipose tissue in lipogenesis and energy metabolism, and have summarized the extensive data gathered over the past twenty years, data which have changed the concept of adipose tissue from that of an inert storage site to that of an extremely active system primarily concerned with the synthesis, storage, and release of lipids.

Looking back at the exciting history of the development of the knowledge of lipid metabolism, one can distinguish three distinct periods. During the first (1935 to 1948), the liver was thought of as the main, possibly the only site of active lipid metabolism (346). The second period (1948-1965) started when it was realized that adipose tissue had an active metabolism, that it was able to synthesize fatty acids and triglycerides, and that its quantitative role in lipogenesis was greater than that of the liver (109, 110, 112, 116, 178, 214, 414). This period reached its explosive phase of development (1956) when three independent groups of workers (93, 162, 259) reported on the existence of a new, metabolically highly active fraction of plasma lipids, the free fatty acids (FFA). The FFA were shown to originate from adipose tissue and to be used throughout the organism as an energy source (144, 214, 425). Since then, the complex regulation of the FFA-releasing activity, and that of the lipogenic function of adipose tissue, has been the subject of a considerable number of papers which eventually resulted in the publication of a special volume comprising well over 4,000 references (359).

A third period has now begun which, as illustrated in Fig. 1, tends to emphasize again the importance of the liver in the general metabolism of lipids. It can be seen that not only free fatty acids produced by adipose tissue but several lipoproteins synthesized in the liver are energetically important. Among them, the very low density lipoproteins (pre- β -lipoproteins), which transport a large quantity of fatty acids in ester form, are of particular significance and best illustrate the relationships existing between liver and adipose

tissue (127, 274, 275). Thus, the liver is able to metabolize free fatty acids and to incorporate them into triglycerides found in pre- β -lipoproteins (127, 179, 180, 438). Furthermore, when the rate of FFA delivery to the liver is increased or when, under certain circumstances, increased quantities of glucose are converted into hepatic triglycerides, the production and release of very low density lipoproteins is concomitantly increased (127). Although the precise role of the different lipoproteins species and the control of their metabolism are still poorly understood, it is certain that they must play a determinant role in energy metabolism, thus completing the FFA-releasing function of adipose tissue (127, 128).

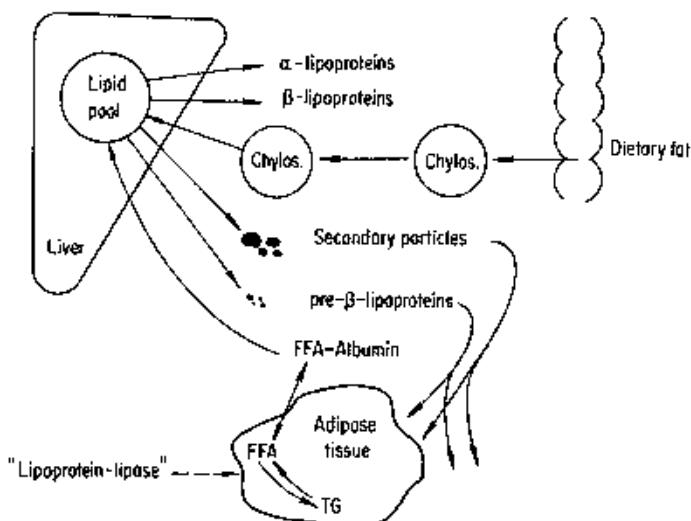


Fig. 1. Relationships between liver and adipose tissue metabolism [scheme modified from E. L. BIERMAN, 1965 (359)]

2. In situ lipogenesis versus triglyceride storage

When considering lipid storage in adipose tissue, it is difficult to assess the relative importance of lipogenesis *in situ* and of mere uptake of triglycerides (e.g. pre- β -lipoproteins) synthetized elsewhere. Until recently, it has been generally accepted, on the basis of experiments *in vitro* and *in vivo* (111, 214, 215, 499), that intrinsic lipogenesis was the major process leading to the growth of adipose stores *in vivo*. Recent experiments cast some doubt on this conclusion and suggest that the fatty acids of plasma triglycerides, rather than plasma glucose, are the major precursors of the lipids deposited in adipose tissue (161, 198, 199). Starting with the observation that fatty acid synthesis in adipose tissue produces mainly saturated acids, whereas tissue fatty acids of mammals are predominantly unsaturated, it was reasoned that if lipogenesis *in situ* were a major way of accumulating lipids in adipose tissue, extensive desaturation or preferential mobilization of the synthetized acids should occur (199). This hypothesis was tested *in vivo* in an incubation technique which permitted one to label tissue triglycerides synthetized from glucose-U- ^{14}C and

to follow for several weeks the fate of the labelled fatty acid triglycerides (198, 199). As illustrated in Fig. 2, no major change in the distribution of the label between saturated and unsaturated acids was observed, which indicated that there was no extensive desaturation of the newly synthesized fatty acids. During a 2 week period, the fat content of the fat pad increased but the fatty acid composition remained constant. Furthermore, fasting did not produce any change in the distribution of fatty acid radioactivity, despite a 50 % fall in total lipid and tissue radioactivity, thus eliminating the possibility of a preferential

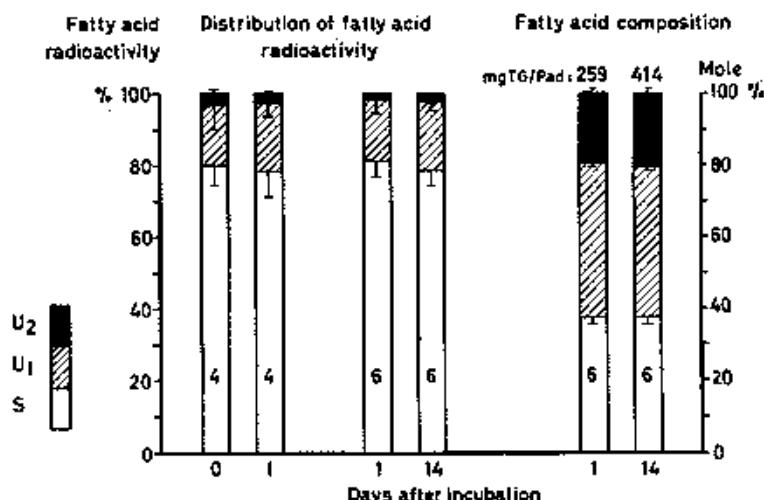


Fig. 2. Radioactivity and composition of adipose tissue fatty acids at varying times (0–1–14 days) after an *in vivo* incubation with glucose-U-¹⁴C following which the fat pads were either excised or replaced in the animal for later study. From C. H. HOLLENBERG, 1966 (199).

mobilization of saturated acids. Other experiments have similarly indicated that little interchange of triglyceride fatty acids occurred *in vivo*, even after two months (161), but this is in contrast to what was reported previously for experiments *in vitro* in which considerable reshuffling of adipose triglyceride fatty acids was observed (163). These *in vivo* experiments suggest that the fatty acids of plasma triglycerides (the composition of which is very similar to that of adipose tissue) may be the major precursors of the lipids deposited in adipose tissue, and that plasma glucose utilization for lipogenesis *in situ* may be of secondary importance. This conclusion is strengthened by other experiments *in vivo*, which have indicated that total fatty acid synthesis from glucose in rats could actually account for only a small proportion of the glucose metabolized, for only 1–4.2 % in some experiments (86, 265, 297, 340), 11–16 % in others (209, 296). Although these experiments do not prove that adipose tissue is not an important site of fatty acid synthesis, a conclusion that would be contrary to too many data (359), they do indicate that a reappraisal is necessary to decide whether adipose tissue or liver (e.g. through its production of plasma triglyceride) is the more important in the building up of the depot

stores. The existence of a complementary role of both tissues is likely to be a reasonable guess! It should be mentioned that fatty acids synthetized within adipose tissue may have a rapid turnover in the cytoplasmic compartment, and may thus be discharged into the blood without entering the fat droplet (340). Indeed, it has been recently found that, following intraperitoneal injection of labelled glucose, the specific activity of the plasma glycerol greatly exceeded that of the glyceride-glycerol of white or brown adipose tissue. This may well reflect the existence of a pool of newly synthetized triglyceride having both a high specific activity and a rapid turnover (188).

3. Adipose tissue versus liver lipogenesis

When considering the problem of the relative importance of liver and adipose tissue in lipogenesis from carbohydrates, it appears that no clear cut answer is yet available. Some experiments have suggested that liver, not adipose tissue, was the more important site of lipid synthesis *in vivo* (209, 340). Others, also carried out *in vivo*, indicated that 90—98 % of the newly synthesized fatty acids was made outside the liver, in the carcass (i.e. all body tissues except liver and epididymal fat pads), a mass of tissues which clearly contains most of the adipose tissue (211). Several factors appear to influence the relative activity of liver and adipose tissue in lipogenesis. Diet is one of them (211). Species is another, and species differences will be discussed now. It has been reported, on the basis of experiments *in vivo*, that mouse adipose tissue synthesized more than fifty percent of the total fat whereas the contribution of the liver was only about three to five percent (109, 110, 112). Rat adipose tissue is usually thought to have high *in vivo* and *in vitro* lipogenic activity (214, 359), although diverging opinions have been expressed (209, 340). On the other hand, the lipogenic activity of human adipose tissue *in vitro* is low (189); adipose tissues of several birds (house-sparrow, white-crowned sparrow, slate-colored junco) incorporate glucose carbon into fatty acids at a very slow rate, a rate which is unaffected by insulin (158). Recent experiments have indicated that these species differences may represent a variable division of labor, which favors liver in some species, adipose tissue in others. This new concept is based on the existence of marked differences in the metabolic activity of liver and adipose tissues from rat and pigeon (160). It was observed that basal lipogenesis from either glucose or pyruvate was much lower in pigeon than in rat adipose tissues. Furthermore, no insulin response could be elicited in pigeon adipose tissue. The adipose tissue acetyl-CoA carboxylase was five times more active in the rat than in the pigeon. The opposite was observed in the liver, pigeon liver acetyl-CoA-carboxylase being 8 times more active than that of the rat. Malic and citrate cleavage-enzyme as well as the pentose cycle dehydrogenases are known to play an important role in lipogenesis (14). It was therefore of interest to observe that, in the pigeon, malic enzyme activity was much

greater in the liver than in adipose tissue, whereas the opposite was true for the rat (160, 503). On the other hand, citrate cleavage enzyme was absent from pigeon adipose tissue but quite active in rat adipose tissue. The activity of the pentose cycle dehydrogenases in pigeon adipose tissue was only 4 % that of the rat (12, 160). Finally, the pigeon was quite different from the rat in its response to fasting and refeeding. Whereas such dietary conditions induced a very marked increase in rat enzyme activities, their effect on those of pigeon were much milder (160). Another difference was the anti-lipolytic action of insulin: easily demonstrable in rat adipose tissue, it was absent in pigeon adipose tissue (159). All these findings are compatible with the idea that, in some species, the *de novo* synthesis of lipids does not occur to any extent in the adipose tissue, but is switched to the liver, which thus becomes the chief site of lipogenesis.

4. Lipoprotein metabolism in adipose tissue

Adipose tissue can take up significant amounts of chylomicron triglycerides and of very low density lipoproteins (372, 381, 416). Initially, most research on the removal and metabolism of triglycerides by adipose tissue has been carried out with chylomicrons or emulsified fat particles (416). However, it was soon realized that although the bulk of post-prandial plasma triglycerides taken up by the liver were chylomicrons, the subsequent uptake of plasma lipids by non-hepatic tissues concerned lipoproteins of higher density. Experiments were therefore aimed at studying the uptake by isolated adipose tissue of triglycerides from various density classes of lipoproteins (290). As illustrated in Table 1,

Table 1. Uptake of triglycerides from different lipoproteins

Lipoprotein TG (μ moles/ml)	Tissue TG uptake (units) from lipoproteins of density class				
	< 1.006 (chylomicrons)	< 1.019	1.019—1.063	1.063—1.21	Whole serum
0.15	98 (2)	114 \pm 9 (4)	95 \pm 8 (5)		
0.25	60 (2)		73 \pm 11 (9)		
0.53		52 \pm 4 (12)	58 \pm 6 (6)		
0.82	41 \pm 2 (5)			45 \pm 3 (10)	
1.10		48 \pm 2 (10)	50 \pm 4 (6)	40 \pm 3 (10)	
1.55	36 \pm 4 (4)	44 \pm 2 (29)			42 \pm 4 (4)
1.97					
2.5	42 \pm 3 (5)	36 \pm 5 (8)	42 \pm 3 (5)		
3.7		50 \pm 6 (4)			
6.3		31 \pm 4 (4)			

The uptake is expressed in units of μ moles triglycerides (TG) recovered in 100 mg tissue after 3 hr of incubation per μ mole of medium TG. The lipoproteins were isolated from human fasting serum and chylomicrons from postprandial serum after a fat-rich meal. Tripalmitin-1-¹⁴C of triolein-1-¹⁴C was used as label. The values presented are means \pm SEM of the number of experiments given in parentheses.

Data of L. MARKSCHEID and E. SHAFRIR, J. Lipid Res. 6, 247, 1965 (290).

adipose tissue could take up triglycerides from lipoproteins of density ranging from <1.006 to 1.063—1.21. The amount of triglyceride taken up by the tissue did not change markedly over a wide range of triglyceride concentrations (from about 0.8 to 6.0 μ moles/ml), and this constant uptake was independent of the nature of the lipoprotein carrier. With low triglyceride concentrations (below 0.5 μ moles/ml) the amount of triglycerides taken up from the medium rose, and this was again observed with all the classes of lipoproteins studied. These experiments therefore suggested that the "site of uptake" on the adipose tissue cells could adapt to the available triglyceride carrier and were able to accommodate either a small number of large particles rich in triglycerides (e.g. chylomicrons) or a large number of small molecules low in triglycerides such as lipoproteins of density 1.023—1.21. The increased fractional uptake observed at low medium concentrations (0.5 μ moles/ml) was attributed to a more efficient trans-esterification, at this low substrate concentration, of the triglyceride taken up (290). The dynamic study of the incorporation of lipoproteins by incubated adipose tissue indicates that two stages are involved (290, 375, 379). The first consists in the uptake of intact molecules of lipoproteins into a compartment which is recovered in the soluble fraction of the tissue homogenate. It is not known, however, whether the lipoprotein carriers which are recovered in this soluble fraction penetrate into the adipocyte, or whether they are only trapped in the extracellular space. During the second stage, lipolysis and subsequent reesterification of the triglycerides taken up occur and, through some loss of solubility, the triglyceride molecules so resynthesized pass into the vacuole. This second stage is probably responsible for the overall rate of triglyceride incorporation, known to decrease, in particular, in fasted or aged animals, and to be highest in young or fasted-refed animals (290, 381).

The importance of the lipoprotein-lipase in the incorporation of circulating lipids into adipose tissue is well substantiated (181, 344, 372, 373, 375, 381, 402, 416). However, the sequence of events mentioned above, namely uptake of intact lipoprotein molecules, hydrolysis of triglycerides taken up induced by lipoprotein lipase, reesterification, and storage, is not universally accepted. Some experimenters favor the concept that hydrolysis of the lipoproteins by lipoprotein-lipase occurs at the capillary wall, and is followed by esterification and storage of the fatty acids thus produced. According to this view lipoprotein lipase is needed for the actual lipoprotein uptake (31, 373). This concept cannot be reconciled, however, with the observation that the inhibition of lipoprotein lipase by diisopropyl-fluorophosphate (DFP, 10^{-4} M) did not appreciably modify the uptake of triglycerides by adipose tissue, although further metabolism of the triglycerides taken up (hydrolysis followed by reesterification) was markedly decreased (375). Furthermore, EDTA, pyrophosphate and triton, substances which, at appropriate concentrations, inhibit

lipoprotein-lipase completely, only partially inhibited triglyceride uptake. On the contrary, NaF, which affects the lipase only mildly, caused a substantial decrease in uptake (289, 413).

The anatomical site of lipoprotein-lipase is still the subject of controversy. Is it located in the capillary endothelium, in the extracellular space, in the interstitium, or in the fat cell? On the basis of indirect evidence it has been suggested that it might be located on the surface of the capillary endothelial cells (371). It was thought that this problem could be easily solved since the stromal-vascular cells of adipose tissue can be separated from fat cells by collagenase treatment and can therefore be studied separately (376). Lipoprotein lipase activity was found in free fat cells and intact adipose tissue, but not in the stromal-vascular fraction. It was therefore concluded that the enzyme was located within the fat cells (377). More recent evidence is at odds with this conclusion. Using a perfusion technique for adipose tissue (191), it was observed that the injection of heparin resulted in an extremely rapid release (within seconds) of lipoprotein lipase into the perfusate, a finding which suggested that the enzyme was located quite near or in the vascular bed (195). Epinephrine injection was ineffective in releasing the enzyme (195). Furthermore, it could be shown that the isolated fat cells treated with heparin did not release lipoprotein-lipase activity, but that the stromal-vascular cells so treated did release it. Thus, the possibility that fat cells were the actual source of lipoprotein lipase appeared unlikely from these experiments which, on the contrary, suggested an association of the enzyme with the stromal-vascular cells (195). It is impossible to reconcile these experiments with the previous ones (377). It should be stressed that in the former experiments (377), acetone-powder extracts of adipose tissue, isolated fat cells or stroma cells were used for the assay of lipoprotein-lipase activity, and that heparin was not added to the extraction mixture. It is therefore not certain that the enzymatic activity measured in the two types of experiments is identical in nature. On the other hand, administration of "Compound 48/80", a substance known to disrupt mast cells, completely depleted rat hearts of their lipoprotein-lipase content, indicating a possible role for mast cells in the production of the enzyme (119, 193). As adipose tissue contains mast cells, it is conceivable that these may also have a physiological role in the release of lipoprotein-lipase and the uptake of circulating triglycerides (193). Clearly, more experiments are needed to determine the localization and source of adipose tissue lipoprotein-lipase!

Besides the influence of age and nutrition cited above (290, 381), very little is known about the metabolic and hormonal regulation of lipoprotein-lipase. The decreased activity observed during starvation could be restored *in vitro* by incubating adipose tissue in a special medium designed as a "Complete Reconstituted Medium" (CRM) (402, 501). The recovery of the enzyme activity appeared to be related to the *de novo* synthesis of a new enzyme protein,

since it did not occur under anaerobic conditions, took place over a period of several hours, and was inhibited by puromycin (501). When fat tissues from starved rats were incubated in CRM from which insulin had been omitted, the recovery of the lipoprotein-lipase activity was markedly decreased. Epinephrine and ACTH markedly inhibited the increase in lipoprotein-lipase activity of adipose tissue incubated in CRM (501). These experiments raise the very interesting possibility that several hormones may indeed play a role in controlling the deposition of triglycerides in adipose tissue, through their regulation of the tissue lipoprotein-lipase activity (402, 501).

As far as the simplest lipoproteins, FFA-albumin, are concerned, one should recall that they appear to be the only form by which lipids leave adipose tissue (with concomitant glycerol output) (290). *In vivo*, FFA release has been shown to be accompanied by a decrease in the blood pH which was unrelated to changes in the plasma levels of lactic acid or ketone bodies (401). A similar finding was observed with adipose tissue *in vitro*; as FFA release took place, the pH of the medium gradually fell from 7.4 to 7.1. The number of micro-equivalents of H⁺ required to produce such a reduction in pH was exactly related to the FFA output, indicating that FFA were released as RCOO⁻, together with an equimolar quantity of H⁺. In addition, the release of H⁺ and FFA was dependent upon the availability of binding sites of albumin for RCOO⁻ (380, 401).

The uptake of FFA by adipose tissue, although observed both *in vivo* and *in vitro* (16, 236, 415, 477), may not be quantitatively very important, since fatty acids probably return to adipose tissue as lipoprotein triglycerides (50, 127, 214). Experiments carried out in adipose tissue particles (362, 363), have given some insight as to the possible mechanism of fatty acid transfer through the membrane into the cell. Membrane lysophospholipids and phospholipids appear to be of prime importance in the process. Lysophospholipids probably serve as factors facilitating fatty acid transport into the cell by loosening the FFA-albumin complex. Once in the membrane, FFA trigger the esterification of lysophospholipids to phospholipids. The latter release FFA inside the cell through the action of some phospholipase, thus regenerating lysophospholipids, which become available again to facilitate the entry of other FFA molecules. Such a process might of course be under the influence of regulatory mechanisms, of which nothing is known at the present time.

II. Mobilization of FFA from adipose tissue

Soon after it was established that adipose tissue lipids were mobilized as free fatty acids (FFA), it was demonstrated that several hormones were stimulating the FFA-releasing process. Today, the underlying mechanism of this stimulatory effect is, at least for some particular hormones, rather well understood.

For clarity's sake, it may be best to divide the FFA-mobilizing hormones into two main categories: 1) the fast-acting hormones (epinephrine, norepinephrine, ACTH, TSH, glucagon); 2) the delayed-type FFA mobilizing hormones (glucocorticoids, growth hormone). The hormones of the first group produce their effect within minutes through the activation of a lipase system, the mechanism of which appears to be quite similar, if not identical, for all of them. On the other hand, the effect of the other hormones is delayed ($1/2$ to several hours), and the underlying mechanism, as yet unclarified, appears to vary from hormone to hormone.

1. The fast-acting hormones

a) Lipolysis and the two-messenger system

One of the greatest advances in recent years pertaining to the mode of action of many hormones on their target tissues has been achieved through the outstanding work of SUTHERLAND and his collaborators. This work has led to a generalized picture of hormone action through the concept of a two-messenger system (458). Although the hormone studied in greatest detail has been epinephrine, it has been shown that several other hormones, norepinephrine, glucagon, ACTH, vasopressin, the luteinizing hormones, the gonadotropins, etc., may produce their effects via the same messenger system (32, 60, 63, 106, 107, 174, 175, 183, 226, 235, 291, 321, 331, 368, 403, 404, 458, 459).

The two-messenger system is now so fundamental that a short outline of it is warranted (64, 287, 321, 456, 457, 458). As illustrated in Fig. 3, the system may be summarized as follows: the hormone, referred to as first messenger, is released from the endocrine gland and interacts with specific target cells. At the site of the interaction, usually the cell membrane, a second messenger is formed that appears to function within the cell, modifying enzyme activities and/or permeability barriers. The second messenger may sometimes stimulate the formation of a third, e.g. a steroid hormone, which is then released and acts elsewhere.

In the case of all the hormones cited above, the sequence of events appears to be that outlined in Fig. 3. The first messenger (ACTH, epinephrine, etc.) reacts with the first target system, membrane adenyl cyclase, which stimulates the synthesis of cyclic 3',5'-AMP from ATP. Cyclic-AMP constitutes the second messenger, which activates a second target system responsible for the physiological response. Both the first messenger and the second messenger can be inactivated by a modulator. In the case of cyclic-AMP, the modulator is a phosphodiesterase specific for the mononucleotide 3',5' bonds. The methyl xanthines (theophylline, theobromine, caffeine) are potent inhibitors of phosphodiesterase, and can therefore mimic hormonal effects by preventing the degradation of synthesized cyclic-AMP (56A, 205).

The fascinating aspect of this messenger system is that the second target system, regulated by cyclic-AMP, changes from tissue to tissue. As a conse-

quence, the response induced by cyclic-AMP varies from tissue to tissue. The expression of the accumulation of cyclic-AMP within each specific cell is thus determined by its own enzymatic pattern. For example, in liver and muscle, increased cyclic-AMP formation produced by glucagon and epinephrine (liver) or epinephrine (muscle), induced the activation of inactive phosphorylase b to active phosphorylase a. This resulted in increased glycogen breakdown in both tissues, which led in the liver to increased glucose output (because of the

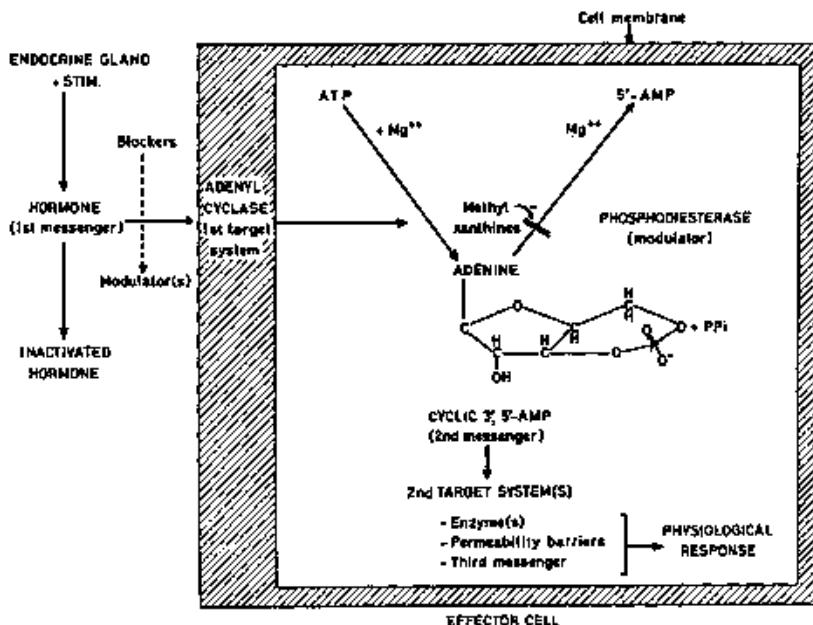


Fig. 3. The "second messenger" theory. From E. W. SUTHERLAND, I. ØYE and B. W. BUTCHER, 1965 (458).

presence of a glucose-6-phosphatase), but, in skeletal muscle, to increased lactate production (lack of a glucose-6-phosphatase) (348, 455, 458). Adrenal slices responded to ACTH by increased accumulation of cyclic-AMP, which stimulated the synthesis of glucocorticoids (third messenger) from acetate-1- ^{14}C and [$\gamma\alpha^3H$]-cholesterol (226). In the heart, cyclic-AMP was the second messenger in the inotropic response induced by epinephrine (458). In the toad bladder *in vitro*, the vasopressin effects on water permeability and sodium transport were also mediated through cyclic-AMP (175). Luteinizing hormone caused a rapid accumulation of cyclic-AMP, which induced progesterone synthesis in bovine and human corpora lutea (291).

All these examples of different hormones producing a similar increase in the level of cyclic-AMP imply that the molecular configuration of the adenyl-cyclase-systems (which appear to be lipoproteins) are probably different from tissue to tissue. Although at the present time cyclic-AMP is the only messenger which has been identified, it is quite possible that other messengers may exist (458).

Let us now consider, using the particular example of epinephrine, the effect of the fast-acting hormones on the lipolytic response of adipose tissue.

It has been well established that the *in vitro* addition of epinephrine to intact adipose tissue or isolated fat cells stimulated lipolysis (i.e. mobilization of both glycerol and FFA), glycogenolysis, as well as the uptake of glucose (88, 196, 214, 443). Further experiments demonstrated that the stimulation of FFA and glycerol release was related to an increase in the activity of a specific lipase, indicating that the enzyme probably existed in two forms, active and inactive, that could be rapidly interconverted (369). The epinephrine-sensitive lipase when measured in tissue homogenate could be activated by the addition of cyclic-AMP (368). Furthermore, it could be shown that glucagon, ACTH and TSH produced the same effect as epinephrine (476), and that all these lipolytic hormones concomitantly increased the phosphorylase activity of isolated adipose tissue (471).

Recently, direct evidence for the role of cyclic-AMP in epinephrine-induced lipolysis of intact tissue has been obtained (63). As illustrated in Table 2, a correlation between the release of FFA by adipose tissue and the intracellular levels of cyclic-AMP was observed. Caffeine, an inhibitor of the phosphodiesterase, when added together with a small amount of epinephrine which by itself was only mildly stimulatory, produced striking increases in both intracellular cyclic-AMP accumulation and FFA release. This clearly indicated the importance of cyclic-AMP in inducing lipolysis, and that of phosphodiesterase

Table 2. Effects of epinephrine, caffeine, and dichloroisopropylarterenol on cyclic 3', 5'-AMP levels and FFA release in epididymal fat pads

Additions	Cyclic 3', 5'-AMP mmoles × 10 ³ /g, wet wt	p versus control	FFA μmoles × 10 ³ /g per min	p versus control
NaCl (control) (29)*	1.8 ± 0.08		1.26 ± 0.08	
Epinephrine, 10.0 μg per ml (6)	3.8 ± 0.3	0.01	4.55 ± 0.25	0.01
Epinephrine, 1.0 μg per ml (6)	2.9 ± 0.3	0.01	4.05 ± 0.25	0.01
Epinephrine, 0.1 μg per ml (6)	2.2 ± 0.2	0.05	2.26 ± 0.24	0.01
Caffeine, 1.0 mM (5)	2.1 ± 0.2	0.1	1.97 ± 0.19	0.01
Caffeine, 1.0 mM, plus epinephrine, 0.1 μg per ml (5)	4.3 ± 1.2	0.01	4.58 ± 0.6	0.01
Dichloroisopropylarterenol, 0.1 mM (3)	2.0 ± 0.06	0.1	1.81 ± 0.54	NS**
Dichloroisopropylarterenol, 0.1 mM, plus epinephrine, 1.0 μg per ml (3)	2.0 ± 0.07	0.2	2.73 ± 0.12	0.01

* Numbers in parentheses represent number of experiments.

** NS, not significant.

Rat epididymal fat pads were incubated in Krebs-Ringer bicarbonate buffer for 20 or 24 minutes, then rapidly homogenized and assayed for cyclic 3', 5'-AMP activity.

Data of R. W. BUTCHER, R. J. HO, H. C. MENG and E. W. SUTHERLAND. *J. Biol. Chem.* 240, 4515, 1965 (63).

as a modulator of the cyclic-nucleotide levels within the cell. The adenyl-cyclase system responded very rapidly to epinephrine. The levels of cyclic-AMP were maximal five minutes after the addition of the hormone *in vitro* and remained high for about twenty minutes. When perfused instead of incubated adipose tissue was used, increased formation of cyclic nucleotide could be elicited within 30 to 60 seconds with low doses of epinephrine (0.03 µg/ml), a metabolic change that was fast enough to account for the rapid lipolytic action of epinephrine. Furthermore, it was shown that the increase in cyclic-AMP levels preceded that of FFA release, thus clearly suggesting that the cyclic compound was a causal factor in the activation of the lipolytic process (63). Finally, when cyclic-AMP or a lipid-soluble analogue of the cyclic compound (N^6 -2'-O-Dibutyryl cyclic 3',5'-AMP), a substance which appears to penetrate the cell easily (349), was added to isolated fat cells or intact adipose tissue, a marked increase in FFA release was observed. This established cyclic-AMP as the intracellular mediator of the hormone. The precise mechanisms relating cyclic nucleotide(s) and actual lipase activation are, however, still to be established. One should add that activation of the phosphorylase, known to occur in adipose tissue treated with lipolytic hormones, is certainly a consequence of increased cyclic nucleotide production, but there is little reason to believe that this activation represents more than a side action, unrelated to the actual breakdown of triglycerides (64).

All fast-acting hormones appear to induce lipolysis in adipose tissue by a mechanism identical or very similar to that just described (56A, 205, 394, 458). It had been previously suggested that ACTH might act by releasing adipose tissue catecholamines, which would in turn increase the mobilization of FFA (334). This appears now to be unlikely since adipose tissue from reserpinized animals, a tissue known to be depleted of norepinephrine (449), responded normally to ACTH (192). Furthermore, a good lipolytic response was also obtained when ACTH was added to isolated adipose cells known to be deprived of the sympathetic nerve endings responsible for the noradrenaline discharge (192).

b) Lipolysis: other modulating factors

— *Peptidases.* Among the ten or so biologically active peptides known to exist in the mammalian pituitary gland, five are believed to possess lipolytic activity in one or more mammalian species, namely ACTH, TSH, α - and β -MSH and arginine vasopressin (394, 397). In addition, the pituitary gland is known to contain other lipid mobilizing peptides. In 1960, an active fraction was obtained from pig pituitaries which was free of the known adipokinetic activities but was highly active in mobilizing FFA. This fraction was named Fraction H (190), and, when obtained in more purified form, Fraction L (397). In 1961, two other pituitary peptides potent in mobilizing FFA, "Peptide I" and "Peptide II", could be characterized (10, 397). "Peptide II" proved to

be identical with a major component in Fraction H (131). In 1964, another different lipolytic peptide, Fraction L', distinct from the recognized pituitary hormones, was isolated from sheep pituitaries (33). In all likelihood, others exist and will be described. The interesting point concerning regulation is that adipose tissues from all the mammalian species studied so far showed a different pattern of responsiveness to lipolytic peptides and catecholamines (397, 398). Adipose tissue from rabbit and guinea-pig was found to be highly responsive to all lipolytic hypophyseal peptides (except for TSH in the rabbit), but it did not respond to the catecholamines. Adipose tissue from hamster, rat and dog responded to a limited number of lipolytic hypophyseal peptides, but was highly sensitive to catecholamines. Adipose tissue from pig did not respond to either hypophyseal peptides or catecholamines. The limited data available for human adipose tissue have also suggested that this tissue was reactive to the catecholamines, but slightly or not at all to the hypophyseal peptides (397). It should be mentioned that in all these experiments, heterologous pituitary hormones were usually employed. However, this does not appear to be the reason for the lack of response, since in several experiments performed with homologous hormones the same pattern was observed (397). Among the possible modulating factors thus influencing the lipolytic response one should cite the following: a) ability of fat cells to take up the hormone from the extracellular fluid; b) presence or absence of an appropriate receptor; and c) presence of intracellular enzymes capable of altering the structure of the hormone, with resulting gain or loss of the lipolytic activity. This last facet has been thoroughly studied and may constitute an important physiological regulatory mechanism (395, 396, 397). It was shown that adipose tissue homogenates from given species cleaved and inactivated certain lipolytic peptides, whereas those from others did not. It was further demonstrated, as illustrated in Table 3, that the capacity of adipose tissue to inactivate the peptide usually correlated well with the inability to respond to the peptide by an increase in lipolysis (397). Two exceptions were observed, namely ACTH in rat and α -MSH in rabbit.

Table 3. Correlation of capacity of adipose tissue slices from different species to respond to adipokinetic peptides, and capacity of homogenates of these tissues to inactivate the peptides¹

Source of adipose tissue	ACTH	α -MSH	β -MSH	Vasopressin	Fraction H
Rabbit	+/0	+/-	+/0	+/0	+/0
Guinea pig	+/0	+/0	+/0	+/0	+/0
Rat	+/-	0/+	0/+	0/+	0/+

¹ Left-hand symbol represents capacity to respond; right-hand symbol represents capacity to inactivate. Thus, for rabbit adipose tissue vis à vis ACTH, +/0 signifies capacity to respond and lack of capacity to inactivate.

Data of D. RUDMAN, Rev. of Physiol. Biochem. and exp. Pharmacol. 56, 297, 1965 (397).

adipose tissues. These two tissues had the capacity to inactivate the peptide but nevertheless displayed accelerated FFA production when incubated with it. Further studies showed, in addition, that the response of rat adipose tissue to ACTH (which contains an enzyme that inactivates ACTH) differed from that of rabbit adipose tissue (which lacks this enzyme). The former exhibited only a brief lipolytic response to a limited exposure to ACTH. In the latter, the response continued at an undiminished rate for at least 2 hours after a similar exposure to the same peptide (395). Extending the meaning of the data, it is quite conceivable that the differences observed between mammals in the responsiveness of their adipose tissue to hypophyseal peptides is related to the amount of peptidases present in the tissue. Furthermore, this regulation may not be restricted to hypophyseal peptides, but may represent a general phenomenon enabling the organism to modulate various hormonal effects, as suggested by the existence of a similar peptidase for insulin in the adipose tissue of certain species (90, 91, 399, 400). In addition, tissues other than adipose tissue have been shown to inactivate lipolytic peptidases (396), thus possibly explaining why some lipolytic agents produced, when administered *in vivo*, a prolonged FFA mobilization with secondary hyperlipemia (393), whereas some others resulted only in a brisk FFA release without inducing lipemia (142).

—*Glucose availability.* Glucose availability constitutes another modulatory mechanism of lipolysis. It has been observed that the release of glycerol from rat adipose tissue stimulated by epinephrine *in vitro* was higher in the presence than in the absence of glucose. As free glycerol cannot be re-utilized by the tissue (442), and as the amount of glucose incorporated into medium glycerol is negligible, this finding was interpreted as representing net increase of lipolysis in the presence of glucose (222). These experiments have been confirmed and extended as follows (17): a) ACTH-induced lipolysis was found to be greater in the presence than in the absence of glucose; b) under these conditions, the FFA concentrations within the tissue were lower in the presence of glucose than in its absence; c) with ACTH, but without glucose in the incubation system, the rate of re-esterification initially increased, then declined, to cease when the levels of tissue FFA had reached a maximum. Simultaneously, the FFA release induced by ACTH decreased; d) upon addition of glucose to the medium, the rate of esterification as well as that of the ACTH-induced lipolysis immediately increased (17). These results indicated that lipolysis was a self-limiting process when the availability of carbohydrate to the cells was restricted. Under those circumstances, as lipolysis proceeded, tissue FFA reached concentrations that were inhibitory for both the esterification and lipolytic processes. On the contrary, glucose relieved this inhibitory influence, thereby increasing lipolysis (17).

It is clear, however, as illustrated in Fig. 4, that when glucose utilization is stimulated above a certain point, lipogenesis from glucose overcomes lipo-

lytic activity, and FFA mobilization comes to an end. Fig. 4 also illustrates the dynamic equilibrium prevailing in adipose tissue. Glycerol is continuously released even in the face of net fat deposition (260, 443), and incorporation of labelled fatty acids occurs under all circumstances, even when there is a net mobilization of FFA (477). Because of lack of phosphoglycerokinase activity, there must be, in order to maintain a steady state, a constant supply of the obligatory glycerophosphate acceptor. This metabolite is therefore generated by either the breakdown of the small stores of tissue glycogen, or glycolysis of the glucose taken up. Finally, it is known that the fast-acting

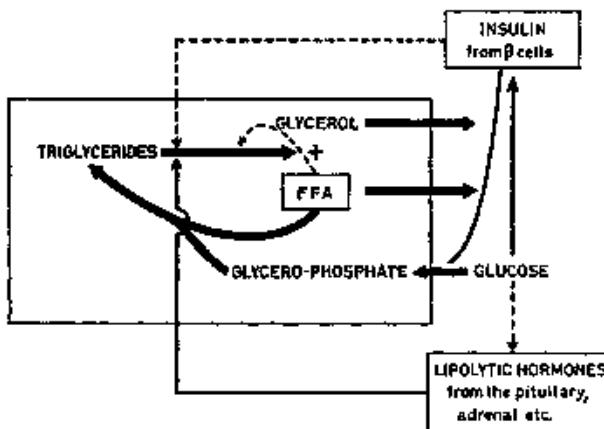


Fig. 4. Possible regulatory mechanisms of lipolysis in adipose tissue. Heavy arrows = substrate changes. Light arrows = regulatory influences (solid lines = stimulation, broken lines = inhibition). From P. R. BALLY, H. KAPPELER, E. R. FROESCH and A. LARHART, 1965 (47).

lipolytic hormones also influence FFA mobilization by increasing simultaneously the uptake of glucose and, as a consequence, the formation of glycerophosphate (14, 67, 121, 214, 282). Although this might be expected to favor triglyceride synthesis from glucose, the lipolytic effect predominates and, through the continuous supply of glycerophosphate, it is actually maintained at the highest (17).

— *Thyroid hormones.* Adipose tissue from hypothyroid animals has been shown to be less responsive than normal tissue to the lipolytic action of catecholamines, whereas adipose tissue from hyperthyroid animals was hyper-responsive (85, 89, 118, 157). Although this "facilitating" action of thyroid hormones is still poorly understood it appears to be a general phenomenon, not one restricted to epinephrine, since FFA release in response to corticotropin, thyrotropin or glucagon was also reduced in adipose tissue from hypothyroid animals (157). Thyroid hormones were ineffective *in vitro* (85, 89) but, upon administration *in vivo*, the facilitating effect could be observed with microgram quantities of triiodothyronine or thyroxine (52). There is no evidence at the present time that the interaction between thyroid hormones and catecholamines

depends upon a direct effect of catecholamines on thyroid gland function, or of thyroid hormones on catecholamine metabolism (177, 484). A common factor relating fast-acting lipolytic hormones and thyroid hormones may be cyclic-AMP, as suggested by the observation that in fat cells incubated with epinephrine, the expected rise in cyclic-AMP was much lower in cells from thyroidectomized than in cells from normal rats (53). It is possible that thyroid hormones contribute to the accumulation of the second messenger by decreasing the rate at which it is inactivated (370, 484). Alternatively, they may increase adenyl-cyclase activity (53, 56A), in particular through the synthesis of additional amount of adenyl cyclase (56A). Finally, as many β receptor effects are secondary to the accumulation of cyclic-AMP, thyroxin may act by enhancing the β -receptor activity to hormones such as epinephrine (455, 484).

— *Glucocorticoid hormones.* The capacity of adipose tissue to respond, both *in vitro* and *in vivo*, to the catecholamines is markedly reduced when animals have been adrenalectomized (286, 361, 411, 412). These results indicate that the catecholamine-sensitive system of adipose tissue also depends in some way on corticosteroid activity (56A). This type of interaction is reminiscent of others, previously described for corticosteroid hormones, and designated as the "permissive" action of these hormones (206). However, as will be seen below, glucocorticoid hormones have other, direct effects, upon adipose tissue metabolism.

c) Lipolysis and the existence of several lipases

The lipase responding to hormonal stimulation has not yet been purified. For that reason it is generally called "hormone-sensitive lipase", and defined on the basis of the assay conditions used (397). It should be stressed, however, that a rapid cleavage of mono- and diglycerides, as opposed to a slow lipolysis of triglycerides, was observed in adipose tissue homogenates, and that this cleavage was related to the presence of other enzyme(s) which are particularly active in splitting lower glycerides (164, 443, 454, 476). The existence of these other lipases appears to be a common finding in tissues obtained from several different species (man, rat, guinea-pig) (164). The lipase acting on lower glycerides was found to differ from that acting on triglycerides in the following respects: a) it was not altered by pre-treatment of the tissue with lipolytic hormones; b) the pH and temperature activity curves were different; and c) it was not inhibited by isopropanol or Tris-buffer. The monoglyceride lipase has been so far best characterized. It is not clear yet whether the rapid lipase acting on diglycerides is distinct from monoglyceridase since both enzymes fractionate together (454, 476). However, they are clearly different from lipoprotein-lipase (454, 476). The respective function of the different lipases found in adipose tissue may be related as illustrated in Fig. 5 (164, 443). It can be seen that the hormonal effect on the overall rate of lipolysis appears to be confined to the first step, as only triglyceride lipolysis is rate limiting. The

hormone-activated lipase splits an ester bond in triglycerides, and the di-glycerides thus generated are very rapidly hydrolyzed to FFA and glycerol. This concept is in accord with the finding of a negligible content of lower glycerides in adipose tissue (474). However, it has been observed that under certain conditions *in vivo* (479), or in perfused tissue (408), accumulation of lower glycerides in the hormone-treated (ACTH, catecholamines) adipose tissue can occur. This may mean that, under those circumstances, the rate of triglyceride splitting is so rapid that the rate of hydrolysis of lower glycerides becomes limiting.

Finally it should be mentioned that lipases other than those previously cited may possibly exist in adipose tissue (406, 407).

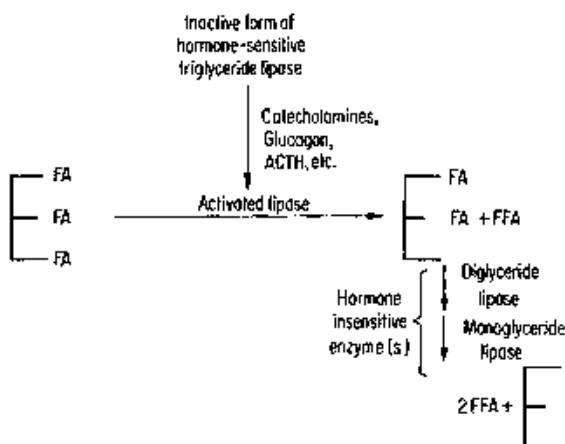


Fig. 5. Lipase activities in adipose tissue. From D. STEINBERG, 1966 (443)

2. The delayed-type FFA mobilizing hormones

a) Effects induced by glucocorticoid hormones (68, 219, 254).

In addition to the "permissive" action noted above, corticosteroids have the ability to stimulate directly FFA release from adipose tissue *in vitro* (213, 218, 219). As will be seen, the mechanism of this lipid-mobilizing effect is quite different from that previously discussed for the fast-acting hormones.

A stimulatory effect on FFA mobilization was initially obtained *in vitro* upon addition of cortisol or corticosterone (3—30 µg/ml) to adipose tissue from normal or adrenalectomized rats, fed or fasted, incubated for three hours in an albumin buffer containing glucose (20 mM). The increment in FFA release was about 70 %. These experiments already indicated an important facet of the fat-mobilizing effect of glucocorticoids: it was of lesser magnitude than that of ACTH or epinephrine; and it took time to become evident (213). Subsequent experiments demonstrated that more prolonged incubations (over 3 hours) were necessary to induce, in addition to the above-mentioned FFA mobilization, clear-cut inhibitory effects of corticosteroids on glucose metabolism

(100, 186, 320). It was further observed that cortisol inhibited the overall glucose metabolism by adipose tissue (262), and that the lowering of the medium glucose concentration (to 2.5—5 mM) made this inhibitory action easier to detect (263). The importance of these observations was greatly extended when it was demonstrated that physiological concentration of cortisol was inhibitory, provided that the duration of incubation was prolonged to five hours (320). Under those conditions, the naturally occurring glucocorticoid hormones markedly decreased glucose oxidation, lipogenesis and protein synthesis from glucose (320, 345). Similar findings were obtained with a synthetic glucocorticoid, dexamethasone (100).

The existence of an inhibitory effect of cortical hormones on glucose metabolism together with a stimulatory effect on FFA mobilization suggested that decreased re-esterification might be responsible for the increase in FFA release. Using a method permitting simultaneous estimation of lipolysis and re-esterification (473), it has been recently shown that decreased FFA re-esterification was indeed induced by a glucocorticoid such as dexamethasone (218). As shown

Table 4. Effect of different concentrations of dexamethasone added *in vitro* on the glycerol-FFA balance in adipose tissue from fed rats

	Total Lipolysis (net glycerol X 3)	Net FFA production	FFA re- esterified	Re-esterification ex- pressed as % of total lipolysis
Controls	19.7 (± 1.0)	3.6 (± 0.8)	16.1 (± 0.4)	82
+ Dexamethasone 0.1 µg/ml	25.3** (± 1.4)	11.6* (± 1.2)	13.7 (± 0.6)	54
+ Dexamethasone 0.01 µg/ml	22.3*** (± 1.2)	9.3* (± 0.9)	13.0 (± 0.7)	58
+ Dexamethasone 0.001 µg/ml	19.5*** (± 1.0)	5.5* (± 0.8)	14.0 (± 0.8)	72

Incubation carried out for 4 hours in a Krebs-Ringer bicarbonate buffer containing 3.5 g/100 ml human albumin, medium glucose 5 mM. All results expressed as µmoles per gram of wet tissue weight. Each figure = mean of 6 values ± S.E. of the mean.

* Significantly different from controls ($p < 0.0025$).

** Significantly different from controls ($p < 0.025$).

*** Not significant

Data of JEANRENAUD, Biochem. J., 103, 627, 1967 (218, 219).

in Table 4, some true lipolytic activity (i.e. mobilization of both glycerol and FFA) was induced by dexamethasone when used at very high concentration; re-esterification was simultaneously decreased. However, with lower concentrations of the hormone (0.001 and 0.01 µg/ml), true lipolysis was unaffected, but FFA production was clearly stimulated, a stimulation that was due solely to a decrease in the re-esterification process (Table 4). Similar findings with both dexamethasone and cortisol were obtained in the isolated fat cells (218).

It would thus appear that, physiologically, glucocorticoids may act, not by activating some specific hormone-sensitive lipase, but rather by decreasing carbohydrate availability, thereby decreasing the rate of FFA re-esterification. Such a process would result in FFA accumulation within the cell followed by increased FFA output. Whether the inhibitory action of corticoids on carbohydrate metabolism is exerted at the level of glucose transport, or at the level of some intracellular step of glucose metabolism remains as yet unsettled (43, 100, 101, 218).

The need for prolonged incubations to obtain reproducible glucocorticoid effects suggested that the action of these hormones was possibly related to *de novo* synthesis of some specific enzyme protein. Such a mechanism has indeed been proposed to explain several effects of cortical hormones upon hepatic gluconeogenesis (115, 485, 486). In agreement with such a concept is the finding that the lipolytic effect *in vitro* of dexamethasone and growth hormone added *together* may be suppressed by puromycin, as will be seen below (104).

Whatever the intimate mechanism of the FFA-releasing effect of glucocorticoids may be, it certainly differs markedly from that of the fast-acting hormones, since FFA release induced *in vitro* by these latter hormones is associated with an increase in the uptake of glucose, the activation of a specific lipase and with a concomitant increase in the re-esterification rate (66, 67, 214, 219, 282, 474). One should add that dexamethasone, which affected the metabolism of epididymal, perirenal, and subcutaneous adipose tissue, failed to alter that of brown adipose tissue (103).

It is now known that part of the plasma glucocorticoids is bound to an α_1 -globulin glycoprotein, referred to as corticosteroid-binding globulin (CBG), or transcortin. Bound glucocorticoids appear to be biochemically inert, whereas unbound steroids are active (42, 422). Similarly, when cortisol, cortisone and corticosterone, as well as prednisone and dexamethasone were bound to a CBG preparation, they no longer had any effect on the metabolism of isolated fat cells. On the contrary, unbound steroids were quite active at physiological concentrations. The binding capacity of CBG was proportional to its concentration. When prednisolone and dexamethasone were used, it was observed that amounts of CBG sufficient to bind natural glucocorticoids completely were unable to bind the synthetic ones to the same degree. As a consequence CBG inhibited the action of these hormones only partially (44).

b) Effects induced by growth hormone

A recent review on growth hormone has stressed the difficulty of understanding the mode of action of this hormone in view of the many different effects which it produces both *in vivo* and *in vitro* (302). The particular relationship between lipid metabolism and growth hormone is perhaps most clearly

seen in the lipid-mobilizing action of the hormone: when administered *in vivo*, it was found to increase the fasting plasma levels of FFA in man, dog, monkey and rats (98, 237, 350, 351, 353, 502).

It should be stressed that the pattern of the growth hormone-induced lipolytic effect which is observed *in vivo* is rather typical, and differs markedly from that induced by the fast-acting lipolytic hormones, which elevate plasma FFA rapidly (302). With growth hormone, on the contrary, the increase in plasma FFA levels begins only after about 2 hours, and maximal concentrations are not reached until at least 4 hours. Furthermore, the observed increase in plasma FFA concentrations is preceded by a slight fall, at 30 minutes, which is concomitant with a decrease in blood sugar (302, 351, 353, 461, 511). These observations make it already clear that the mechanism of action of this hormone is certainly a complex one, and that it must differ markedly from the other hormones previously discussed. Despite the prominent fatty acid-mobilizing activity *in vivo*, the effects of growth hormone added *in vitro* are not yet clear. Quite often large or rather large amounts of the hormone are required *in vitro*, casting doubt about purity of the preparation used, and consequently about both the specificity and the physiological meaning of the effects observed. Furthermore, one of the greatest difficulties appears to be that the response of adipose tissue *in vitro* to growth hormone does not simulate precisely, or completely, that of the tissue *in vivo*; thus explaining the many conflicting data obtained *in vitro* under various incubation conditions, and with adipose tissues taken from animals in different nutritional states (214, 302).

— *Effects of growth hormone on carbohydrate metabolism.* Early experiments with high concentrations of growth hormone (0.1—1.0 mg/ml) have shown that, concomitant with the observed increase in FFA mobilization, there was a marked stimulation of glucose oxidation to CO₂, and of glycerol-glyceride synthesis from glucose, although lipogenesis from glucose was either decreased or unaffected (173, 214, 261, 498). The increase in glucose metabolism thus observed was interpreted as representing events secondary to an accelerated rate of lipolysis. According to this view, FFA accumulated within the cells and was then partly released into the medium, partly re-esterified, a process for which glycerophosphate derived from glucose was needed (173, 261). More recently, it has been observed that small concentrations of human, rat, porcine, bovine and ovine growth hormone (0.01—10 µgm/ml), stimulated the oxidation of glucose, as well as lipogenesis from glucose, in adipose tissue from hypophysectomized rats (151). These experiments further indicated that the metabolism of specifically labelled glucose was increased by insulin or by growth hormone, in a way that was qualitatively rather similar for both hormones. Furthermore, the increase in glucose metabolism induced by growth hormone was not accompanied by any stimulation of FFA release. Clearly under those

conditions, growth hormone behaved as an insulin-like hormone (151), not as a lipolytic one (261). It is therefore not unlikely that the pattern of glucose stimulation, linked with increased FFA output, observed previously with high concentrations of growth hormone (a metabolic pattern that resembled closely that observed with the fast-acting lipolytic hormones) (261, 498), may have resulted from the presence in these hormonal preparations of trace amounts of ACTH or TSH (151, 221).

Finally, it should be stressed that the diet given to the rats prior to sacrifice as well as the hormonal balance appear to be of importance. Tissues from hypophysectomized rats fed a high carbohydrate, fat-free diet, responded better to growth hormone than those from hypophysectomized rats fed an ordinary laboratory chow; tissues from normal rats responded only when they were obtained from rats fed a high carbohydrate, fat-free diet (151).

At the present time, the effects of growth hormone on adipose tissue lipogenesis from glucose may possibly be divided into two phases: a) an early insulin-like one; and b) a late anti-insulin-like one. Thus 30 minutes after the administration of the hormone *in vivo* to hypophysectomized rats (50 µg per rat), the subsequently isolated adipose tissues exhibited higher oxidation of glucose and higher lipogenesis from glucose than the control tissues. Three and a half hours later, the opposite was true (153). This late inhibitory effect lasted for about 24 hours, and could be prevented by simultaneous administration of actinomycin-D prior to killing. This suggested that growth hormone had possibly evoked the transmission of some kind of genetic "information", thereby bringing the late inhibitory response (153).

Other experiments have shown that repeated daily treatment of hypophysectomized rats with growth hormone leads not only to a marked reduction in the fat stored in adipose tissue, but also to a severe impairment in the capacity of this tissue to synthesize lipid from labelled glucose or leucine *in vitro* (147).

— *Effects of growth hormone on lipolytic activity.* It is evident that the relationships between findings such as the increase in plasma FFA levels following growth hormone administration, the late-occurring decreased lipogenesis of adipose tissue from animals treated with the hormone, and the increase in FFA release observed in adipose tissue *in vitro* upon addition of the hormone, are far from clear.

Early experiments *in vitro* have shown that growth hormone at high concentrations stimulated FFA release from adipose tissue (173, 261). As mentioned before, this effect might have been due to the presence of some contaminant, since later, lower concentrations of the hormone usually proved ineffective even after prolonged incubation (104, 151). However, a significant lipolytic effect could be obtained in intact adipose tissue when growth hormone and dexamethasone, at concentrations as low as 0.01 µg/ml and 0.016 µg/ml

respectively, were added *together* to a glucose-containing medium (104). In isolated fat cells, the presence of glucose in the medium was unnecessary, which indicated that the joint effect of growth hormone and dexamethasone was unrelated to the inhibitory action of dexamethasone on glucose metabolism (104, 105, 134). This joint lipolytic action of growth hormone *and* dexamethasone has also been observed *in vivo* (409), as well as in perfused adipose tissue (244). In all cases it was a slow process, which took one (244) to several hours to occur (104, 134), suggesting that it might be related to the synthesis of some enzyme. Indeed, it was completely blocked by the addition *in vitro* of either puromycin or actinomycin-D. These protein inhibitors were completely ineffective in suppressing the lipolytic action of ACTH (104), further indicating that the intimate site of action of growth hormone must differ markedly from that of the fast-acting lipolytic hormones. However, this intimate mode of action of growth hormone remains to be elucidated. The difficulty in understanding the underlying mechanisms is evident from the available data which successively show that growth hormone is lipolytic by itself (84, 173, 261); that it is lipolytic only in the presence of glucocorticoid hormone (104, 105, 134); that the lipolytic effect can (104) or cannot (84) be suppressed by puromycin; that the lipolytic effect can be totally absent (104, 151); but that, when present, it may (173, 261) or may not (104) be accompanied by concomitant stimulation of glucose metabolism; that growth hormone has (500) or has not (257) a specific stimulatory effect on the glucuronic acid pathway within adipose tissue; that all growth hormone-induced effects may vary from species to species (76); and finally, that an intact pituitary appears to be required for normal responsiveness of adipose tissue to several lipolytic hormones including growth hormone, a finding which in itself may be more complicated to understand than all the above-mentioned discrepancies (156).

III. Lipogenesis and anti-lipolysis

The primary function of adipose tissue is that of storing fat in time of plenty, and to release it in a controlled manner when endogenous energy is needed. We have seen previously, that adipose tissue was able to take up circulating lipoproteins, as well as to synthesize lipids *in situ* from various substrates; we have discussed the complex hormonal regulation of the FFA-releasing function. Let us now consider the controls that operate to achieve optimal lipid storage, either by increasing lipogenesis or by reducing lipolytic activity.

Fig. 6 schematizes what can now be considered as common knowledge, namely that adipose tissue can take up glucose, metabolize glucose-6-phosphate through the pentose and the Embden-Meyerhof pathways, synthesize long chain fatty acids from acetyl-CoA, and esterify them into triglycerides, which are accumulated into the vacuole. Insulin is the main, possibly the sole, lipo-

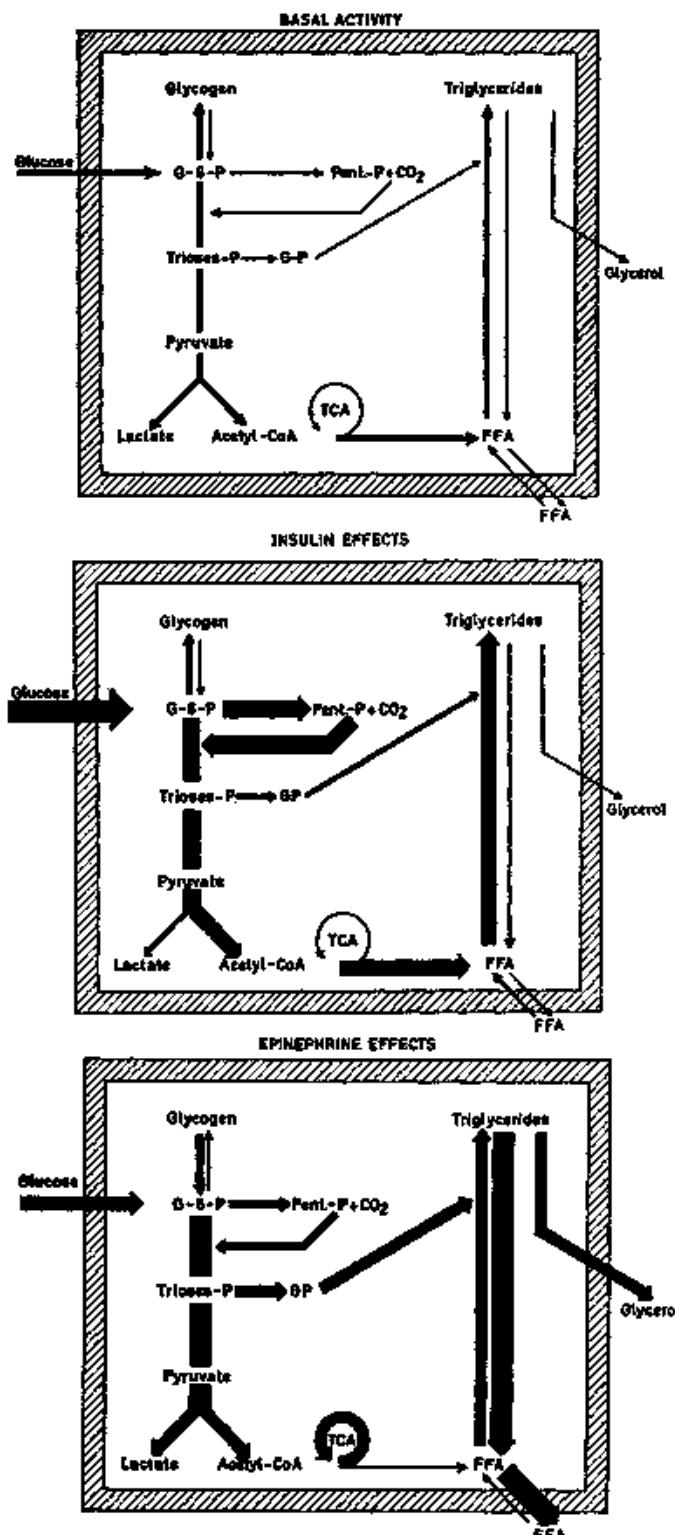


Fig. 6. Diagrammatic representation of glucose metabolism in adipose tissue. Metabolic pattern induced by insulin or epinephrine. The thickness of the arrows schematizes the relative rates of metabolism. Abbreviations: FFA, free fatty acids; G-6-P, glucose-6-phosphate; G-P, glycerophosphate; Pent-P, pentose-phosphate; TCA, Tricarboxylic acid cycle. From B. JEANNERNAUD (214, 215)

genic hormone of physiological importance. It has been shown to increase markedly the flow of glucose through all these pathways, an observation that clearly suggests that the primary action of the hormone must be an early one, presumably at the level of the transformation of extracellular, free glucose into intracellular glucose-6-phosphate. The increase in glucose uptake induced by insulin and, as a consequence, the increased formation of glycerophosphate, acetyl-CoA and reduced coenzymes, results in an accelerated lipogenesis. Simultaneously, FFA release is decreased through a mechanism that involves, at least in part, increased esterification of the newly synthesized fatty acids, and of the FFA, which are continuously produced within the cells. Although many of the effects of insulin on adipose tissue metabolism are probably the result of an accelerated rate of glucose entry into the cell (1, 121, 123, 214, 273, 446), other effects of the hormone are present in the absence of glucose, indicating that sites of action other than the cell membrane must be present (133, 222, 273).

We shall discuss successively: a) the effects of insulin on the transmembrane penetration of glucose; b) the "non-transport" effects of insulin; c) the influence on lipogenesis and anti-lipolysis of agents other than insulin.

1. Effect of insulin on the transmembrane penetration of glucose

Many of the effects of insulin on the metabolism of adipose tissue can be attributed to a facilitation of the uptake of glucose. However, it has been very difficult to decide whether the primary action of the hormone was at the membrane level, at the level of the hexokinase reaction, or at both (212, 214, 359). From studies carried out with tissues other than adipose tissue, with striated muscle in particular, it has been established that in cells from which glucose was excluded by a diffusion barrier, as presumably in adipose cells, there exists a transport system in the membrane which facilitates diffusion by the use of a carrier that is both specific and mobile (272, 314, 336, 355, 492, 493). The carrier system was shown to exhibit stereospecificity, competition among pairs of sugars with structural similarities, and saturation kinetics of the Michaelis-Menten type. Furthermore, the steady state concentration of a transported but not metabolizable sugar (such as 3-O-methyl-glucose) was altered by the existence of a transmembrane concentration gradient of a transported and metabolizable sugar (α -D-glucose) in such a way that the phenomenon of "countertransport" could be observed, as illustrated in Fig. 7. These studies further demonstrated that, in cardiac or diaphragmatic muscle, the transport of glucose from extracellular to intracellular space was the major rate-limiting step of glucose uptake, and the principal site of insulin action (234, 246, 315, 337, 355).

In adipose tissue, the phosphorylation of glucose is now known to be controlled by an enzyme that is similar to the hexokinase of brain or muscle.

The affinity of this enzyme was found to be sufficiently high (i.e. K_m sufficiently small) to provide for its saturation at concentrations well below the physiological concentrations of glucose. It was further shown that glucose phosphorylation could not be a rate-limiting step for glucose utilization, and a site of hormonal regulation (92, 358). Another step to be considered, likely to be rate-limiting and hormone-dependent, was that of the actual transport of glucose across the adipose cell membrane. To prove the existence of such a

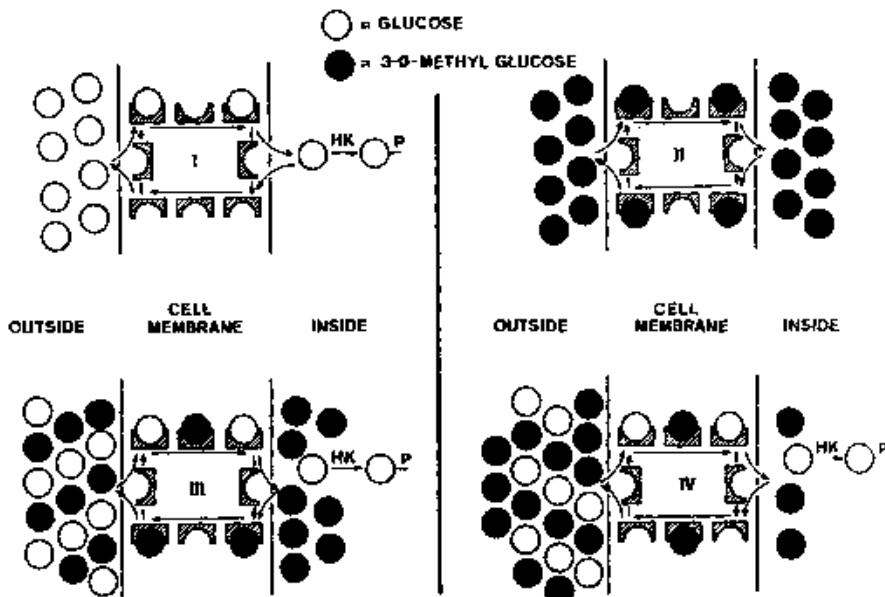


Fig. 7. Schematic representation of the countertransport theory

regulatory site, it was obvious that the measurement of the spaces available for the distribution of different substances was necessary. Although this task is difficult in adipose tissue since the intracellular water, by the very nature of the adipocyte, is exceedingly small, a technique has been recently developed that permitted the measurement of the concentrations of substances within intracellular water (80, 81). It could be shown, that the total water space of adipose tissue was quite small (15 μl per 100 mg of tissue compared with 75 μl in muscle), and that only one third was intracellular, compared with two thirds in muscle (80, 81, 358). These experiments further demonstrated that the membrane of the adipose cell was similar to that of the muscle, and was characterized by the presence of a transport system involving specific and mobile carriers (81, 82). The transport system exhibited: a) clear-cut stereospecificity, as was shown by the very slow rate of L-glucose transport compared with that of D-glucose; b) competition, illustrated by the decreased metabolism of glucose in the presence of increasing concentrations of 3-O-methylglucose, a sugar that is transported but not phosphorylated; c) saturation kinetics of

the MICHAELIS-MENTEN type; d) inhibition by substances such as phloretin or phlorizin, known inhibitors of sugar transport in several tissues (81, 130, 358). Finally, as illustrated in Fig. 8 the experiments demonstrated that the "counter-transport" phenomenon was present in adipose tissue, thus further substantiating the existence of the carrier system (81). As theoretically explained in Fig. 7 and demonstrated for adipose tissue in Fig. 8, the addition of glucose to a medium in which the tissue had previously come into equilibrium with 3-O-methylglucose, resulted in the subsequent decrease of the intracellular 3-O-methylglucose concentration. This was the result, since the glucose within

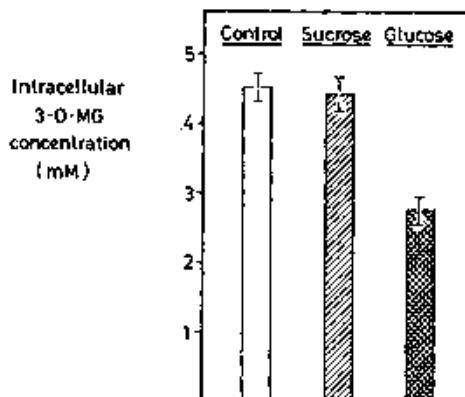


Fig. 8. Countertransport in incubated rat epididymal adipose tissue. Intracellular 3-O-methylglucose concentration after preincubation in 5 mM 3-O-methylglucose for 120 minutes, followed by incubation in 5 mM 3-O-methylglucose + 80 mM sucrose or glucose for 30 minutes. From O. B. CROFFORD and A. E. REYNOLDS, 1965 (81).

the cell was rapidly metabolized, of the concentration gradient set up between glucose outside and inside the cell. Glucose therefore competed to a lesser degree for the outflow than for the inflow of 3-O-methylglucose. As a consequence, the outward transport of 3-O-methylglucose was increased and its intracellular concentration decreased. On the contrary, sucrose, a sugar that is neither transported nor metabolized, was without influence upon the intracellular 3-O-methylglucose concentration (81). The influence of insulin upon the transport system was studied in other series of experiments summarized in Table 5 (80, 358). In these studies, the sorbitol space was used as an expression of the extracellular space of adipose tissue. When control tissues were incubated at 37.5° C, the space occupied by glucose was smaller than the sorbitol space, suggesting that there was no free intracellular glucose, and that the diffusion for glucose from the medium to the surface of the tissue cells could not keep up with glucose metabolism by the cells. The addition of insulin resulted in a striking increase in glucose uptake, and, simultaneously, in a shrinking of the glucose space, indicating that glucose diffusion into the tissue had become still more inadequate when cellular utilization of glucose had been accelerated. The finding clearly suggested that insulin must act at

Table 5. Effect of insulin on glucose space (GS) and sorbitol space (SS) of adipose tissue from fed rats

Incubation temperature	Insulin	Glucose uptake μmoles	(GS-SS)		Insulin effect	
			amount	p*	amount	p*
37.5°	+	22.9 (3.2)	-3.1	< 0.01		
37.5	0	3.8 (0.2)	-1.0	< 0.01	-2.0	< 0.01
17.5	+	6.1 (1.7)	+1.8	< 0.01		
17.5	0	2.4 (0.3)	+0.7	0.02	+1.0	0.01

(GS-SS): glucose space minus sorbitol space (μl). Medium glucose concentration was 20 mM. Each value is the mean of 9 paired experiments \pm SEM. The insulin effect on (GS-SS) is the (GS-SS) of the insulin-treated fat pad minus the (GS-SS) of the control.

Data of CROPPORD and RENOLD, J. Biol. Chem. 240, 14, 1965 (80).

the surface of the adipose tissue cell or beyond. In other experiments the temperature of the incubation medium was decreased in order to decrease the metabolic rate, and thus to achieve conditions where the regulating importance of glucose diffusion into tissue would become less. Under those conditions (Table 5), glucose space was slightly greater than the sorbitol space in the absence of insulin, and it became definitely greater in its presence. This finding means that the presence of insulin resulted in the accumulation of free glucose within the cell, and thus that the insulin-induced acceleration of glucose metabolism was exerted not at the level of glucose phosphorylation, but just prior to intracellular free glucose, that is at the level of glucose transport through the cell membrane (80, 83, 217, 358). The fact that insulin thus "opens the gate" of adipose tissue to glucose certainly accounts for, and is consistent with, most of the known effects of insulin upon this tissue, i.e. increased glycogen synthesis, enhanced glucose oxidation, rapid lipogenesis and decreased FFA mobilization. It would therefore appear that the transport effect may well be an event that is physiologically very close to the molecular action of insulin. However, as will be seen below, several observations on the effects of insulin are difficult, if not impossible, to account for simply on the basis of an initial action on the trans-membrane penetration of glucose.

Adipose tissue contains no detectable magnesium, and more sodium than potassium (124, 165, 295); which is in contrast with what has been observed in diaphragm muscle, which contains magnesium and has more potassium than sodium (295). The relationships between these various cations and lipogenesis, either the basal or the insulin-stimulated one, have been little studied. Previous and more recent experiments have indicated that K^+ depletion had a stimulatory effect on the basal metabolism of glucose both in intact tissue (266, 510), and in isolated adipose cells (196, 267). In the presence of insulin, results have been more contradictory. The absence of Na^+ has been reported

to decrease insulin responsiveness of intact adipose tissue (169). Similar results have been obtained in some experiments with isolated fat cells (267, 268), but not in others (378). Similarly the absence of K^+ did not alter the insulin effect in some experiments (267), but decreased it in others (169, 378). Recently, a systematic study has been carried out to re-examine in more detail the effects of cations upon the metabolism of glucose by the isolated fat cells, and upon the responsiveness of these cells to insulin (267, 268). The following was observed: a) the omission or Ca^{++} or Mg^{++} from the medium, and the replacement of these cations by Na^+ did not change the basal metabolic activity nor did it alter the insulin responsiveness of the cells; b) when K^+ was omitted and replaced by Na^+ , the basal activity of the cells markedly increased, but the insulin responsiveness remained unchanged. The basal activity was restored to normal upon addition of K^+ to the medium; c) the omission of Na^+ from the medium, and its replacement by K^+ , also resulted in an increase in the basal activity. In addition, however, there was a marked *decrease* of the insulin effect on glucose metabolism. Similar effects were observed when Na^+ was replaced by choline or by Tris, thus indicating that they were not due to K^+ overloading but to the actual reduction of Na^+ . The decreased insulin responsiveness induced by lowering Na^+ of the medium was partly abolished upon addition of Na^+ ; d) finally, kinetic studies suggested, in direct contradiction with previous studies (11, 84), that all these alterations were related more to changes in V_{max} than to changes in K_m , which remained rather unaffected. In control cells, the V_{max} was increased by the absence of K^+ as well as by that of Na^+ . In the presence of insulin Na^+ lack decreased the V_{max} , K^+ lack being ineffective. These (267, 268) and other experiments (194, 196) suggest that there may exist a link between glucose metabolism and the activity of the Na^+-K^+ pump. Indeed, several conditions known to decrease the activity of this pump (ouabain; K^+ or Na^+ lack) have been shown to stimulate the uptake and the further metabolism of glucose (196, 267). However, the relationship between carbohydrate metabolism and pump activity is certainly not a direct one since, as indicated above, the omission of only Na^+ but not that of K^+ , decreased the insulin responsiveness of the isolated adipose cells (267). From the above-mentioned experiments a hypothetical model of glucose transport, as related to the carrier system and the cationic environment has been designed, and is illustrated by Fig. 9. In the absence of insulin, it is conceivable that the mobile carrier for glucose simultaneously binds Na^+ and K^+ . These electrolytes would then be transported inside the cell together with glucose. Once unloaded, the empty carrier would move back to the outer surface of the membrane and carry additional glucose molecules and cations. It is further conceivable that by omitting cations in the medium, more binding sites become available on the carrier, and that more glucose molecules can therefore be transported inside the cell. Insulin may act by increasing the number of sites available for glucose

on another mobile carrier. This carrier would be inactive in the absence of the hormone, and would require Na^+ before accepting glucose for transport across the membrane. Liberation of both glucose and Na^+ would then occur at the inner surface of the membrane. Once activated by insulin and provided Na^+ is present, this carrier would be able to carry more glucose molecules per unit time. This would be due to the presence of a greater number of binding sites for glucose and/or to a faster shift of the loaded carrier across the membrane (267, 268).

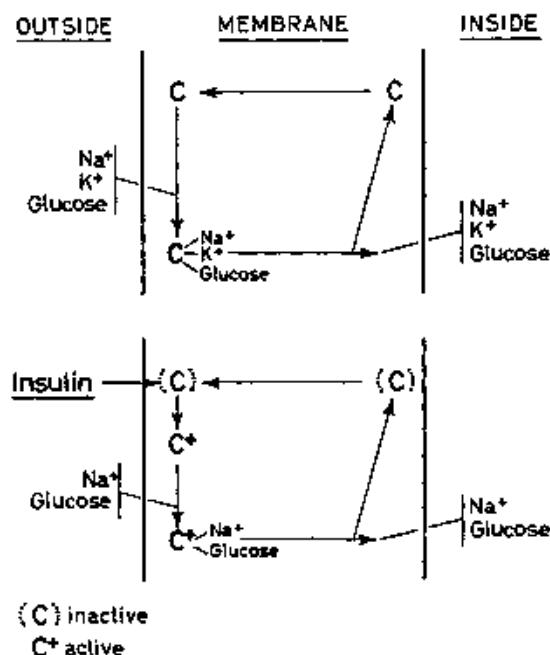


Fig. 9. Hypothetical carrier model for glucose transport in isolated fat cells. From J. LETARTE and A. E. RENOLD, 1967 (267)

The basal uptake, and the effect of insulin on the entry of carbohydrates into adipose cells can certainly be viewed as a very complex phenomenon, once one further realizes that there probably exist several sites for the uptake of the different hexoses taken up by the cells (101, 102, 132, 168). Finally one should mention that the process by which insulin interacts with the cell membrane, a process that has been studied mostly in muscle tissue, is still unknown. Does a binding of insulin occur in or on the cell? Is it a tight or a transient binding? Is it a specific or a non-specific process? Most of these questions are not settled yet (41, 62, 135, 136, 310, 325, 339, 429, 430, 435, 504, 509).

2. The "non-transport" effects of insulin

The majority of the effects of insulin on adipose tissue metabolism are probably related to its action on the transport of carbohydrates across the cell membrane, as discussed above. However, it is unquestionable that several

exceptions exist, which cannot easily be reconciled with this "transport hypothesis". Several examples of these "non-transport" effects will now be recalled:

a) — Insulin has been shown to increase the retention of K^+ and to decrease that of Na^+ in adipose tissue, an effect that was small and unrelated to the presence of glucose in the incubation medium (165).

b) — The existence of resting membrane electric potentials has been demonstrated in rat epididymal adipose tissue. These potentials decreased as K^+ in the medium was increased, or as Na^+ was decreased (20). Furthermore, an increase in the potentials could be induced by insulin even in the absence of glucose in the incubation medium (19). This insulin effect could be obtained with low doses of the hormone; it was quite specific since it was suppressed by anti-insulin antibodies (21).

c) — Other experiments have suggested that insulin may act intracellularly by facilitating the rate of interaction of a given hexose with its phosphorylation site (9).

d) — The decrease in FFA mobilization brought about by insulin can be readily understood on the basis of its action on the transmembrane penetration of glucose since increased glucose uptake is followed by increased lipogenesis and re-esterification (Fig. 6). However, it has now been demonstrated unequivocally that an inhibitory effect of insulin upon lipolysis can be observed in the *complete absence of glucose* in the incubation medium (133, 222, 223, 285, 343, 463). This direct anti-lipolytic action of insulin appears to be a general phenomenon for it has been shown to occur with various lipolytic hormones (epinephrine, norepinephrine, glucagon, ACTH) when used at small concentrations (105, 222, 285). Insulin was also inhibitory, in the absence of glucose, on the joint lipolytic effect of dexamethasone plus growth hormone, and on lipolysis induced by theophylline (105). Of further interest was the finding that the direct anti-lipolytic effect of insulin differed from species to species, an observation that had been made previously for the glucose transport effect of the hormone (400). Thus, insulin was a potent anti-lipolytic agent upon hamster adipose tissue, but it failed to act upon adipose tissues from rabbits and guinea-pigs. As previously discussed, adipose tissue from various species appears to have, or to lack, peptidases that are able to cleave lipolytic hypothalamic peptides. The capacity to cleave these peptides was found to be inversely related to the capacity to respond to their lipolytic action. In contrast, the relationship between responsiveness to the anti-lipolytic action of insulin and the presence of insulin-cleaving enzymes was shown to be direct! Clearly more experiments will be needed to understand the significance of such a relationship (400).

e) — Other experiments with adipose tissue *in vitro* have shown that, in the absence of glucose in the medium, insulin prevented glycogen breakdown

and markedly diminished the activation by epinephrine of the tissue phosphorylase (223).

Both the anti-lipolytic and the anti-glycogenolytic effects of insulin are of interest in view of the new concept of hormonal action via a "second messenger", since it can be hypothesized that they could be related to a decrease in the tissue level of cyclic AMP. Recent experiments have shown that this hypothesis may well be correct (65). Thus, a marked increase in the levels of cyclic-AMP was induced by the addition of epinephrine or caffeine to adipose tissue *in vitro*. The presence of insulin resulted in a substantial and rapid (within 5 minutes) reduction of these cyclic-AMP levels (65). However, the reduction was not great enough to fall into the range where cyclic adenylylate could be expected to be limiting for lipolysis; the correlation between the anti-lipolytic effect of insulin and the levels of tissue nucleotide was not always satisfactory (65). One should recall at this point that adipose tissue contains substantial amounts of cyclic-AMP, which appear to be almost inactive in promoting lipolysis, possibly because of binding or sequestration (63, 65). It is thus conceivable that cyclic-AMP must be in a specific form (or location) to be physiologically active. Furthermore, the actual effective variations in the nucleotide levels may occur in a range that is too narrow to be easily detectable and correlated with the lipolytic activity (65). Other experiments on adipose tissue phosphorylase and glycogen synthetase have indicated, although indirectly, that insulin and epinephrine had opposite effects on the tissue levels of cyclic-AMP (224). When adenyl cyclase activity was measured, tissues from insulin-treated animals were found to exhibit a lower cyclase activity than those from control animals (224).

f) — Lipogenesis, as well as protein synthesis from various amino acids has been shown to occur in adipose tissue, and to be the subject of complex metabolic regulation (5, 24, 51, 75, 117, 148, 150, 187, 270, 284, 309). From evidence obtained in other tissues, in muscle in particular, it would be conceivable that insulin might accelerate the entry of amino acids into adipose tissue (506). However, the precise site of action of insulin upon amino acid metabolism by this tissue has not been elucidated yet, and contradictory results have been obtained (150, 155). Whatever the effect of insulin on the actual penetration of amino acids may be, several experiments have indicated that the hormone may exert some action beyond the membrane level (187). In particular, in the absence of added glucose, insulin stimulated the incorporation of labelled carbon of pyruvate-3-¹⁴C or of acetate-4-¹⁴C into adipose tissue proteins; it also stimulated that of histidine-2-¹⁴C into proteins, provided that pyruvate was present (245, 247).

g) — Another "non-transport" effect of insulin appears to be exerted on the lipoprotein lipase of rat adipose tissue. It was demonstrated that following cessation of insulin treatment in alloxan diabetic rats, lipoprotein lipase activity

decreased rapidly. Simultaneously a rise in plasma triglycerides was observed, which suggested that a deficient lipoprotein lipase could be partly responsible for elevated plasma triglycerides of the untreated diabetic rats (405).

From all the experiments mentioned above, one may conclude either that insulin has several distinct sites of action within adipose tissue, which appear to be related in a rather loose way, or that a single reaction possibly underlies transmembrane transport, *as well as* the other seemingly unrelated events. Experiments favoring the second possibility have been carried out recently in isolated fat cells (43, 382, 384). It was observed that many of the effects seen with insulin could be mimicked by exposing the adipose cells, not to insulin but to phospholipase C, an enzyme known to hydrolyze several phospholipid components. When the cells were exposed to high concentrations of phospholipase C, cell membranes were damaged, and insulin responsiveness disappeared. The action of the phospholipase was then "buffered" by supplementing the incubation medium with lipoproteins which represented additional substrates for the enzyme, and therefore protected the cell membranes. Under those conditions, increasing the phospholipase concentrations resulted in increased glucose uptake, increased oxidation of glucose to CO₂ and lipogenesis from glucose. The incorporation of labelled amino acids was also stimulated. Furthermore phospholipase C was able, *in the absence of glucose*, to block completely the FFA release induced by lipolytic agents such as ACTH, epinephrine, glucagon and theophylline. In other words the metabolic pattern induced by phospholipase C was completely similar to that of insulin (382, 384). On the basis of these observations, it was suggested that insulin might act by altering the lipid portion of the plasma membrane, perhaps in the direction of transforming a stable, double-leaflet membrane into a micellar one, thereby exposing previously unexposed portions of the underlying layer of the membrane. The latter, in turn, might well be the site of a "carrier", which would thus become more available to its substrate. Similar views underlie other observations in which an insulin-like effect of proteolytic agents on isolated muscle has been observed (365, 366, 367).

In an attempt to reconcile the "transmembrane transport" effect, and the "non-transport" effects of insulin on adipose tissue, one might further hypothesize that the changes induced in the cell membrane (by insulin or by substances such as phospholipase C) might result in signals that would have far-reaching influences on intracellular events. They might for instance produce effects similar to those described by SUTHERLAND, namely the appearance of second, possibly third "messengers", which would then be responsible for the many metabolic effects observed with insulin ranging from actual uptake of glucose and subsequent lipogenesis, to anti-lipolysis, protein synthesis, etc., observed in the absence of added glucose. This general hypothesis of insulin action would imply a very reactive cell membrane. Actually, such a reactivity

of the cell membrane is rather well suggested by the existence of a rapid turnover of phospholipids in adipose tissue, and by the strong stimulatory effect of insulin on the incorporation of ^{32}P -phosphate into phospholipids, phosphatidylinositol in particular, both in adipose (466) and muscle tissue (288). Furthermore the structure of the cell membrane does change upon addition of agents such as phospholipase C, as recently studied electronmicroscopically. Under those circumstances, the membrane leaflets were shown to be actually transformed into many ring-like structures apparently formed by several globular subunits (22).

3. Effects of agents other than insulin

a) Lipogenic agents

All the *growth hormone* preparations used so far, regardless of the species from which they were obtained, have been shown to promote glucose metabolism (151, 153, 154, 173, 261, 498, 500). However, the pattern of glucose metabolism so induced differed markedly from preparation to preparation. Some of them induced changes in carbohydrate metabolism similar to those observed with epinephrine (Fig. 6), changes that were the likely consequences of a primary lipolytic effect of the hormone (173, 261). Others were insulin-like, i.e. they stimulated glucose metabolism and lipogenesis (Fig. 6) (151, 153, 154). Although these discrepancies have been attributed, as previously mentioned, to contamination of some of the hormonal preparations by other lipolytic hormones, this has not yet been clearly proven. Furthermore, the discrepancy may be only superficial since the observed insulin-like effect induced by growth hormone appears to be transient, and to be followed by a marked depression in lipogenesis from glucose (153), a change that could well result in a secondary increase in the FFA output from adipose tissue. The conflicting effects observed with growth hormone *in vivo* become perhaps more understandable if one realizes that the hormone is distributed in many organs (muscle, liver, intestine, kidney, bone, adipose), which it may therefore influence metabolically (77).

Prolactin is another hormone that controls lipogenesis in adipose tissue. Here again, as for growth hormone, some hormonal preparations were lipogenic, whereas others stimulated the lipolytic activity of the tissue (160, 173, 214). These differences appear to depend upon the method of purification used (173). Thus, employing three different procedures, three different prolactin preparations (PLR, PLC, PLF) may be obtained. When added to adipose tissue *in vitro*, PLR and PLC produced effects similar to those of epinephrine, namely increased glucose metabolism, decreased (or unchanged) lipogenesis, and increased FFA output. PLF had no lipolytic activity but was insulin-like, stimulating both glucose metabolism and lipogenesis from glucose (173). One should mention that in the pigeon, prolactin was found to stimulate the activity

of liver malic enzyme (160), an action that was of particular interest in connection with the ability of the hormone to induce an increase in both body weight and liver size of normal pigeons, mostly through increased fat deposition (364). Furthermore, it has been demonstrated that prolactin also induced an increase in body weight and fat deposition in the migratory white crowned sparrow (307).

Thyroid hormones have been reported to enhance total fat metabolism *in vivo*, i.e. both synthesis and breakdown (214). *In vitro*, the sensitivity to insulin was higher in adipose tissue from hyperthyroid than that from normal rats (170); a similar finding was observed with epinephrine, which induced a higher FFA release in tissues obtained from hyperthyroid rats (85, 170). More recently, it has been suggested that thyroid hormones may play a specific role in adipose tissue metabolism. Thus, the activities of hexokinase, citrate-cleavage enzyme, malic enzyme, NADP-linked isocitrate dehydrogenase, four enzymes playing an important role in lipogenesis, were sharply decreased in adipose tissue, adrenals and testes from hypophysectomized rats. Treatment of the hypophysectomized animals with thyroxine restored in part the normal activity of all four enzymes in adipose tissue, but was without effect in either adrenals or testes (58).

Various *oxytocin preparations* and *lysine-vasopressin* stimulated the incorporation of labelled glucose into adipose tissue triglycerides (308, 311, 347). The pattern of glucose metabolism induced by these hormones was qualitatively similar to that obtained with insulin (311, 347). Quantitatively, however, the effect produced by oxytocin was much smaller than that of insulin (308). Furthermore, whereas insulin stimulated both parametrial and epididymal fat tissue, oxytocin had clear-cut effects only on epididymal fat tissue, suggesting that the effect might be sex-dependent (308). When tested *in vivo*, various oxytocin and vasopressin preparations produced a rapid decrease in the FFA concentrations of the plasma of nondiabetic and diabetic dogs (312). Here again, the effect observed appeared to be sex-dependent, since FFA levels decreased after hormone administration to normal male dogs, whereas they increased in female animals (61).

Estrogens (estrone, estradiol, estriol) have also been demonstrated to increase lipogenesis in adipose tissue from female rats *in vitro* (141). The lowest effective concentration was 10^{-9} μ mole/ml in tissue from female rats, whereas much higher concentrations were ineffective in tissue from male animals. Of particular interest was the finding that estradiol and insulin were synergistic in female adipose tissue; no such effect could be obtained in rat epididymal adipose tissue (141). As far as sex hormones are concerned, one should mention that the addition of *testosterone* at high concentrations (30 μ g/ml) to rat epididymal adipose tissue *in vitro* resulted in decreased glucose oxidation to CO_2 and

lipogenesis from glucose. FFA mobilization was stimulated, although not very markedly, by much lower concentration of the hormone (0.003—0.3 µg/ml) (216).

Finally, lipogenesis from glucose by adipose tissue can be stimulated by *hyperosmolar concentrations* of sucrose, mannitol, NaCl and sorbitol. The pattern of glucose metabolism so induced was similar to, although not identical with, that due to insulin, suggesting that hyperosmolarity may have influenced in some way the permeability of the cell membrane (251).

b) Anti-lipolytic agents

Prostaglandins. The prostaglandins are acidic lipid compounds with vaso-depressor and smooth-muscle stimulating properties. They were first reported in 1935 in human semen (99, 145), and were purified and characterized from 1946 on (28, 200, 201). Initially two forms of prostaglandins were isolated, prostaglandin E₁ (PGE₁), and prostaglandin F_{1α} (PGF_{1α}). Subsequently, six "primary" prostaglandins were structurally characterized, as well as several "secondary" prostaglandins presumably derived from PGE₁ and PGE₂. The prostaglandins are not restricted to the male accessory genital glands and their secretion. They have been found, in addition, in menstrual fluid, lungs, brain, thymus, pancreas and kidney (28).

When added to adipose tissue *in vitro*, PGE₁ at low concentration (0.1 µg/ml) reduced the basal release of both FFA and glycerol (441). PGE₁ also markedly diminished the lipolytic effect of epinephrine, norepinephrine, glucagon and ACTH. TSH-induced lipolysis was more difficult to reduce: a second addition of PGE₁ during the incubation of the tissue was necessary to obtain the inhibitory effect (441). PGE₂ was less effective than PGE₁, and PGE₃ was inhibitory only at very high concentrations (17 µg/ml) (441).

In human adipose tissue *in vitro*, very low doses of PGE₁ (0.01 µg/ml) could inhibit both the basal and the norepinephrine stimulated lipolysis. No concomitant effect on glucose metabolism was observed (27, 71). PGE₁ also inhibited lipolysis induced by theophylline (319, 335, 424). The effectiveness of PGE₁ in inhibiting lipolysis was much greater for theophylline-induced than for epinephrine-induced lipolysis. The actual mechanism of inhibition of PGE₁ is still unknown. In the particular case of epinephrine, the effect could be related to a decrease in the degree of activation of the hormone sensitive lipase (440, 441). On the other hand, prostaglandins have been reported to inhibit epinephrine either competitively (451) or non-competitively (319). It has also been suggested that the inhibition may be of a mixed type, competitive and non-competitive, depending upon the concentration of calcium in the medium: lack of Ca⁺⁺ would favour non-competitive inhibition (108). Although the type of interaction between PGE₁ and theophylline is not determined yet, it

appears to be quite different from that observed with epinephrine (319, 335). Finally, it has been observed that PGE₁ has no effect on FFA and glycerol release in adipose tissue obtained from fasted rats (73). A similar observation has been made *in vivo* (30), which fits the concept that FFA mobilization during fasting may not involve hormonal and nervous control (447).

Observations *in vivo* have confirmed the above-mentioned experiments *in vitro*. Intravenous injection of PGE₁ in anaesthetized dogs reduced the high levels of plasma FFA obtained by a continuous infusion of norepinephrine (25). Similar results have been obtained in unanaesthetized dogs (444). In both cases PGE₁ did not counteract the effect of epinephrine on the level of blood glucose (25, 444). In unanaesthetized human subjects, contrary to the finding in dogs, PGE₁ was found to increase plasma FFA and glycerol levels, although blood sugar was unaltered. Furthermore, PGI₁ infused intravenously had no inhibitory effect on the increase in plasma levels of FFA caused by the administration of norepinephrine (26, 28). This unexpected phenomenon may be related to the rate of PGE₁ infusion, as recently suggested by experiments carried out in anaesthetized or unanaesthetized dogs in which, depending upon the rate of PGE₁ infusion, an increase (slow rate) or a decrease (higher rate) in plasma FFA could be obtained (28). The increase in the plasma FFA levels induced by a sympathetic ganglionic-stimulating agent was abolished by concomitant infusion of PGE₁, while the dog still responded to the agent with increase in blood glucose, heart rate and arterial pressure. These results indicated that PGE₁ specifically inhibited the effect of catecholamines on lipid mobilization, but not that on the cardiovascular system or on glucose metabolism (25, 28, 441, 444).

Nicotinic acid. It is now well established that nicotinic acid produces a decrease in the basal level of plasma FFA, and inhibits the rise in plasma levels induced by hormones such as the catecholamines and glucagon (69, 71, 72, 478). When added *in vitro*, nicotinic acid inhibited the norepinephrine-induced release of both FFA and glycerol in rat as well as in human adipose tissue (70, 71), a finding that could be related to a decrease in the hormone-sensitive lipase activity (38, 40). On the other hand, nicotinic acid stimulated the uptake of glucose (38), the oxidation of glucose to CO₂, and the incorporation of glucose-carbon into lipids (40, 196). Concurrently, it appeared to increase the re-esterification rate of free fatty acids (264, 332). The anti-lipolytic action of nicotinic acid may thus be due to complementary mechanisms: a) increased rate of glucose metabolism followed by decreased FFA output; b) decreased activation of the hormone-sensitive lipase.

Ouabain. This compound has been found to produce insulin-like effects in adipose tissue cells, not only on glucose metabolism and lipogenesis from glucose, but also on FFA release (anti-lipolysis) in the absence of glucose in the incubation medium (194, 196, 197). These findings, because of the known

action of ouabain on the Na-K pump, are of interest since they suggest a link between carbohydrate, lipid, and electrolyte metabolism (421).

— *Effects of ouabain on carbohydrate* (196). When added to isolated fat cells, ouabain (concentrations as low as 10^{-4} M) stimulated glucose uptake and oxidation, as well as lipogenesis and glycogen synthesis from glucose. Simultaneously, both basal glycogenolysis, and ACTH- or epinephrine-induced glycogenolysis were reduced by the presence of this compound. When comparing the pattern of glucose metabolism induced by ouabain or by fast-acting

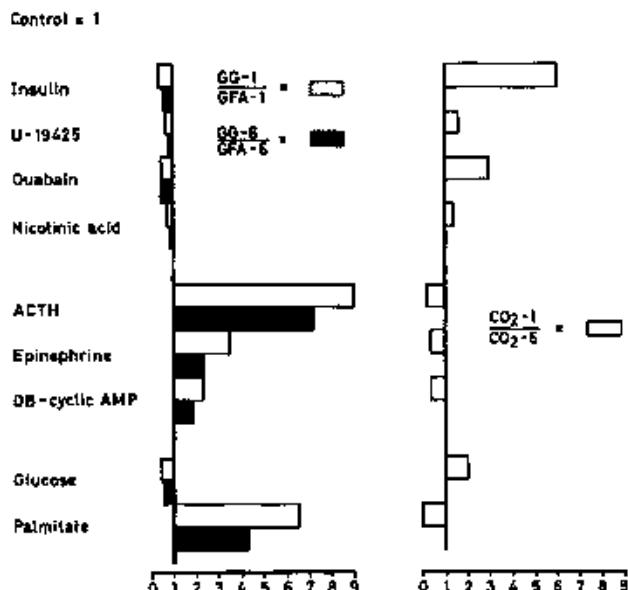


Fig. 10. Effect of various anti-lipolytic and lipolytic agents, of glucose and FFA, on glyceride-glycerol to glyceride-fatty acids ratios derived from glucose- 1^{14}C and glucose- 6^{14}C , and on the 1^{14}C to 6^{14}C ratios for CO_2 produced from labelled glucose. Ratios are compared to that of the controls which are considered as 1.

From R. J. Ho and B. JEANRENAUD, 1967 (196)

lipolytic hormones, the main differences observed were as follows: a) glyceride-glycerol synthesis was decreased by ouabain, increased by the hormones; b) glyceride-fatty acid synthesis was stimulated by ouabain, inhibited by the hormones. As a consequence, the ratios glyceride-glycerol/glyceride-fatty acid were decreased by ouabain, but markedly increased by the fast-acting lipolytic hormones (ratios: controls = 0.8, ouabain = 0.44, ACTH = 2.7) (196). The respective effects of ouabain, and of the fast-acting lipolytic hormones, were further characterized by using specifically labelled glucose as substrate (glucose- 1^{14}C , glucose- 6^{14}C). In addition, these experiments permitted comparing the pattern of glucose metabolism induced by several anti-lipolytic agents such as insulin, U-19425 (5-methylpyrazole-3-carboxylic acid, an anti-lipolytic and hypoglycemic agent), nicotinic acid and ouabain, to that induced by lipolytic agents such as ACTH, epinephrine and cyclic-AMP. As illustrated in Fig. 10, it is readily apparent that the anti-lipolytic agents on the one hand,

the lipolytic hormones on the other, behaved as two homogeneous but quite distinct groups, each having characteristics of its own (196). Thus, all anti-lipolytic agents decreased the glyceride-glycerol/glyceride-fatty acids ratios, while increasing the ratios of the CO_2 derived from glucose-1- ^{14}C to that derived from glucose-6- ^{14}C ($\frac{\text{CO}_2,1}{\text{CO}_2,6}$ ratios). Detailed calculation showed that all these agents increased glucose metabolism through the pentose cycle. On the contrary, all fast-acting lipolytic hormones decreased glucose metabolism through the pentose cycle. They all increased the glyceride-glycerol/glyceride fatty acids ratios, while decreasing the $\frac{\text{CO}_2,1}{\text{CO}_2,6}$ ratios. Finally (Fig. 10), it was observed that glucose mimicked the anti-lipolytic group, palmitate the lipolytic one. These observations suggest that the differences observed between the two groups of compounds may be due to different FFA concentrations within fat cells, which in turn would control the metabolic pattern of glucose utilization. Indeed, all agents or conditions that may be expected to decrease intracellular concentration of FFA exerted similar effects upon glucose metabolism. On the other hand, agents such as lipolytic hormones, which may reasonably be assumed to increase the intracellular levels of FFA, exerted effects on carbohydrate metabolism that were clearly different from those of the anti-lipolytic group. It may be further hypothesized that the level of two metabolites, namely that of FFA and of cyclic-AMP, are closely related, and are the cause of the observed metabolic pattern of glucose. Thus, fast-acting lipolytic agents, by increasing the levels of cyclic-AMP (63), may increase lipase activity, and therefore intracellular FFA levels. Increased FFA levels may in turn decrease glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and acetyl-CoA carboxylase activities, thereby bringing about a decrease in lipogenesis (48, 239, 300, 329, 374, 462). Simultaneously, increasing levels of cyclic-AMP may activate phosphofructokinase, thus channeling glucose metabolism through the Embden-Meyerhof pathway (87). These metabolic events would explain rather well the pattern of glucose metabolism induced by lipolytic agents, as illustrated in Fig. 6 and 10. Anti-lipolytic agents would do just the opposite. As demonstrated for insulin (65, 224) and suggested for ouabain (197), they would decrease intracellular cyclic-AMP and FFA levels, therefore channeling more glucose through the pentose cycle and increasing lipogenesis.

Although all anti-lipolytic agents may act, at least in part, by decreasing cyclic-AMP levels, this does not imply that the intimate site of action is identical for all of them. As far as ouabain is concerned, the effect may well be related to its effects on electrolyte transport, since an ouabain effect can be mimicked by omitting K^+ from the medium (194, 267). The possible sequence of events linking ouabain, K^+ lack, and cyclic-AMP is discussed below: the important point to stress here is that insulin and ouabain show some degree of similarity, and that this property may be due to a common effect on cyclic-AMP

level. However, one should keep in mind that, in addition, insulin probably has properties that it probably does not share with ouabain, e.g. a "membrane effect", which has been previously discussed.

— *Inhibitory effect of ouabain on FFA release.* As illustrated in Fig. 11, when ouabain was added to isolated fat cells incubated in the *absence of glucose*, a marked inhibition of the lipolysis induced by several fast-acting hormones

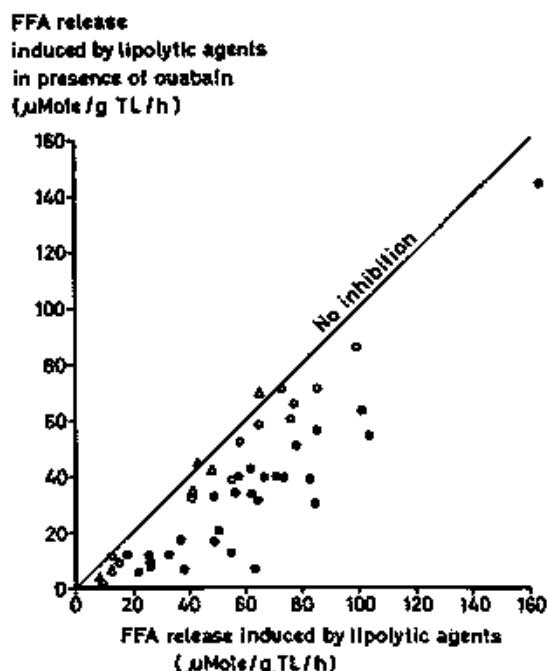


Fig. 11. Comparison of the inhibitory effect of ouabain on the lipolysis induced by lipolytic hormones, a cyclic-AMP derivative, and caffeine, in the absence of glucose. Each point is the mean of 4–8 paired values. ● lipolytic hormones (ACTH 0.4 to 2.0 mU/ml; epinephrine 0.05 to 0.1 µg/ml; or glucagon 1 µg/ml). ○ N⁶C₁-dibutyryl 3', 5'-AMP (0.05 to 2.0 mM). △ caffeine (10⁻⁴ to 10⁻³ M). Ouabain, when added, was 3.1 × 10⁻⁴ M. The 45° line is the theoretical identity line corresponding to no inhibition. From R. J. Ho, B. JEANRENAUD, TH. POSTERNAK and A. E. REINOLD, 1967 (197).

was observed (194, 197, 318). By contrast, the presence of ouabain resulted in little or no inhibition of lipolysis induced by dibutyryl-3', 5'-AMP or caffeine. These results therefore suggested that the inhibitory effect of ouabain on lipolysis was related to an action at the level of the formation of cyclic-AMP but that, once cyclic-AMP had been formed, the compound was essentially without effect. Indeed, when adenyl cyclase and phosphodiesterase activities were measured, it was found that adipose tissue that had been incubated with ouabain exhibited a marked decrease in adenyl cyclase activity, phosphodiesterase being unaffected (197). Finally, omission of K⁺ from the incubation medium resulted in ouabain-like effects, namely: a) ACTH- or epinephrine-induced lipolysis was sharply reduced; b) adenyl cyclase activity was decreased (Table 6). These results indicate that the process of the hormone-stimulated

Table 6. Adenyl cyclase activity in adipose tissue homogenate: effect of preincubation with ouabain or K⁺-free medium

	1	2	3	4	Mean ± S.E.	P values
Controls	32.0	32.0	38.6	41.8	36.1 ± 2.5	
+ ouabain (6.9 × 10 ⁻⁴ M)	15.2	17.9	17.4	20.6	17.8 ± 1.1	
% difference	47.6	55.8	45.2	48.6	49.3 ± 2.3	< 0.01
Controls	56.5	16.2	30.3	11.3	28.6 ± 10.1	
K-free medium	18.0	9.0	15.8	8.1	12.7 ± 2.5	
% difference	33.2	55.4	52.2	71.1	52.9 ± 7.8	< 0.01

Cyclic-3', 5'-AMP formation is expressed as μmole/50 mg per 15 min. ± S.E. Percent difference is calculated as $\frac{\text{ouabain or K-free treated tissue}}{\text{control}} \times 100$.

Data of R. J. HO, B. JEANRENAUD, TH. POSTERNAK and A. E. RENOUD, Biochim. biophys. Acta (Amst.) 144, 74, 1967 (197).

mobilization of FFA appears to require an intact active transport system for electrolytes, and that the anti-lipolytic effect of ouabain may be linked to the action of sodium and potassium transport (197, 318). A possible sequence of events might be as follows: ouabain would decrease the formation of cyclic-AMP by interfering at, or just before, the adenyl cyclase reaction. It is further conceivable that the affinity between lipolytic hormones and adenyl cyclase, or between lipolytic hormones and the cell membrane, could be regulated by the concentration of cations. Such an affinity could be decreased either by the presence of ouabain, or by the lack of K⁺ in the medium, thus explaining the similarity of effects observed under those two different conditions (197). Other cations, such as calcium, appear to be important modulators of the lipolytic process, increasing or decreasing FFA release depending on the relative concentration of sodium and potassium (46).

Other anti-lipolytic agents. Many other agents, the precise mechanism of action of which is as yet unknown, have been shown to decrease FFA release from adipose tissue. Among them one should cite the following substances; a) The salicylates brought about a marked inhibition of the free fatty acid release *in vivo*, even when small quantities were administered; *in vitro*, however, large doses were necessary to obtain an inhibitory effect (34, 36, 137). b) Both α- and β-adrenergic blocking agents could inhibit the lipolytic effect of noradrenaline and ACTH, although the β group was shown to be the more potent (182, 451). It has been suggested that the α and β adrenolytic compounds may have different sites of action on the lipolytic system (450). The β-adrenolytic compounds would primarily inhibit the formation of cyclic-AMP, whereas the α group would inhibit the actual action of the cyclic nucleotide (451). A very good review pertaining to the adrenergic receptors, the adrenergic α and β

functions, and the adrenolytic agents as related to the effects of catecholamines on lipid mobilization has recently been published (488). c) Experiments *in vivo* have suggested the possible existence of a direct antilipolytic effect of the *sulfonylurea drugs* (139, 452). Tolbutamide (10–50 mg/100 ml) decreased the net release of glycerol and FFA from adipose tissue *in vitro*, even when glucose was absent from the incubation medium (453). The magnitude of the anti-lipolytic effect was similar to that obtained with insulin (222). A non-hypoglycemic sulfonamide, sodium sulfadiazine, was ineffective (453). d) *Ketone bodies*. β -hydroxybutyrate inhibited both the basal and the norepinephrine-stimulated release of glycerol from adipose tissue *in vitro* by decreasing lipase activation. The inhibition was more pronounced in the absence than in the presence of glucose. Acetoacetate had similar effects. On the contrary, acetone increased glycerol release, as well as lipase activity brought about by norepinephrine (39). In addition, ketone bodies stimulated the conversion of glucose-U- ^{14}C to CO_2 , as well as lipogenesis from glucose. It is likely that these effects were partly responsible for the decrease in FFA and glycerol release induced by ketone bodies in the presence of glucose (176). e) *Lactic acid* has also been reported to inhibit the activation of adipose tissue lipase (37). f) It has been previously reported that *nucleic acids*, *nucleotides* and *nucleosides* inhibited the lipolytic action of epinephrine on adipose tissue, whereas purine and pyrimidine bases increased it (94). More recent experiments carried out with human and rat adipose tissue have shown that some purines were actually inhibitory. Of the major purine bases, only adenine was stimulatory, whereas hypoxanthine, xanthine and guanine were potent inhibitors of the epinephrine-induced lipolysis (352). The inhibition by purines and their conjugates was found to be associated with an increased destruction of epinephrine (352). g) NAD was also a potent inhibitor of lipolysis *in vitro*, as were a number of products of NAD hydrolysis. The similarity of the inhibitory effects of NAD, 5'-AMP and adenosine suggested that the antagonistic action of NAD was dependent upon the presence of adenosine, rather than that of the nicotinic acid moiety. In addition, ADP, ATP and NADP were as potent inhibitors as NAD, suggesting a similar dependence upon the presence of the adenosine moiety. The physiological role of these effects is not established (342). h) *Caffeine* inhibited glucose oxidation and lipogenesis from glucose by rat adipose tissue *in vitro*. Furthermore, it markedly reduced the stimulatory effect of insulin on glucose metabolism (6).

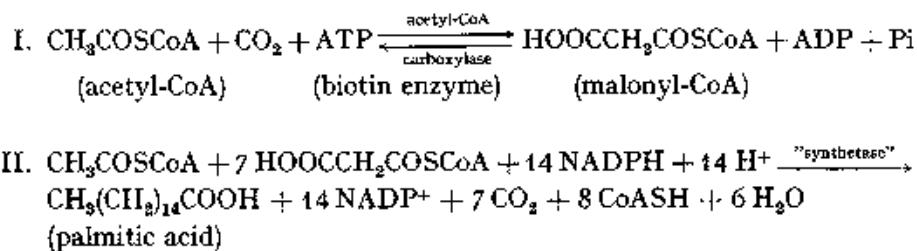
IV. Metabolic pathways and intracellular regulation

1. Synthetic pathways

The synthesis of long chain fatty acids and subsequently of triglycerides is the "raison d'être" of adipose tissue. Although the biochemical pathways of lipogenesis have been studied mostly in the liver (278, 280, 281), experiments

carried out in adipose tissue clearly indicate that the pathways are essentially similar in the two tissues (113, 125, 293).

For many years it had been recognized that CO_2 was playing a central role in lipogenesis (49, 114, 283). This function, however, was better understood when it was realized that HCO_3^- was playing the role of a catalyst and was not incorporated into the fatty acids (301, 480, 481). Two fundamental reactions were then characterized (278, 281, 469, 482).



In reaction I, HCO_3^- , in the presence of biotin (Vitamin H), Mn^{++} and ATP, is incorporated in acetyl-CoA to produce malonyl-CoA. This carboxylation reaction actually consists of two sequences: in the first, HCO_3^- , in the presence of ATP and Mg^+ , reacts with biotin to form carboxybiotin; in the second carboxybiotin reacts with acetyl-CoA, and malonyl-CoA is formed (281). The whole sequences of reaction II, studied in greatest detail in yeast (281) and *Escherichia coli* (3, 468, 469), is performed by a multienzyme complex. Acetyl-CoA serves as "primer" of the process. Its C_2 unit is recovered only in the methyl end of the fatty acid produced, whereas C_2 units from malonyl-CoA are added to the acetyl residue during the synthetic reaction. In addition, the "synthetase" enzyme system has two different types of thiol groups, referred to as "central" and "peripheral" thiol groups. The synthetic process is first initiated by the transfer of an acetyl-CoA molecule to the "peripheral" thiol group. Incorporation of malonyl-CoA is achieved through intermediates that are covalently bound to the "central" thiol group. This allows them to come in close contact with the several enzymes of the "synthetase" unit, enzymes that are arranged around the "central" thiol group. During reaction II, acetyl-CoA condenses with malonyl-CoA to form acetoacetyl-CoA, acetoacetyl-CoA is reduced to β -hydroxybutyryl-CoA, which is then dehydrated to form crotonyl-CoA; crotonyl-CoA is reduced and butyryl-CoA formed. Butyryl-CoA then replaces acetyl-CoA in a condensation reaction with another molecule of malonyl-CoA, and a compound of longer chain length, which is similarly reduced, dehydrated and reduced, is synthetized. Repetition of this series of reactions leads, by consecutive additions of 2-carbon units from malonyl-CoA, to the long chain acyl-CoA derivatives (C-16, C-18). At this point, the saturated acids are cleaved from the "central" thiol group of the enzyme system. It is

not known at the present time why this system produces mainly palmitic and stearic acids, and no carboxylic acids of shorter chain length (281, 468).

As far as adipose tissue lipogenesis is concerned it is important to recall that the enzymes of this synthetizing system are present in the tissue. They can be recovered in the "particle-free" (i.e. free of mitochondria and microsomes) supernatant of tissue homogenates after one hour centrifugation at $105,000 \times g$ (293). Furthermore, several experiments have confirmed the importance of CO_2 in adipose tissue lipogenesis. Thus, by depriving animals of part of their bicarbonate reserve through the administration *in vivo* of an inhibitor of carbonic anhydrase, acetazolamide, a marked decrease in fatty acids synthesis was observed (47, 114, 388, 389, 391). On the contrary, the intravenous administration of bicarbonate to 4–6 hours fasted mice restored to normal the decrease in lipogenesis that had been induced by fasting. Lipogenesis from acetate was restored more clearly than that from glucose, but no effect was obtained when the fasting was prolonged to 24 hours, indicating that other alterations had occurred under the latter condition (114, 392). Finally, decreased lipogenesis induced by acetazolamide *in vivo* could be restored to normal following intravenous administration of bicarbonate (389).

2. Intracellular control of acetyl-CoA carboxylase activity

Reaction I is much slower than reaction II. The acetyl-CoA carboxylase may therefore constitute a rate limiting step in adipose tissue lipogenesis, and an important one in regulation (294, 327). This conclusion, however, is still open to discussion, for experiments with intact adipose tissue *in vitro* have shown that the capacity of the tissue to convert acetyl-CoA to fatty acids did not appear to be limited (14, 123).

It is well established that fasting causes a marked loss in the fatty-acid-synthetic activity of mammalian tissues, in adipose and liver tissues in particular (78, 210, 298, 390). Although many experiments have been carried out to study the mechanisms underlying this decreased lipogenesis, none has explained satisfactorily all aspects of the problem (390). Furthermore, as mentioned previously, most of the work pertaining to the control of lipogenesis has been done in liver rather than adipose tissue, because the former is easier to handle when enzyme systems are studied.

Acetyl-CoA carboxylase activity has been found to be markedly depressed by fasting (300, 326) and diabetes (491). However, this could not explain fully the decreased lipogenesis observed under those conditions (279, 300), indicating that other factors must be involved. The disappearance, during fasting, of a lipogenic stimulator, "lipogenin", has been incriminated (74). It has also been proposed that fasting might favour the appearance of lipogenic inhibitors derived from the microsomes (microsomal ATP-ase) (238, 299), or enhance mitochondrial inhibitors that would interfere with acetyl-CoA carboxylase

(238). On the contrary, microsomes obtained from fed rats appear to have a stimulatory effect on lipogenesis (238, 276, 300). The physiological significance of these factors remains to be established, all the more that several experiments are still contradictory (114, 390). For instance, normal mitochondria have been shown either to inhibit (238, 300) or to stimulate (294) fatty acid synthesis when added to particle-free supernatant.

An interesting property of acetyl-CoA carboxylase, which may provide for another site of regulation, is that it can be inhibited by long chain acyl-CoA derivatives; the unsaturated derivatives were found to be less inhibitory than the saturated ones, whereas the FFA were ineffective (48, 300, 329). More recently, it could be shown that FFA, and not only fatty acyl-CoA derivatives, had an inhibitory effect on the activity of the enzyme (239, 300). These findings may help understanding of the decrease in adipose tissue lipogenesis that is known to occur upon the addition *in vitro* of lipolytic hormones such as ACTH (67, 420).

Another characteristic of acetyl-CoA carboxylase is its ability to be activated by di- and tri-carboxylic acids, particularly by citrate (293, 301). It has been shown that the increase in enzymatic activity was associated with an aggregation of the enzyme, which changed from a monomer to a trimer form (328, 329, 467). This may constitute a rather general physiological mechanism, since it has been described for other enzymes, in particular for glutamic dehydrogenase, which has been found to aggregate and disaggregate under the influence of various stimuli (465). As yet the physiological significance of this citrate effect is unknown.

3. Relationship between carbohydrate and lipogenesis

a) Production of suitable reduced coenzymes

It has long been recognized that the process of fatty acid synthesis is influenced by concurrent changes in carbohydrate metabolism. For instance, the decrease in the metabolism of glucose observed during starvation or in diabetes is accompanied by a marked decrease in the rate of fatty acid synthesis (214, 359). Several mechanisms have been suggested to explain these observations; among those most commonly cited are: a deficiency of NADPH secondary to a fall in glucose-6-phosphate dehydrogenase activity; a fall in the level or activity of the necessary lipogenic enzymes; and a decrease in the availability of substrates (215, 359). The role of NADPH in lipogenesis has been particularly stressed (129, 214, 464), and this has been at the origin of many experiments aimed at evaluating the activity of the pentose cycle relative to that of the Embden-Meyerhof pathway, since the pentose cycle represents an important source of NADPH (121, 123, 214, 230, 231, 252, 253, 256). Most of these studies have been based upon comparison of the yield of radioactive CO₂ or other metabolic products from specifically labelled glucose. The many

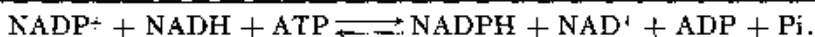
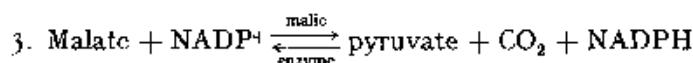
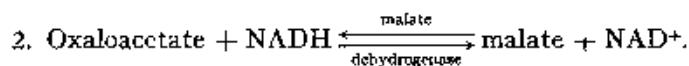
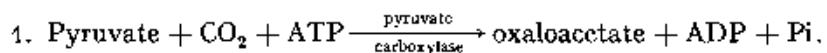
pitfalls underlying this type of approach have been thoroughly discussed and they will not be considered in this review (121, 227, 228, 229, 230, 252, 253, 255, 256, 505). From data obtained *in vitro*, it appears that the contribution of the pentose cycle to total metabolism is of the order of 15—25 % (196, 214, 230, 252), although data obtained *in vivo* would indicate that pentose cycle activity might be higher (close to 50 %) (341), (or 30 to 35 %).¹

Another question to be answered when considering pentose cycle activity is the following: what is the quantitative role of this pathway in providing NADPH for lipogenesis? Several studies have suggested that the pentose cycle was increased by insulin (121, 196, 252, 497) and decreased by adrenaline (121, 196, 230), thus explaining the increased lipogenesis observed with the former hormone, and the decrease with the latter. More recently, careful experiments have been designed to study the fate of the reduced coenzymes produced during glucose catabolism in adipose tissue (121, 123). It was found that the amount of NADPH produced by the pentose cycle was sufficient, in control tissues, to provide the total reducing equivalents necessary for fatty acid synthesis (121). However, in the presence of insulin, and despite the fact that the NADPH thus formed was used quantitatively for lipid synthesis (231), the pentose cycle furnished only 63—65 % of the NADPH needed for lipogenesis. This clearly demonstrated that when fatty acid synthesis was proceeding swiftly, other reduced coenzymes, namely those produced during the oxidation of triose phosphates to acetyl-CoA (NADH), must somehow be called upon to furnish the electrons necessary for this increased synthesis (121). When NADH production was estimated, it was found that these coenzymes were always formed in excess of those actually needed to complete, not only the synthesis of fatty acid, but that of lactate and glycero-phosphate as well (121, 122). As it is likely that during fatty acid synthesis, NADPH is the primary and perhaps the only effective reductant (14, 123, 278), some mechanisms must apparently exist in adipose cells, whereby NADH would, through a transhydrogenation process, be converted into NADPH. The pyruvate carboxylase—malate dehydrogenase—malic enzyme system appears to be precisely responsible for this transhydrogenation, as will be seen now (14).

It should first be recalled that previous experiments had shown that adipose tissue *in vitro* could convert pyruvate to fatty acid in the absence of added glucose even when the tissue was obtained from starved animals (213, 496). This indicated that the oxidation of pyruvate to acetyl-CoA resulted in the production of reduced coenzymes utilizable for fatty acid synthesis (14, 503). In a recent series of experiments, the possible pathways open for the metabolism of pyruvate were therefore investigated. It was discovered that adipose tissue far exceeded all other tissues in its content of malic enzyme. Furthermore, the activity of this enzyme was highest in adipose tissue from fasted-

¹ S. Rous, L. LÜTHI and P. FAVARGER, personal communication.

refed rats, lowest in that from fasted animals, and varied therefore directly with conditions known to affect lipogenesis (14, 172, 258, 269, 271, 503). This was a very important finding, for it had been previously suggested that malic enzyme might play a role in providing NADPH for lipogenesis (120, 508), and that marked changes in malic enzyme activity could be induced by dietary or hormonal conditions known to affect lipogenesis in liver (418, 464, 470), as well as in mammary gland (202, 357). In addition to malic enzyme, it was shown that adipose tissue also had pyruvate carboxylase and malate dehydrogenase activities (503). The following sequence of reactions was therefore proposed (14, 503):



In other words the pyruvate carboxylase - - malate dehydrogenase - - malic enzyme system represents an ATP — requiring transhydrogenation, and can account for the transformation of reducing equivalents (NADH formed during the conversion of glucose to acetyl-CoA via the Embden-Meyerhof pathway) into NADPH suitable for lipogenesis (14, 333, 503, 508). Since nearly 2 NADPH molecules are required for the reduction of each acetyl-CoA molecule to fatty acid, the transhydrogenation process is capable theoretically of supplying at best 50 % of the total NADPH needed. Actually, experiments have shown that it supplied about 35 % of the total, and that the balance was furnished by the pentose shunt (123).

As mentioned above, reduced coenzymes (NADH) are produced in excess, and they must be reoxidized if acetyl-CoA production and fatty acid synthesis are to proceed. Part of this reoxidation occurs through the pyruvate carboxylase-malate dehydrogenase-malic enzyme system just described. The remaining excess of NADH is directly oxidized by oxygen, thus explaining the 80 % increase in oxygen consumption observed when lipogenesis is initiated by insulin (121, 123). Furthermore, in the absence of insulin, 72 % of the oxygen consumed by adipose tissue can be accounted for by electrons produced by oxidation of acetyl-CoA in the TCA cycle. On the contrary, in the presence of the hormone, the TCA cycle is shut down, only 16 % of the oxygen is so used, but a large portion of the reduced coenzymes produced during the formation of acetyl-CoA replace, as a source of energy for the cell, those generated from acetyl-CoA in the TCA cycle. Thus, with insulin, the adipose cells not

only appear to channel more acetyl-CoA into fatty acids but, by making use of what may be termed by-products of lipogenesis, they achieve a greater efficiency in the energy cost of converting glucose into lipids (15).

The rate at which NADPH can be furnished by the pentose cycle does not appear to be a rate limiting factor in intact adipose tissue: the rate of operation of this cycle is, on the contrary, geared to the rate at which NADPH is needed (15, 123). This concept is illustrated by the following experiments: when insulin-stimulated adipose tissue was incubated in the presence of glucose, 65 % of the NADPH was provided by the pentose cycle. When acetate was added to glucose, an increased glucose uptake and glucose flow through the pentose cycle was measured, with the result that 75 % of the NADPH was now produced by the pentose cycle. This clearly suggested that a precursor of fatty acids such as acetate, the metabolism of which, contrary to that of glucose, utilizes reduced coenzymes without producing them, had increased the demand for NADPH, and had therefore stimulated the activity at the pathway responsible for its production (123).

b) Controlling effects of metabolites

The conversion of glucose to long chain fatty acids is a process in which several reactions are so tightly integrated that it is extremely difficult to tell which one is cause and which one is effect. One controlling factor may be the rate at which reduced coenzymes, known to be formed in excess, can be re-oxidized by oxygen (123). The energy-consuming reactions involved in the transhydrogenation of NADH to NADPH might also assume an important regulatory role of lipogenesis (15, 123). Other metabolites may be of importance:

Citrate. As illustrated in Fig. 12 citrate is of interest because, in addition to its ability to activate acetyl-CoA carboxylase (293, 301), it represents a vehicle for the transfer of acetyl-CoA from the mitochondrion where it is formed, to the cytoplasm where it is utilized for lipogenesis (14, 330, 428). This transfer process may not be the only one operative in adipose tissue, as others are known to exist in tissues, namely: a) hydrolysis of acetyl-CoA, and diffusion of acetate out of the mitochondria (277, 330); b) conversion of acetyl-CoA to acetyl-carnitine, followed by diffusion of this substance out of the mitochondria into the cytoplasm where it is reconverted to acetyl-CoA (54, 277, 330). However, citrate may be of particular importance for the transport of acetyl-CoA in adipose tissue since the activity of the *citrate cleavage enzyme* has been shown to be very high in this tissue (57, 241). As shown in Fig. 12, the citrate cleavage enzyme, first described in liver (242, 243, 427), is extra-mitochondrial, and catalyzes the transformation of citrate to acetyl-CoA and oxaloacetate. This reaction may well be crucial for lipogenesis in adipose tissue since, in addition to furnishing acetyl-CoA for the extra-mitochondrial lipid-synthesizing system, it constitutes a link with the pyruvate carboxylase-malate

dehydrogenase-malic enzyme process described previously. The existence of a parallel in the alterations of adipose tissue malic enzyme and citrate cleavage activities brought about by dietary conditions may indicate that the response of both enzymes is elicited by a common trigger, or is under the control of a common operational genetic unit (14, 241, 503).

3', 5' cyclic AMP. This cyclic nucleotide has been shown to be quite potent: a) in activating adipose tissue phosphofructokinase; and b) in reversing the inhibitory effect of citrate on phosphofructokinase (87). These findings may partly explain the accelerated glucose uptake, glycolysis and flow through

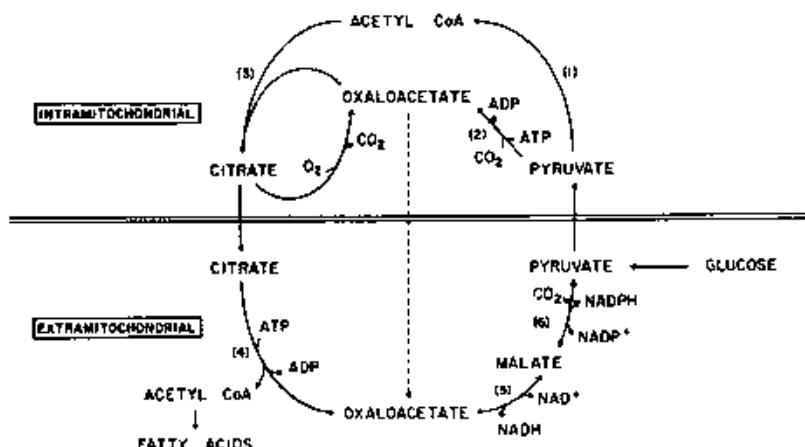


Fig. 12. A possible relationship between citrate cleavage and malic enzymes in adipose tissue lipogenesis.
From M. S. KORNACKER and E. G. BALL, 1965 (14, 241)

phosphofructokinase known to occur in adipose tissue stimulated by adrenaline, in spite of the fact that the hormone simultaneously increases the concentration of citrate (87, 88). It is conceivable that the overall rate of fatty acid synthesis may be governed to some extent by phosphofructokinase activity. This enzyme may in turn be influenced by the cellular concentration of metabolites such as citrate, and ATP (inhibitory); or ADP, cyclic AMP, and phosphate (stimulatory) (87, 88, 338). However, it is not yet possible to assess with accuracy the relative importance of phosphofructokinase, and the above-mentioned citrate-malate cycle in controlling lipogenesis (14, 123).

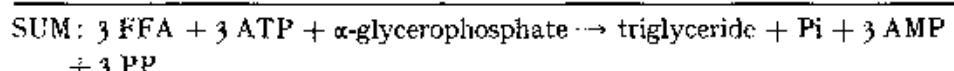
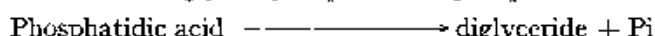
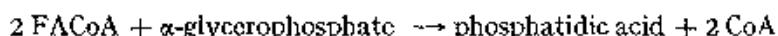
Phosphorylated sugars. They have been reported to increase the hepatic synthesis of palmitic acid from acetyl-CoA and malonyl-CoA by stimulating the activity of the fatty acid synthetizing system (483). This stimulatory effect appears to be mostly due to allosteric effects of phosphorylated sugars such as fructose diphosphate, glucose-1-phosphate, glucose-6-phosphate, on one or more enzymes of the lipogenic system. Although similar studies have not yet been done in adipose tissue, such a mechanism may well exist in this tissue, and could explain the stimulatory influence of carbohydrate upon lipogenesis.

Furthermore, if the fatty acid synthetizing system does respond to phosphorylated hexoses *in vivo*, as it does *in vitro*, then the stimulation would parallel the uptake of glucose. On the contrary, in starvation or in diabetes, where the cellular concentration of the phosphorylated sugars is low, fatty acid synthesis would consequently fall (483).

4. Role of some adipose cell organelles

Triglyceride synthesis and most of the other biochemical reactions taking place in adipose tissue are thought to occur in the cytoplasm of the adipose cell (214, 472). When the fine structure of adipose cells is studied by electron-microscopy (Fig. 13), it becomes evident that the rim of cytoplasm, which surrounds the large central lipid vacuole, although extremely thin, is quite rich in a variety of organelles such as mitochondria, endoplasmic reticulum, small vesicles and lipid droplets (323, 324). The way in which these organelles participate in glyceride synthesis and breakdown has not yet been clearly defined (8, 495). Some experiments suggest that the FFA formed during lipolysis may be complexed with a protein, and transported as microvesicles from the central vacuole to the surface of the cell. These microvesicles would then fuse with the plasma membrane and burst, thus releasing FFA into the extracellular fluid (495). This outward transport of FFA as microvesicles, the content of which remains isolated from the surrounding cytoplasm, is consistent with some biochemical evidence suggesting that the intracellular free fatty acids derived from lipolysis are anatomically separate from those coming in (95, 512).

Extensive studies have demonstrated that adipose tissue readily synthetizes neutral glycerides (8A, 214, 472). Once fatty acids are synthetized from glucose, or are taken up from the extracellular space, the formation of triglycerides is effected via the following sequence of reactions (439):



Recently, the intracellular localization of these processes has been investigated. Following a series of centrifugations, nuclei, mitochondria, endoplasmic reticulum, and the soluble supernatant fraction could be isolated from isolated fat cells (8). These preparative steps made it possible, in particular, to isolate numerous membrane-enclosed lipid droplets that would float when centrifugated at $10^4 \times g$ for 15 minutes, and that closely resembled chylomicra under

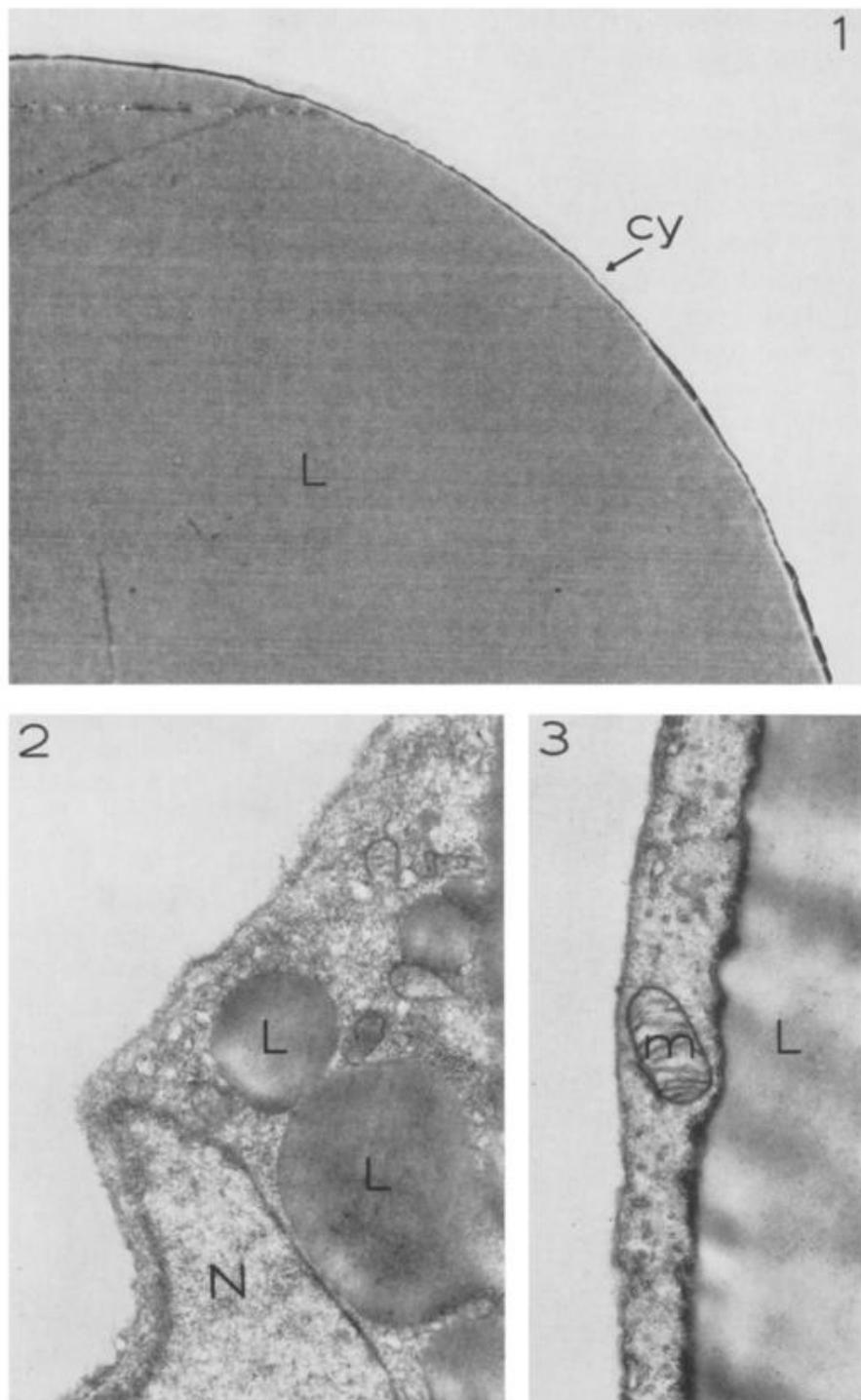


Fig. 13. Electronmicrographs of the fine structure of isolated fat cells. 1. L = the fat vacuole; Cy = cytoplasm ($\times 4,000$). 2. N = nucleus; L = lipid droplets ($\times 18,000$). 3. L = the fat vacuole; m = mitochondrion ($\times 22,000$). Unpublished micrographs of R. PIETET, B. JEANRENAUD, CH. ROUILLER and A. E. RENOLD,
1967

the electron microscope. Furthermore, metabolic studies with isolated fat cells were carried out using glucose-U-¹⁴C as substrate, and the distribution of ¹⁴C-lipid in the various organelles was investigated. It was found that the specific activity of the lipids contained in the mitochondria and the microsomes was highest; that of the lipid droplets was intermediate; and that of the vacuoles was lowest. This clearly indicated that these organelles were arranged in a succession of precursor to product relationship. As non-specific physical association of ¹⁴C with the different fractions could be ruled out, it was concluded that the mitochondria and the microsomes were intimately associated with the process of glyceride synthesis (8). More recently, other experiments have demonstrated that the esterifying activity of adipose tissue was indeed confined to the mitochondria and the microsomes, and that the mitochondria were the more active. With both mitochondria and microsomes, it was further shown that phospholipids (probably phosphatidic acid) were the main ester products. The soluble fraction alone did not stimulate the esterification process, but, when added to particulate preparations, it altered the distribution of the esters synthetized in such a way as to increase the proportion of di- and triglycerides, and to decrease that of the phospholipids. It appears that the soluble fraction may have exerted its effect by increasing the activity of diglyceride-acyl-transferase or, more likely, that of phosphatidatephosphohydrolase (385). It is thus interesting to observe that in adipose tissue the intracellular localization of the esterification reaction is similar to that reported for liver (436, 437, 494), intestinal mucosa (55), and lactating mammary gland (184).

The small, membrane-enclosed droplets may well have a specific functional significance and represent a vehicle for the intracellular transport of neutral lipids from the sites of synthesis to the vacuole (8). From the above-mentioned experiments one might summarize the present knowledge of glyceride synthesis and transport in adipose tissue as follows: conversion of glucose to fatty acids and α -glycerophosphate in the soluble fraction of the cell → esterification in association with the mitochondria and the microsomes → formation of lipid microvesicles, a vehicle that allows the hydrophobic, newly-synthetized, glycerides to be transferred into the central vacuole of the adipocyte.

V. Conclusion

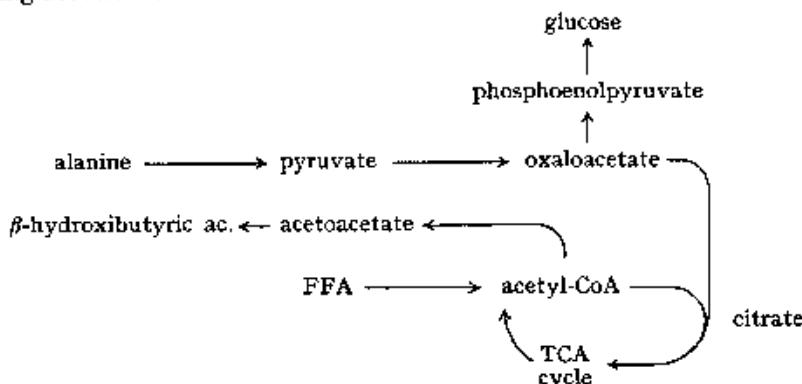
In this review, emphasis has been deliberately placed on adipose tissue metabolism and its control, and no effort has been made to link biochemical facts to possible clinical consequences. However, from the now recognized importance of adipose tissue in bioenergetics, an importance that the present work has attempted to summarize, it is evident that this tissue is bound to play an important role in several aspects of pathophysiology. A brief mention

of several aspects of human pathology in which adipose tissue is or could be implicated will now be made.

A major pathophysiological importance must of course be given to *obesity*. Although the existence of different obesities (regulatory, metabolic) is well substantiated in laboratory animals, human obesity is more difficult to characterize (432, 433, 490). As recently pointed out, the energy surplus represented by human obesity may reflect direct failure of the mechanisms responsible for energy balance, or a blurred combination of a variety of neurological, endocrine, enzymatic, psychological, genetical, and environmental disorders, of which little is known (303).

Diabetes and adipose tissue metabolism are also linked (434, 360). To cite but one example, an hypothesis of the pathogenesis of diabetes mellitus in man is based upon the assumption of a primary acceleration of FFA release from adipose tissue, a defect that would result in an inhibition of the glucose transport mechanism, and modify several physiological actions of insulin, catecholamines, growth hormone, and corticosteroids (356).

A close interrelation exists between *ketosis*, and lipolytic activity of fat tissue. The primary event of ketosis appears to be a rise in the plasma FFA concentrations. Fatty acids being probably the main source of ketone bodies in the liver, FFA production closely parallels ketone bodies production. However, during carbohydrate deficiency (starvation, diabetes), increased amino acid output from muscle occurs concomitantly with increased lipolytic activity (258). Furthermore, in the liver (and the kidney) oxaloacetate is an intermediate for both the gluconeogenesis and the disposal of acetyl-CoA as illustrated by the following reactions:



As the rate of phosphoenolpyruvate synthesis increases when the demand for carbohydrates is increased (diabetes, lactation, fasting), increased gluconeogenesis is accompanied with a drop in the steady state level of oxaloacetate, therefore with a fall in TCA cycle activity. Under those conditions, the FFA which reach the liver in increasing quantity are oxidized to acetyl-CoA but

cannot be oxidized completely in the TCA cycle (lack of oxaloacetate). Acetoacetate is therefore formed through condensation of acetyl-CoA, and ketosis results (249). Although the concept of a close relationship between FFA output and ketogenesis is rather widely accepted, one should mention that recent experiments have indicated that ketogenesis can be varied independently of plasma FFA and acetyl-CoA in the liver, thus suggesting that starvation ketosis is not causally related to increased mobilization and oxidation of FFA! (126).

Several authors have suggested that *heat production* may be an important function of adipose tissue in general, and of brown adipose tissue in particular. It is not quite clear, however, as to whether or not the heat production so produced is of quantitative importance (220).

Finally, one should recall the relationships which exist between adipose tissue metabolism and lipoproteins. Among the several types of *hyperlipoproteinemia* described so far, some appear to be due to a defect in their removal by extrahepatic tissue, by adipose tissue in particular; others might be related to an increased mobilization of FFA to the liver, resulting in an increased synthesis of the pre- β lipoproteins (127).

These few examples of human disorders do indicate that considerable work will continue to be required from both biochemists and physician-scientists to fully unravel the thread of the multiple control mechanisms possibly relating adipose tissue metabolism, and the many facets of human physiology and human pathology.

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Erratum to Figure 1, page 59:

It appears now that secondary particles are formed, *not* via the liver, but directly from the chylomicrons in the plasma as a result of the interaction of lymph chylomicrons with a plasma triglyceride — containing lipoprotein. (E. L. BIERMAN and D. E. STRANDNESS, *Am. J. Physiol.* **210**, 13, 1966.)

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Glucagon*

By

PIERO P. FOA**

With 12 Figures

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Abbreviations

ACH	= Adrenal cortical hormones	NADPH = TPNH = Nicotinamide-adenine
ACTH	= Adrenocorticotropin	dinucleotide phosphate
3'-AMP	= Adenosine 3'-phosphate	PC = Pyruvate carboxylase
5'-AMP	= Adenosine 5'-phosphate	PEPCK = Phosphoenolpyruvate
Cyclic AMP	= Adenosine 3', 5'-phosphate	carboxykinase
FFA	= Free fatty acids	PFK = Phosphofructokinase
IRG	= Immunoreactive glucagon	UDPG = Uridine diphosphoglucose

I. Introduction

The time elapsed since the publication of my most recent reviews on glucagon (227, 228, 232) has been relatively short, but very productive, and it seems desirable to reconsider the many actions and the possible physiologic role of this essential hormone. The selection of the material has been arbitrary as I have attempted to write a logical rather than a complete summary of the newer knowledge. In so doing, I have yielded to the temptation of offering new hypotheses, most likely just as ephemeral as the old ones, but perhaps stimulating to the reader, whom I urge to acquire a balanced perspective (and additional references) by reading several other recent reviews on glucagon and closely related matters in English (728); French (432, 435, 801); Italian (46—48, 100, 231) and Japanese (562).

II. The chemistry of glucagon

Methods for the extraction of glucagon have been reviewed (230). Glucagon and insulin can be effectively separated from serum by means of chromatography on resin-impregnated paper (851) or electrophoresis on strips of cellulose acetate (459). Studies by x-ray diffraction, optical rotatory dispersion and concentration-difference spectra suggest that glucagon crystals have helical structure and that glucagon molecules can change from an associated, helical form in solutions approaching saturation to a dissociated, random-coil form upon dilution (58, 400). The synthesis of several polypeptide chains, representing the complete glucagon molecule, has been achieved (355, 687—691, 847, 874—884). Other studies led to the realization that the molecule of glucagon resembles that of insulin in several respects. For example, if histidine, the NH₂-terminal amino acid of glucagon, is placed next to the histidine residue in the B-chain of insulin, then three of the four OH-groups in the insulin ring coincide with similar groups in glucagon. Furthermore, a comparison of the large ring of insulin with that proposed for glucagon shows that about one-fifth of all the amino acids in glucagon have exact counterparts in insulin and, when the two chains are aligned in a fully extended configuration, there are some striking similarities in the distance between amino acid residues. These findings suggest that the two hormones may have comparable configurations *in vivo* and may, therefore, have common binding sites (694). Glucagon,

a polypeptide with 29 amino acids and secretin, a polypeptide with 27 amino acids, have 14 amino acid residues located in the same position, a fact which may help explain the immunologic similarities between pancreatic glucagon and the glucagon-like material secreted by the mucosa of the gastrointestinal tract (16, 305, 809). A study of the ionization characteristics of the two tyrosine residues in the glucagon molecule revealed that Tyr 10 and Tyr 13 ionize equally, but Tyr 13 is less reactive with cyanuric fluoride. The reactivity of Tyr 13 is increased by tryptic digestion, suggesting hydrogen-bonding of Tyr 13 with other residues in the peptide chain (499). The differential specificity of chymotrypsin α and chymotrypsin β toward glucagon was investigated (187) and glucagon was hydrolyzed by proteinase C of snake venom (677) and by carboxypeptidase (358a). Glucagon reacts with di-isopropyl-phospho-fluoridate which phosphorylates several of its serine radicals (236) and with a variety of quinones which decrease its hyperglycemic activity (789). No information is available on the amino acid composition of glucagon derived from different animal species or from different organs of the same animal (see p. 147).

III. The assay of glucagon

Glucagon can be assayed using an isolated perfused rat liver preparation capable of detecting 10 μ g of the hormone (732). Glucagon can be assayed also by its ability to stimulate the formation of adenosine 3', 5'-phosphate (cyclic AMP) and hence the conversion of inactive dephosphophosphorylase to active phosphorylase *in vitro* (480). Although this method is sensitive to as little as 0.005 μ g of glucagon per ml and is suitable for the determination of glucagon in biologic materials, its specificity leaves something to be desired. For this reason, immunologic methods are generally preferred (656, 848). These methods are based on the competition between unlabeled glucagon in the sample and ^{131}I -labeled glucagon for binding by ant glucagon serum and consist of three major steps: first, labeled glucagon is incubated with glucagon antiserum, then part of the labeled glucagon is displaced from its binding sites by the unlabeled glucagon present in the standard solution or in the sample and, finally, the radioactivity of antiserum-bound and free labeled glucagon is measured after separation by electrophoresis (14, 656, 804—807), chromatography (424), or precipitation with a second antibody (365, 710) or with ammonium sulfate (613). These methods require the preparation of ^{131}I - or ^{125}I -labeled glucagon with high specific activity, free of radiation damage and with well defined stability, antibody-binding capacity and other properties (310, 365, 424, 613, 686). Essential among these is that the glucagon used as standard be immunologically identical with the glucagon being measured. Fortunately, rat, dog, bovine-porcine, porcine, and human pancreatic glucagon fulfil this requirement so that the easily available bovine-porcine glucagon may be used as a standard for experiments in dogs, rats, and men. Anti-

glucagon sera with high energy and good binding capacity are difficult to prepare, but have been prepared in rabbits, guinea pigs and ducks by means of repeated injections of glucagon in FREUND's adjuvant, of mixtures containing polyvinylpyrrolidone or of glucagon conjugated with serum albumin (14, 365, 403, 424, 512, 613, 656).

Part of the difficulty may be caused not by a low antibody concentration of the antisera, but by the failure of some labeled glucagon to react with the antibody. Therefore, suitable labeled glucagon may be concentrated by preliminary antibody binding, isolation of the antigen-antibody complex by gel filtration and dissociation of the "active" portion of the labeled glucagon from the complex (686). Proteolytic damage to glucagon during incubation of the samples interferes with the determination and must be prevented. This may be done by adding a trypsin inhibitor, such as Trasylol¹ (679, 794, 797). Although this recently recognized fact may cast some doubt on the results of earlier experiments, recovery studies, transhepatic measurements of endogenous and of ¹³¹I-labeled glucagon and measurements of glucagon in tissue extracts seem to confirm their validity (794, 797). Using immunologic methods it is possible to measure as little as 20—50 µg of glucagon, with a reproducibility of ± 2 to 10% and with recoveries varying between 87 and 125% (424, 710).

IV. The site of origin of glucagon and the histology, histochemistry and embryogenesis of the A cell

The various types of islet cells, their staining properties, their morphology and A and B cell composition have been reviewed (28, 101, 319). Efforts have been made to improve the specificity of A cell staining methods by means of toluidine blue (322), dark field luminescence (318, 324, 737, 738), aldehyde thionine (326), aldehyde fuchsin and silver impregnation (833). New methods of silver impregnation have helped dispel the confusion between "argentaffine" cells, which reduce silver salts directly and "argyrophilic" cells, which become impregnated with silver only in the presence of a reducing agent. Both types have been described in the pancreatic islets and in the mucosa of the gastrointestinal tract (280, 719). Using BOUIN fixation and silver impregnation, HELLERSTRÖM and HELLMAN have established that the A cells can be divided into silver-positive (A_1) and silver-negative (A_2) cells. It is now believed that the A_2 cells are the source of glucagon, while the A_1 cells, which are probably identical with the D cells, secrete gastrin, other gastrointestinal hormones capable of acting as mediators for the secretion of insulin and glucagon (see p. 149, 174) (101, 191, 249, 537, 599, 600), or lipocalic (190). The A_2 cells may be differentiated from other cells also by the characteristic histochemical reactions of tryptophan, an amino acid present in the glucagon, but not in the

¹ FBA Medical Research, Division of Metachem, Inc., 425 Park Avenue, New York 22, New York.

insulin molecule. Glucagon has been identified and localized in tissue sections, although not in individual cells, by its fluorescence after treatment with fluoresceine-conjugated antiglucagon serum (29). Criteria for the electron microscopic identification of various types of islet cells and granules have been described (32, 415, 515, 775). Two types of A cells have been recognized by means of electron microscopy: one type arranged as a syncytium, the other surrounded by a membrane (55), one type light and the other dark (96). An ultramicroscopic study of the secretory cycle of the A cells has shown that the α granules form in the midst of the GOLGI apparatus and that cell degranulation consists in the disintegration of the granule with formation of small particles which pass through the plasma membrane and can be traced through the cytoplasm of the capillary endothelium (515, 536). In addition to the A₁ (or D) and to the A₂ cells, the islets of LANGERHANS contain cells and fibers of the autonomic nervous system. These structures appear to be functionally related to the A cells (774) and, perhaps mediate the nervous stimuli which are believed to modify the secretion of glucagon (see p. 148), of the catecholamines and of serotonin. These substances have been found in the blood of the pancreatic-duodenal vein and in the pancreatic islets (206, 207, 227, 465).

It has been reported that injections of neutral red in the rat cause complete disappearance of the A cells, followed by over-regeneration (561), while a significant reduction in the percentage of A cells has been noted also in monkeys treated with thyroxine (256). A variety of enzymes have been demonstrated in the A cells. Among them are lactic and malic dehydrogenases (160, 161), glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, isocitric dehydrogenase, succinic dehydrogenase, cis-aconitase, NADPH reductase, glutamic-oxaloacetic transaminase, alkaline phosphatase and esterase (70, 274, 320, 356, 598, 600, 602, 736, 768, 840). The presence of the dehydrogenases indicates that the hexose monophosphate shunt is active in the A cells, although the relationship between these enzymatic activities and the secretion of glucagon is unknown. An approach to the solution of this problem has been made using quantitative histochemical methods in the duck, an animal in which A and B cells are located in separate groups (70) and by the use of the isolated islet of fish (343) or of intact isolated islets of mammals (32, 160, 317, 369, 388, 416, 597, 767). A cells have been identified in the pancreatic islets of all animals studied, including the monkey, (255, 867), the horse (324), the viper (6), amphibians (322, 392, 426), in most, but not all, fish (208—210, 393, 607, 722, 866) and other species (8, 55, 318, 326). These studies leave little doubt that the A₂ cells of the islets of LANGERHANS are the most important, although perhaps not the sole source of glucagon (391, 395, 480). The distribution and relative abundance of the A cells vary significantly from species to species and it is interesting to speculate that an abundance of A cells may be a factor in the relative resistance of certain animal species to the hypo-

glycemic effects of insulin. Thus, the A cell-rich birds and lizards can tolerate doses of insulin many times larger than those which cause death or profound hypoglycemia in the relatively A cell-poor fish, frogs, salamanders and mammals (272, 393). The tunny, *Orcynus (Thunnus) thynnus* L., a teleost fish which has two types of islets, one composed mainly of A cells and the other richer in B cells (607) should be a very useful animal for this type of study.

The ontogeny of the endocrine pancreas was reviewed recently (56, 190, 233, 282, 284, 321). Alpha granules may first be recognized in the chick embryo pancreas on the third day of incubation by means of the electron microscope (159), but only on the seventh day by light microscopy (777). In the rat, argyrophilic cells are present only at birth, although dark field luminescence, which could be due to either insulin or glucagon granules, appears on the eighteenth day of gestation (11). In the guinea pig, A₁ and A₂ cells can be first seen in the pancreas of a 26 day-old fetus (599). In man, the first differentiation of A cells is believed to occur at the 30 mm stage. During the fifth month of gestation there is a marked proliferation of the A cells, which tend to decrease in number thereafter, reaching a minimum during the seventh and eighth month of gestation (121, 457). A detailed electron microscopic study of A₁, A₂ and B cells in the pancreas of human fetus obtained by legal abortion has been published recently (56). Measurements of glucagon-like activity in tissue explants or tissue extracts suggest that significant amounts of the hormone begin to accumulate in the pancreas during the fourteenth day of prenatal life in the rat and during the eighth or ninth day of incubation in the chick embryo. This development of pancreatic glucagon-like activity parallels that of hepatic phosphorylase, reaching a maximum at or near birth, when glycogen synthetase content is low and glucose 6-phosphatase activity increases (23, 79, 123, 124, 144, 159, 284, 285, 406, 563, 566, 604, 829, 892). Clearly, these hormonal and enzymatic changes favor glycogenolysis and, by utilizing liver glycogen which had been accumulating during the last few days of prenatal life, give the newborn the means to prevent serious hypoglycemia in the critical period between the loss of placental support and the beginning of adequate food intake (446, 511, 604, 696).

V. The secretion of glucagon, its regulation and its effect on the secretion of insulin

The introduction of sensitive and specific immunoassay methods for the determination of glucagon in blood, tissue extracts and other biologic materials has helped clarify the multiple factors which seem to regulate the secretion of this pancreatic hormone (15, 39, 424, 656, 710, 801, 804, 805, 807). Thus, the concentration of immunoreactive glucagon (IRG) in normal human serum varies between 1 and 8 m μ g/ml (424, 670, 671, 673, 674), although considerably lower values (ave. 0.3 m μ g/ml) have also been reported (15, 710, 800, 801,

803, 807), especially after removal of the pancreas (424). Calculations based on the biologic half-life of glucagon suggest that its secretion rate in the fasting man is between 50 and 200 µg/hr (672). A glucagon-like material has been found in the gastrointestinal mucosa of man and animals in low concentrations, but significant total amounts in relation to the pancreas (16, 480, 670, 675, 797, 809, Table 1). The nature of this extra-pancreatic glucagon is uncertain: it has been reported that the immunologic properties of pancreatic glucagon

Table 1. Immunoreactive glucagon content of tissues µg equivalents*

	Pancreas per g wet wt.	Stomach		Duodenum		Jejunum		Ileum		Colon per g wet wt.
	total	per g wet wt.	total	per g wet wt.	total	per g wet wt.	total	per g wet wt.	total	
Rat	2.4	2.0	0.0031	0.05	0.006	—	0.14	0.5	—	0.1
Dog	4.5	127.0	0.2—0.47	44.5	0.043	1.2	2.1	67.0	—	10.9
Beef	8.0	—	—	—	—	—	1.2	—	0.24	—
Man (biopsy)	0.4—9.0	—	0	-0.004	—	—	0.061	—	—	—
Man (autopsy) (Ave. 0.24)	0—0.8	23.0	—	—	0.004	0.7	0.009	2.2	0.032	0.94 to 0.006

* These values have been obtained in several laboratories, represent small samples and must be considered preliminary. Statistical evaluation is not possible. All values are expressed as bovine-porcine glucagon equivalents since bovine-porcine glucagon was used as a standard for the assay.

are different from those of glucagon extracted from gastric, duodenal and jejunal mucosa, but not of glucagon extracted from the mucosa of the colon and that "enteroglucagon" may differ from pancreatic glucagon in molecular structure, molecular weight and biologic properties (670, 675). Indeed, enteroglucagon appears to share the insulinogenic, but not the cyclase-activating, glycogenolytic and hyperglycemic properties of the pancreatic hormone (579, 801). Measurements of plasma IRG have demonstrated that glucagon secretion is stimulated by insulin-, tolbutamide- or phlorizin-induced hypoglycemia (15, 805, 806, 807), confirming and extending the results of pancreatic-femoral cross-circulation experiments (232). Starvation is an effective stimulus to glucagon secretion in both dog and man (424, 800, 803). Indeed, in young male volunteers, after three days of total starvation, the level of circulating IRG rose progressively to 3 times its normal value while their blood glucose levels gradually declined and their tolerance for glucose deteriorated (800, 803). Oral glucose, in doses of 1 g/kg reverses the stimulation of glucagon secretion caused by insulin or starvation. On the other hand, oral glucose, given in relatively large doses (1.75 g/kg), and the direct instillation of glucose into the duodenum stimulate, rather than inhibit, the secretion of a glucagon-like material, possibly "enteroglucagon" (579, 801, 808) (see p. 174). The effects

of intravenous glucose are uncertain: a decrease of normal serum IRG levels (710), and of IRG levels after they had been raised by insulin hypoglycemia (807), or by the intravenous injection of amino acids and pancreozymin (15, 170, 798), as well as no significant effect (424), have been reported. Oral as well as intravenous amino acids and pancreozymin evoke a prompt and sharp rise in plasma IRG level (670, 795, 808). Chronic hyperglycemia, as in alloxan-diabetes, causes a reduction in the total weight of the glucagon-secreting A₂ cells or, at least, a marked increase in the A₁ to A₂ cell ratio (323). The secretion of glucagon is inhibited also by the administration of glucagon itself, perhaps because of the hyperglycemia which it produces. Thus, treatment with glucagon causes atrophy of the A₂ cells (600, 601), degranulation of the A cells (706, 845) and decreases their zinc content and their metabolic and enzymatic activities (751, 819, 845). In contrast to the effects of glucagon, prolonged insulin treatment decreases the insulin content of the pancreas (269) and causes degranulation of the B cells, while the A cells remain fully granulated (463). Although the anterior pituitary may have an indirect influence on the activity and microscopic appearance of the A cells (100, 112, 232) and although large doses of human growth hormone may release glucagon from its immunologically inactive protein-bound form (888), the available evidence for a direct relationship between growth hormone and glucagon secretion is, at best, circumstantial. Indeed, it seems probable that the blood sugar-raising substance found in the blood of the pancreatic vein of dogs treated with growth hormone is a catecholamine or serotonin, rather than glucagon (119, 251, 252, 452, 720, 721). Similarly, the rapid increase in hepatic phosphorylase activity, glycogenolysis and gluconeogenesis following hypothalamic stimulation resembles that obtained by splanchnic nerve stimulation, does not occur in adrenalectomized animals and, probably, is the result of epinephrine, rather than glucagon secretion (213, 712, 713), although structures suitable for the transmission of nerve impulses to the A cells have been described (see p. 145). Other experiments suggesting that glucagon secretion may be under control of the autonomic nervous system have been reviewed (232, 728), but have not been repeated or extended.

Many new experiments have confirmed the fact that the administration of either glucagon or glucose causes the release of insulin from the pancreas *in vivo* (92, 103, 134, 149, 172, 263, 395, 396, 427, 443, 444, 491, 492, 510, 663, 671, 674, 678, 717, 718, 761, 769) and have provided new evidence for the thesis that the secretion rates of insulin and glucagon can be mutually regulated. However, the concept that this reciprocal regulation is mediated through changes in the concentration of blood glucose must be modified in view of the observation that glucagon stimulates insulin secretion also under conditions in which glucose is not present or changes in glucose concentration are not possible, such as in the isolated rabbit and rat pancreas (155, 287, 289, 760, 786), in cultures of pancreatic explants from rat fetus (815) and in patients with

VON GIERKE's disease (33, 134), hepatitis or cirrhosis (670). Furthermore, the insulinogenic effect of glucagon appears to be more prompt and greater than that of glucose at comparable blood glucose levels (396, 492, 663, 674). It should be pointed out also that hyperglycemia is not necessarily always an insulinogenic stimulus and that epinephrine, which shares with glucagon a glycogenolytic and hyperglycemic action, inhibits rather than stimulates insulin release (481, 483, 609, 610, 611). Cervical vagotomy does not suppress the insulinogenic action of glucagon (247), but vagotomy suppresses and vagal stimulation enhances insulin release (248, 262). It would seem, therefore, that glucagon can stimulate the secretion of insulin directly, although this effect may be enhanced by previously established hyperglycemia (670, 672) or by the simultaneous administration of glucose or tolbutamide (663). Using perfused

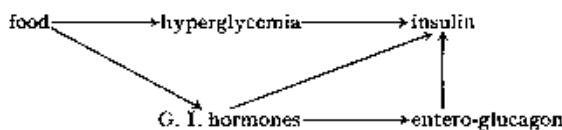


Fig. 1. Effect of oral glucose on insulin and entero-glucagon secretion. \rightarrow stimulation

pancreas preparations it has been calculated that glucagon may cause the release of about 1 to 2% of the total pancreatic insulin content (287). Since the omission of sodium ion inhibits the stimulation of insulin secretion by a variety of agents (526) and since potassium is a stimulant of insulin release in the perfused pancreas (288) and glucagon causes prompt release of potassium from the liver (see Section VII, 5), increases in intracellular sodium or in the extracellular potassium may be mechanisms for the insulinogenic action of glucagon. This action is enhanced or mimicked by caffeine and theophylline, two inhibitors of cyclic AMP degradation (289, 418, 481) and by cyclic AMP itself, although the latter appears to be effective only in relatively high doses (481, 760). Recent experiments have demonstrated that secretin and pancreozymin also are potent insulinogenic stimuli, suggesting that, under normal conditions, the food-induced secretion of insulin may be the result of a variety of stimuli, including alimentary hyperglycemia, increased production of "enteroglucagon" and other gastrointestinal hormones, as well as the pre-existing levels of insulin and glucagon itself (68, 169—173, 241, 346, 395, 505, 530, 546, 603, 808, 810). This "entero-insular axis", activated by the intake of food, would accelerate the disposal of glucose, amino acids and fatty acids and prevent their excessive accumulation in the blood (801, 808; Fig. 1).

VI. The metabolic fate of glucagon

Studies with ^{131}I -labeled glucagon indicate that the half-life of intravenously injected glucagon varies from less than 10 to about 15 minutes. Although urinary excretion may contribute in small part to this rapid dis-

appearance, the most important factors seem to be organ fixation and enzymatic destruction, occurring mainly in liver, kidney and skeletal muscle (40, 128, 432, 805). Indeed, glucagon may be destroyed effectively by perfusing it through the liver (442) or incubating it with chick embryo hepatocytes cultured *in vitro* (313). Two apparently different enzymes capable of inactivating glucagon have been isolated from beef liver: one causes the cleavage of the seryl-glutamyl bond, while the other promotes the cleavage of the NH₂-terminal dipeptide, histidylserine. The latter enzyme has an absolute requirement for SH-groups, an optimum pH of about 6.5 and maximal activity in the presence of NaCl and KCl (381, 781).

VII. The physiologic effects of glucagon

1. Effects of glucagon on carbohydrate metabolism

a) *Actions of glucagon on the liver.* Perhaps the best known effect of glucagon is an increase in blood glucose concentration. This has been observed in most animals in which it was sought, although with varying degrees of intensity and duration, depending upon the species and the amount of hepatic glycogen available. Most recently, hyperglycemia has been noted in normal, obese, cirrhotic and diabetic human subjects, in patients with CHAGAS' disease and with non-diabetic ketosis (90, 95, 215—217, 306, 359, 397, 398, 464, 553, 605, 635); in infants (547, 548, 559); in adult dogs (145, 199) and newborn puppies (7); in normal rabbits (816); in normal (130, 162) and alloxan-diabetic rats (244); in the domestic fowl (312); in normal and depancreatized South American iguanas [*Tupinambis teguixin* and *T. rufuscens* (344, 582)]; in normal and depancreatized alligators (587), in normal, depancreatized and hypophysectomized toads [*Bufo arenarum* HENSEL (583)]; in the frog [*Rana tigrina*] and the lizard [*Varanus monitor* (628)] and in some (771), but not all (35, 210) species of fish. In normal human subjects, glucagon causes about a four-fold increase in the transhepatic arterio-venous difference in glucose concentration and raises hepatic glucose production from about 50 to about 150—200 mg/min per m² within five minutes (398). In man, this effect occurs without significant changes in hepatic blood flow [about 750 ml/min per m² (397)], although hemodynamic changes have been described in the dog (374, 577). Glucagon causes glycogen depletion in cultures of chick embryo hepatocytes as shown in Fig. 2 [see also (817, 818)] and in the perfused liver of the toad (591, 592) and of the rat (1, 451, 522, 523, 730, 735, 860). In this preparation the effect of glucagon is proportional to the logarithm of the hormone concentration in the perfusing fluid, according to the formula $y = 1.95 + 3.6 \log x$, where y is glucose output and x is the dose of glucagon. A substantial increase in circulating glucose level accompanied by a decrease in glycogen content has been obtained with as little as 4 µg of glucagon per liter of perfusing fluid, corresponding approximately to a 10⁻⁹ M concentration of the hormone. A meas-

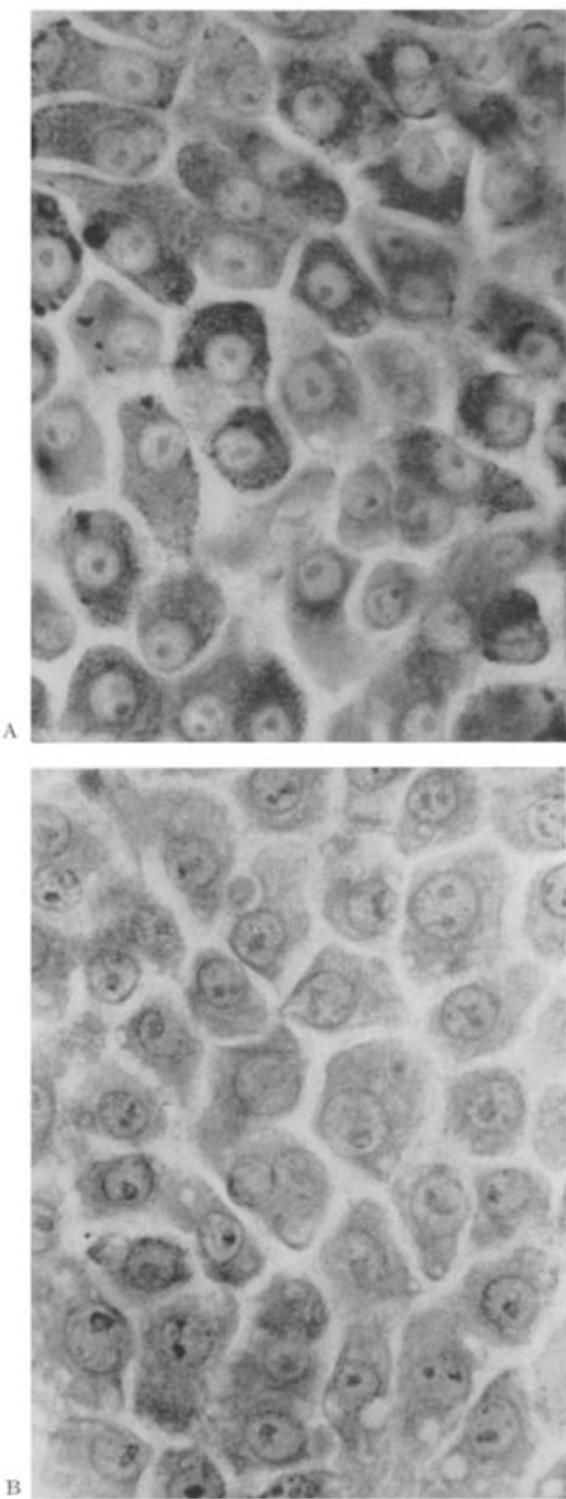
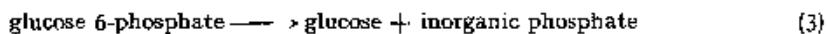
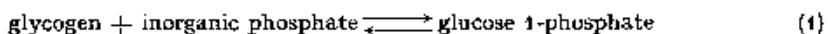


Fig. 2A and B. Hepatocytes in tissue culture; periodic acid-Schiff glycogen stain. A Control. B After treatment with glucagon (313)

urable glycogenolytic response has been obtained with a glucagon concentration of 10^{-10} M and it is likely that the smallest active concentration is somewhat lower still (728), possibly in the range of concentrations found in the blood of the pancreato-duodenal vein of dogs (807). The effect of glucagon is smaller in patients with liver cirrhosis than in normal subjects (245-217), in brittle than in stable diabetics (359). On the other hand, cortisol administered to the mother prior to elective cesarean section augments the subsequent hyperglycemic response to glucagon in the newborn infant (559).

Glucagon causes hyperglycemia by stimulating the breakdown of hepatic glycogen, the most abundant source of readily available carbohydrate. Three major reactions are involved in hepatic glycogenolysis:



Reaction 1 is catalyzed by phosphorylase (α -1,4-glucan: orthophosphate glucosyl transferase; EC 2.4.1.1) and is reversible. When conditions favor glycogen breakdown, phosphorylase removes a glucosyl unit from the outer branches of the glycogen molecule breaking an α -1,4 linkage, with formation of glucose 1-phosphate. This process continues until the α -1,6 linkage of a branching point is reached, leaving a residual polysaccharide called the "first limit dextrin." The branching point is then broken by amylo-1,6-glucosidase or "debranching enzyme," a process which exposes the α -1,4-linked units of the inner branches and allows the phosphorylase reaction to proceed, with formation of more glucose 1-phosphate and of a polysaccharide residue called the "second limit dextrin." Reaction 2 is catalyzed by phosphoglucomutase (D-glucose 1,6-diphosphate: D-glucose 1-phosphate phosphotransferase; EC 2.7.5.1) and is also reversible. Reaction 3 is irreversible and consists in the hydrolysis of glucose 6-phosphate by a specific phosphatase (D-glucose 6-phosphate phosphohydrolase; EC 3.1.3.9) found primarily in liver and kidney. Glycogen may be debranched also by the action of an oligotransferase which transfers α -1,4-linked maltotrioseyl and maltosyl, but not glucosyl, residues from one chain to another (73). This non-phosphorolytic pathway can continue to degrade glycogen in the theoretical total absence of phosphorylase and appears unrelated to the action of glucagon. Of the three phosphorolytic reactions involved in glycogen breakdown, reaction 1 is the slowest. Therefore, the rate of glucose production is determined by the amount of active phosphorylase available. This amount, in turn, is not constant, but depends upon its distribution within the hepatic cells and upon the balance between enzyme inactivation and reactivation. Active phosphorylase, or phosphorylase α , may be inactivated by conversion to dephosphophosphorylase, or phosphorylase β . This inactivation is brought about by a specific phosphatase which may destroy almost all

enzyme activity in 15 minutes (495). Enzyme reactivation consists in the rephosphorylation of phosphorylase *b* by ATP, in the presence of Mg⁺⁺ and of a specific dephosphophosphorylase kinase (ATP: phosphorylase phosphotransferase: EC 2.7.1.38), which, at least in muscle, attaches phosphate to serine residue of the inactive enzyme. Activation of pig liver phosphorylase requires the incorporation of two moles of phosphate per 100,000 g of protein (13, 223, 264, 554). Phosphorylase kinase also must be activated and this is done by adenosine 3',5'-phosphate (cyclic AMP), a thermostable nucleotide which may be obtained by incubating cell-free particulate preparations of liver and other tissues with ATP, in the presence of magnesium ions and other additives.

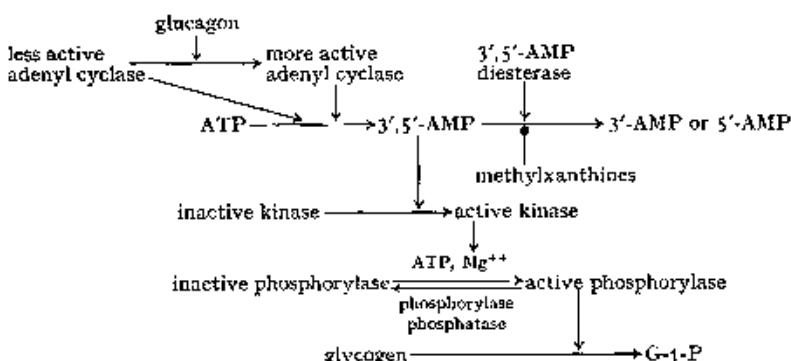


Fig. 3. Diagram illustrating hormone-induced activation of phosphorylase. —> stimulation or increase;
—● inhibition or decrease

The concentration of cyclic AMP in tissues, in turn, depends upon the relative rates of its production by a "cyclase", and of its destruction by different phosphodiesterases which convert it to adenosine 5'-phosphate (5'-AMP) or to adenosine 3'-phosphate (3'-AMP). The diesterase obtained from dog or cattle heart is inhibited by caffeine and theophylline. It is generally believed that glucagon acts in this complex series of reactions by stimulating the formation of cyclic AMP and hence the reactivation of hepatic phosphorylase (232, 646, 762—764). Indeed, intraportal infusions of cyclic AMP in the dog mimic the action of glucagon and cause an immediate hepatic vein hyperglycemia (36), while glucagon injections cause an increase in the hepatic turnover of ³²P-labeled orthophosphate (152, 153). The activation of phosphorylase has been likened to a "cascade reaction" in which a weak initial stimulus is amplified in successive stages, because the product of one reaction influences the rate of the next. This concept, illustrated by Fig. 3, was proposed to explain the action of epinephrine (67), but since the initial step is stimulated also by glucagon, it may apply to the action of this hormone as well.

Various agents such as 5'-AMP, glucose 6-phosphate and ATP, not produced specifically in response to the original stimulus, also act on one or more of the enzymatic stages, perhaps as control systems distinct from, or complementary

to, the hormones. For example, 5'-AMP increases the activity of phosphorylase by an allosteric effect (325, 478), Ca^{++} and a kinase-activating factor stimulate the activation of kinase (516), thus producing the same effect as the hormones, but at the same time pre-empting the amplification of the hormonal signal to the extent that the latter may no longer have any effect on the production of glucose from glycogen (67).

Most of the information on the mode of action of glucagon summarized above has been obtained using liver slices and homogenates and appears applicable to the intact organism. However, there is a major difference between the behavior of phosphorylase *in vivo* and *in vitro*. *In vitro* the phosphorylase reaction is reversible and can bring about glycogen synthesis from glucose 1-phosphate. Indeed, phosphorylase activity may be measured by the amount of inorganic phosphate liberated during the incorporation of glucose units into the glycogen molecule. However, conditions in the living animals rarely, if ever, favor this synthetic reaction and phosphorylase activation always results in a breakdown of liver glycogen, whereas glycogen accumulates in the liver when tissue phosphorylase activity is low (328, 558) (see Table 5, p. 178). Glycogen synthesis *in vivo* depends upon the activity of uridine diphosphoglucose-pyrophosphorylase (uridine triphosphate: α -D-glucose 1-phosphate uridylyltransferase; EC 2.7.7.9) and of glycogen synthetase (uridine diphosphoglucose: α -1,4 glucan α -4-glucosyltransferase; EC 2.4.1.11), acting on the same 1,4-linkages which are split by phosphorylase (821).²

Recent experiments have shown that uridine diphosphoglucose (UDPG) is a competitive inhibitor of phosphorylase in extracts of *Agrobacterium tumefaciens* (476, 477) as well as in normal liver (473, 474). Thus, UDPG which is at the same time an activator and a substrate for glycogen synthetase may limit the rate of glycogen degradation, even as it increases the rate of glycogen synthesis. Under normal conditions, the liver contains enough UDPG to inhibit that fraction of phosphorylase which is active at all times as a result of the hormone-regulated equilibrium between active and inactive forms. If this were not so, the ever-present active phosphorylase could, theoretically, deplete all glycogen stores in about 20 minutes (612). Indeed, in the unstimulated rat liver, in the steady state, when hepatic glycogen is constant and very little turnover occurs, the liver contains enough phosphorylase α to produce 8 to 15 μ moles of glucose per minute per gram of tissue and to support a rate of glycogenolysis significantly higher than that which has been observed during maximal glucagon action. These observations indicate that the total amount of active phosphorylase may not be the controlling factor of hepatic glycogenolysis and that glucagon may act not by increasing total phosphorylase α activity but, perhaps, by facilitating the contact of glycogen with the active

² For details regarding the pathways of glycogen synthesis and breakdown the reader should consult recent reviews (72, 219, 264, 347, 638, 778).

enzyme already present (728). In this way, glucose production by perfused rat liver could approach the rate of approximately 1 mg/min per gram of tissue, an amount near the maximum which can be supported by the glucose 6-phosphatase activity of the liver (about 7 to 8 μ moles or 1.2 to 1.4 mg/g per minute (89, 835).

The balance between phosphorylase and glycogen synthetase and, consequently, the relative rates of glycogen synthesis and breakdown is influenced also by the adrenal cortical hormones, and by glucose which, under suitable circumstances, inhibit phosphorylase and favor the activation of synthetase (52, 53, 139, 157, 299, 379, 394, 421, 646, 697, 711), and by the concentration of cyclic AMP which not only stimulates phosphorylase (372, 379), but also decreases the total activity of synthetase (379, 764) and increases its glucose 6-phosphate-dependent fraction (12, 52, 53, 139, 157, 264, 299, 352, 372, 379, 394, 421, 646, 652, 697, 711). Thus glucagon, by stimulating the synthesis of cyclic AMP and by reversing the action of insulin (53) would promote glycogenolysis and inhibit glycogen synthesis (422). These observations suggest the existence of a feedback regulation of phosphorylase activity (Fig. 4). In addition, glucagon prevents the induction of hepatic glucokinase by glucose and, therefore, limits the production of glucose 6-phosphate necessary for synthetase action (551, 741). Glucagon administration may lead also to the inhibition of hexokinase and of glucose utilization in extra-hepatic tissues, by promoting the release of free fatty acids from adipose tissue (see p. 158). Free fatty acids are converted to acetyl CoA and to citrate; and citrate, by inhibiting phosphoglucomutase, phosphofructokinase and glucose 6-phosphatase (555, 581, 608, 666, 792, 793, 863, 896), can lead to an accumulation of glucose 6-phosphate (214, 580, 682, 683, 684, 791) and, hence to a decrease in hexokinase activity. A decrease in the activity of phosphofructokinase, the rate limiting enzyme for the conversion of glucose 6-phosphate to lactate and of hexokinase would, of course, reduce glucose utilization. Conversely, when glucose utilization is accelerated by insulin, glucose or food intake, the release and oxidation of free fatty acids are reduced and the activity of hexokinase and phosphofructokinase increases (62, 64, 66, 212, 214, 254, 315, 384, 514, 624—627, 681, 682, 700, 753, 773, 836, 837, 856). (Fig. 5). This "glucose-fatty acid cycle" provides a theoretical pathway for an indirect action of glucagon on skeletal muscle, even though glucagon has no direct effect on muscle phosphorylase.

It is interesting to contrast these actions of glucagon with some of the actions of insulin. For example, insulin promotes the synthesis of glucokinase³ and its induction by glucose and, possibly, inhibits the destruction of the enzyme

³ It has been shown that in normal rat liver, hexokinase (EC 2.7.1.1) accounts for no more than 30% of the total glucose-adenosine triphosphate phosphotransferase activity, the rest being due to glucokinase (EC 2.7.1.2), but that this ratio may vary greatly, without significant changes in total enzymatic activity (506).

and increases its effectiveness and, hence the production of glucose 6-phosphate (42, 64, 357, 387, 549, 550, 551, 606, 660, 665, 702, 746, 766, 820), increases the activity of glycogen synthetase (52, 53, 139, 421) and of pyruvate kinase (838) and promotes the conversion of the glucose 6-phosphate dependent to the independent enzyme (129, 140, 422), suppresses the generation of cyclic AMP in the liver (194, 195, 578) and the release of hepatic glucose induced by nucleotides (383), decreases lipolysis and ketone production and enhances

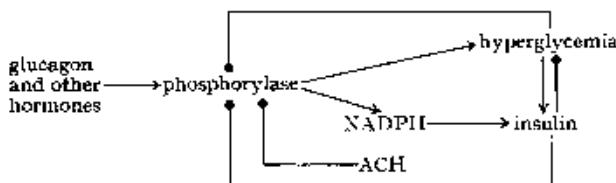


Fig. 4. Possible feedback regulation of phosphorylase activity in the adrenal cortex and in the B cells. —→ stimulation or increase; —● inhibition or decrease; ACH = adrenal cortical hormones; NADPH = coenzyme II = TPNH = reduced nicotinamide-adenine dinucleotide phosphate

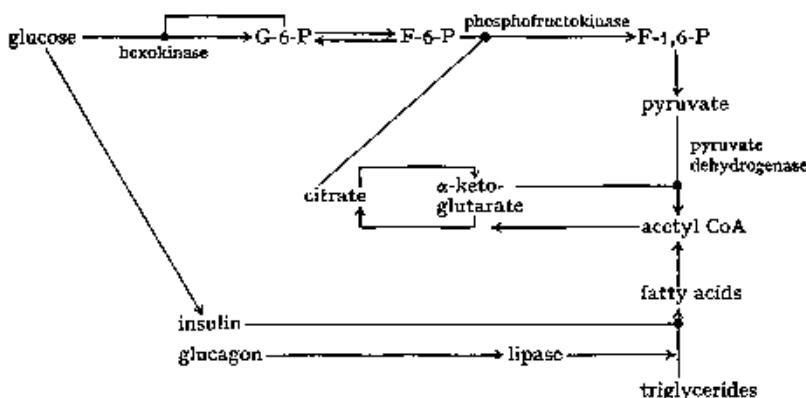


Fig. 5. The glucose-fatty acid cycle. —→ stimulation or increase; —● inhibition or decrease

phosphofructokinase activity (315, 384, 551, 836, 837). Glucose 6-phosphate, in turn, activates glycogen synthetase (242, 783) and inhibits hepatic phosphorylase (242). Thus, insulin stimulates the utilization of glucose by stimulating many of the pathways which are inhibited by glucagon (Fig. 6). Examples in which insulin and glucagon appear to have similar, rather than opposite, effects should also be cited. Glucagon increases the rate of production of hepatic ketone bodies; and ketones, while inhibiting glucose utilization in isolated organs, stimulate insulin secretion (475) and, therefore, decrease the concentration of serum glucose and of free fatty acids (214, 475, 509). Insulin stimulates the esterification of free fatty acids while glucagon activates tissue lipase, yet both hormones often lower the concentration of serum free fatty acids (see p. 160). According to some investigators, glucagon and cyclic AMP increase glucose utilization by the rat diaphragm (175, 176, 477, 596) and adipose tissue (302) *in vitro*, which is a characteristic effect of insulin. The

reasons for these apparent discrepancies are not clear; in some cases, increased glucose uptake may be the result of glucagon-induced release of potassium into the extracellular fluid (135) or of insulin-contamination of allegedly "insulin-free" glucagon preparations (850).

b) Actions of glucagon on skeletal muscle. While the effects of glucagon on liver phosphorylase, liver glycogen and glucose output have been established reasonably well, its effects on skeletal muscle have been harder to ascertain (227, 232). Most recent evidence seems to indicate that glucagon has no direct effect on phosphorylase activity, glycogen content and glucose metabolism of

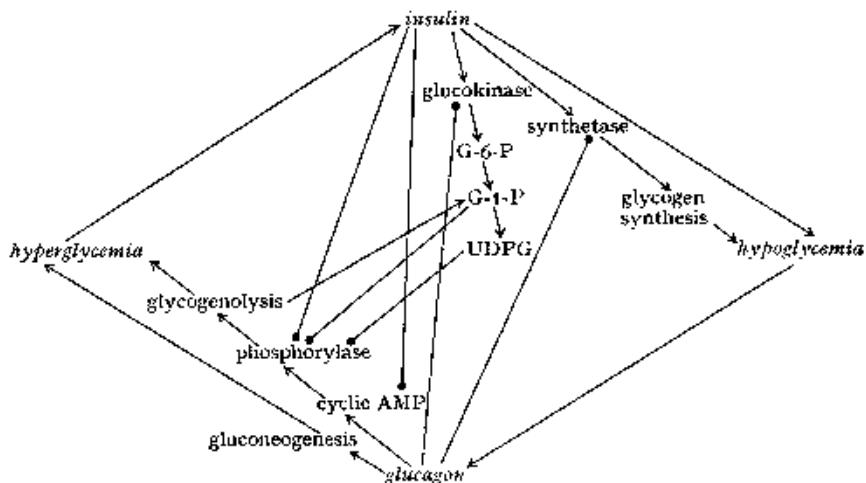


Fig. 6. Effect of insulin and glucagon on glycogenesis and glycogenolysis. —→ stimulation or increase; —● inhibition or decrease

skeletal muscle. For example, glucagon does not modify glucose tolerance and oxidation in eviscerated liverless animals (167, 358), although it suppresses glucose uptake in the intact dog (145, 746); glucagon increases peripheral arterio-venous difference in glucose concentration, which is considered a measure of glucose uptake (432, 433, 436, 444), but this may be due to the accompanying hyperglycemia or to the secondary increase in available insulin. Indeed, this effect is enhanced by the simultaneous administration of glucose and/or insulin and it is absent or significantly reduced in depancreatized animals or severely diabetic patients. In evaluating these results, one should remember that a correct interpretation of arterio-venous differences, measured when the concentration of blood glucose changes rapidly, is very difficult and that, in most of the reported experiments, blood flow was not measured (232). Undoubtedly, another difficulty lies in the fact that glucagon may influence carbohydrate metabolism in muscle indirectly, not only by increasing the amounts of available glucose and insulin, but also by increasing the plasma level of metabolic modifiers such as epinephrine, free fatty acids and ketone bodies (see p. 155).

c) *Actions of glucagon on the heart.* The breakdown of myocardial glycogen, like that of hepatic glycogen, is regulated by the relative concentration of active and inactive phosphorylase (370, 371, 570, 764) and, although not all the enzymatic steps leading to phosphorylase activation in the heart muscle have been elucidated, the myocardium contains a cyclic AMP-splitting enzyme (541) and a phosphorylase kinase (389) subject to stimulation by cyclic AMP (165) and by epinephrine (166). Glucagon enhances the conversion of myocardial phosphorylase β to phosphorylase α , resulting in a rapid breakdown of tissue glycogen which may drop to 60% of its initial value within five minutes. Following this initial phase of rapid glycogenolysis, the steady state concentration of hexose- and triose-phosphates increases 300%, reflecting an increased rate of glucose phosphorylation. It has been reported also that glucagon decreases myocardial uptake of fatty acids and causes a fleeting increase in phosphofructokinase activity and lactate production and a more durable increase in the uptake of glucose, acetate and oxygen (125, 410, 531, 532, 580, 632, 853, 858).

Most of the metabolic effects of glucagon in the perfused heart are qualitatively indistinguishable from those of the catecholamines (304, 330, 342, 502, 644, 764) and of anoxia, although anoxia has a greater effect on glycogenolysis and lactate production and a smaller effect on phosphorylase (125, 409, 631). Indeed, glucagon shares with the catecholamines a chronotropic and intropic action (158, 632, 788, 849), perhaps facilitated by an increase of coronary blood flow (577) and by a glucagon-induced release of catecholamines from the tissues (407). However, it should be noted that the latter is not a universal phenomenon (104) and cannot be the sole reason for the effects of glucagon on myocardial contractility (742). Furthermore, these effects remain unaltered after catecholamine depletion by reserpine pretreatment (125). Recent evidence showing that cyclic AMP and an unidentified protein constitute a cardiac relaxing system (341) and suggesting that cyclic AMP is the beta receptor (645) or that its intracellular concentration is an immediate consequence of the activation of beta receptors (185), may be clues to the mode of action of glucagon on the heart and other tissues as well.

2. Effects of glucagon on lipid metabolism and on adipose tissue

The primary effect of glucagon on lipid metabolism appears to be a cyclic AMP-mediated activation of tissue lipase, resulting in a net mobilization of free fatty acids (FFA) and glycerol in the adipose tissue of the rabbit and of man, in the interscapular (brown) and the epididymal (white) adipose tissue of the rat and the mouse (309, 375, 385, 430—432, 445, 461, 462, 479, 661, 662, 750, 843) and in the abdominal adipose tissue of the house sparrow (*Passer domesticus*) and of other birds (270, 271, 662). The effect of glucagon on ketone body production is still a matter of controversy. According to one investigator,

this effect, if at all demonstrable, is very small (633) while, according to other workers, glucagon causes a fall in hepatic triglyceride and coenzyme A, an increase in the supply of FFA, acyl CoA and acetyl CoA and, consequently, an increase in the formation of acetoacetate and of β -hydroxybutyrate (43, 45, 407, 567, 861). Lipase activation by cyclic AMP does not require the simultaneous activation of phosphorylase (83, 84, 240). This independence is confirmed by the observation that epinephrine and glucagon activate adipose tissue phosphorylase almost to the same extent, although the effect of epinephrine on fatty acid release is almost seven times that of glucagon (302).

Several hormones, in addition to epinephrine, share the lipolytic action of glucagon. Among them are: norepinephrine, ACTH and the adrenal cortical hormones, vasopressin, thyroid-stimulating hormone and thyroxine, growth

Table 2. Effect of glucagon (0.1 mg/kg intravenously) on serum free fatty acids concentration ($\mu\text{Eq/l}$) in unanesthetized trained dogs. Number of experiments in parentheses

	Control concentration	Immediate rise (5–10')	Secondary fall (40–60')	Final rise (3–4 h)
Normal (6)	705	+ 247	- 462	+ 588
Depan. On I (5)	1600	+ 290	- 633	+ 134
Depan. Off I (9)	3096	+ 259	- 565	- 254
Depan. Untreated (4)	2603	+ 176	- 288	- 346

I = Insulin.

hormone and serotonin (24, 28, 49, 86, 201–205, 211, 222, 224, 268, 271, 309, 329, 375, 405, 466, 479, 616, 641, 642, 750, 762, 813, 814, 868) (Table 2). Cyclic AMP, or its preservation by the caffeine- or theophylline-induced block of esterase also cause lipolysis (784, 813) and so does the electrical stimulation of the sympathetic nerves of adipose tissue (651) and of the peripheral end of the vagus (454). Beta sympatholytic agents, such as dichloroisoproterenol and pronethalol, antagonize the effect of the catecholamines on cyclic AMP accumulation and lipase activation, in adipose tissue. On the other hand, alpha sympatholytic agents, such as phenoxybenzamine are less effective, although they are capable of blocking the lipolytic effect of growth hormone and of the glucocorticoids (44, 81, 86, 203, 354, 462, 846). The lipolytic action of glucagon is unaffected by these drugs (462). Starvation (21, 367) and diabetes, such as that produced acutely by the injection of anti-insulin serum (769, 864), induce lipolysis and inhibit lipogenesis, while lipase activity and the lipase-stimulating action of glucagon, of the catecholamines, of theophylline, of thyroid-stimulating hormone and of ACTH are effectively inhibited by insulin, by prostaglandin E and by several nucleotides (22, 38, 44, 82, 201, 203, 204, 205, 267, 271, 379, 385, 405, 428, 479, 579, 637, 648, 662, 701, 704, 725, 748, 749). Insulin may reduce lipolysis also by stimulating the enzymatic destruction of cyclic AMP (693). Glucagon-induced lipolysis should

result in a rise in serum free fatty acid concentration and although this rise has been noticed in normal human subjects (430, 432), in patients with essential hyperlipemia (9), in chickens (312), dogs (295, 431, 448), rabbits (259, 260) and birds (517), it is by no means a constant finding; and a decrease in serum free fatty acids or a biphasic effect have been obtained in dogs (92, 148,

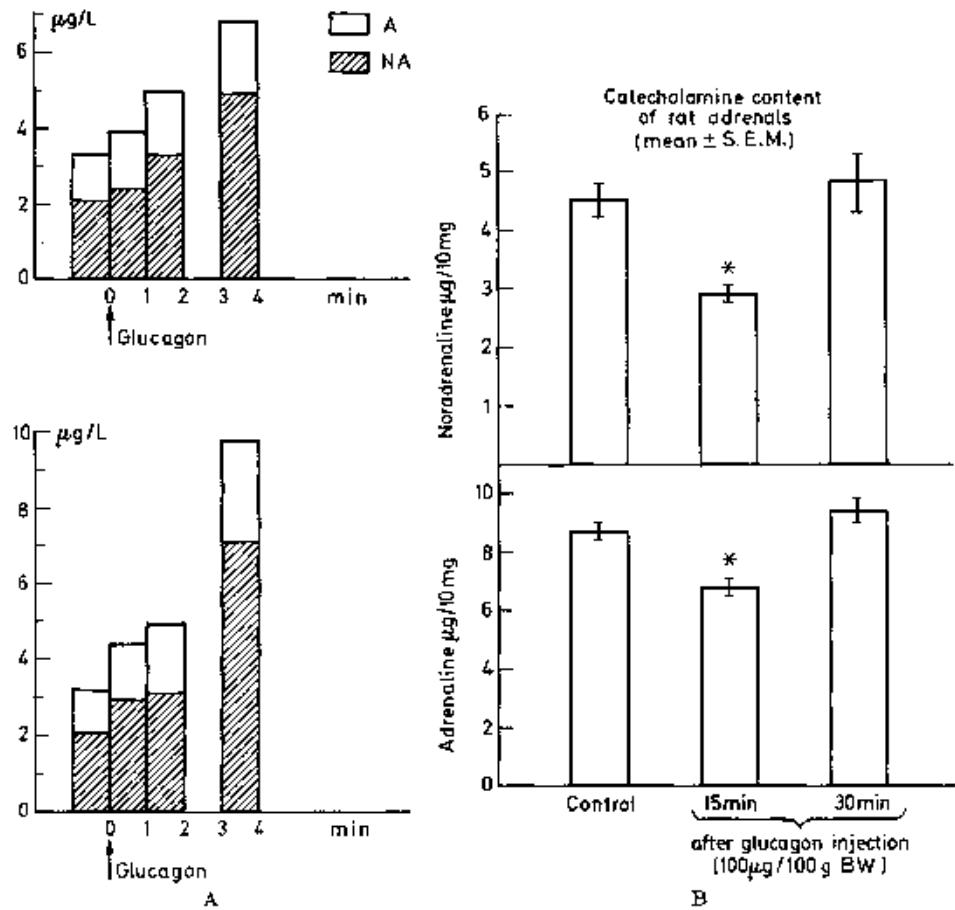


Fig. 7. A Effect of glucagon (1 mg i.v.) on the plasma catecholamine concentration in two normal subjects (A = adrenaline; NA = noradrenaline). B Effect of glucagon on the catecholamine content of rat adrenals (438).

294, 731) and in man (134, 164, 527). The interpretation of these results is complicated by the possibility that a release of epinephrine may contribute to the increase of serum FFA (259, 260, 729) and that a release of insulin may contribute to a FFA decrease (134, 148, 731), since either of them could occur following glucagon injections. This problem was reinvestigated recently in normal and depancreatized dogs (850). The results indicate that the response of serum FFA to intravenous injections of glucagon has three phases: a prompt increase, reaching a maximum in 5 to 10 minutes, a decrease lasting 2 to 3 hours, and a final prolonged increase (see Table 2). It is believed that the initial rise may be due to the rapid glucagon-induced release of epinephrine

(432, 438, 439, 445, 470) (see Fig. 7 A and B), the secondary decrease to reactive insulin secretion or to the small amount of insulin present even in the best glucagon preparation and that the final increase may reflect the true lipolytic effect of glucagon or, possibly, the combined effects of glucagon and epinephrine. Perhaps related to the lipolytic effect of glucagon and to the lipogenic effect of insulin is the observation that in the blackbird (*Turdus merula*) during winter fattening, the activity of the A cells is lower than that of the B cells (188). This brief review would not be complete without mentioning the observation that, in man, the mere sight of food may lower the plasma content of FFA (589).

Insulin increases the concentration of glucose 6-phosphate in adipose tissue, possibly by increasing glucose transport (147, 450) and, since glucose 6-phosphate increases the activity of glycogen synthetase, the net results of glucagon and insulin on the glycogen content of adipose tissue are opposite to one another. Investigators wishing to study the effects of glucagon and insulin on lipolysis may be interested in several recent papers describing the effect of insulin on lipogenesis in rat liver (301, 385) and rat (146) and chick embryo heart (235), the purification, properties and assay of lipase (57, 486) and of the hexokinases (292), the decrease of adipose tissue phosphorylase (296) and hexokinase (74) after prolonged fasting, the suppression of fatty acid release from adipose tissue by a lipid-bound inhibitor found in the epididymal fat of rats with hypothalamic obesity (300), the isolation of a lipid mobilizing substance from calf midbrain (337), the activity of several enzymes in the adipose tissue of obese-hyperglycemic mice (10, 243) and the relationship between growth hormone, exercise and fat mobilization in man (308).

3. Effects of glucagon on protein metabolism

Glucagon hyperglycemia is primarily the result of hepatic glycogenolysis. However, gluconeogenesis from protein may also be a factor, since glucagon promotes urinary nitrogen excretion (362, 669, 787) and, if administered repeatedly or in large doses, causes a marked increase in nitrogen excretion with negative nitrogen balance, loss of weight and decrease in the total mass of liver and muscle (258, 362, 382, 667, 669, 787). Glucagon added *in vitro* increases the release of amino acids from skeletal muscle (30, 596) and of protein from isolated fat cells (647), decreases the incorporation of ^{14}C from labeled leucine or glycine into protein (87, 368) and increases its incorporation into liver glycogen (382). Recent experiments have demonstrated that glucagon stimulates the uptake of α -amino-isobutyric acid by the isolated perfused rat liver, under conditions which suggest a hormonal effect on the membrane transport system for amino acid (106). Thus, glucagon may stimulate gluconeogenesis from protein by increasing the supply of amino acids to the hepatocyte. The net result of these effects of glucagon on the release of amino acids from the peripheral tissues and their uptake by the liver is a reduction in blood

amino acid concentration (361, 364, 368, 472, 841, 842). It has been calculated that the maximal glucose production from gluconeogenesis in the perfused rat liver is approximately 0.05 mg/min per gram of tissue. This value may appear small in comparison to the amount of glucose produced by glycogenolysis, but it corresponds to a possible production of 700 mg of glucose per rat per day, or 2 to 3 times the entire hepatic glycogen content of the fed rat and is sufficient to provide 10 to 15 % of the animal's basal energy requirement (728). The gluconeogenic action of glucagon is not quite as prompt as its glycogenolytic action, but is prolonged and may cause an increase in hepatic glucose output even if the glycogen reserve has been depleted (168, 730). This action of glucagon may be important for the maintenance of blood glucose levels in starvation. The protein catabolic effect of glucagon is prevented by hypophysectomy and restored by growth hormone (363), it is abolished by insulin (521, 522) but not by the anabolic androgens; according to some investigators, it requires an intact adrenal cortex (842), while according to others (258), it may be demonstrated also in adrenalectomized rats maintained on saline. Under certain conditions, the injection of glucagon causes an increase in plasma corticosteroids (404) which, in turn, are potent gluconeogenic hormones (333, 653, 746, 790, 825, 834). Glucagon increases the activity of hepatic glutamic dehydrogenase (552) and that of three major enzymes of the urea cycle: arginino-succinate synthetase, carbamyl phosphate synthetase and arginino-succinase (507), induces the premature development of tyrosine aminotransferase in fetal rat liver (279), stimulates the synthesis of this enzyme in adrenalectomized rats and enhances its induction by hydrocortisone (137, 278) and, in general, increases hepatic transaminase activities (113, 136, 138, 278, 390). In the isolated perfused liver, glucagon causes a rapid increase in the rate of urea nitrogen production (180, 485, 521, 522, 523, 586, 655, 730, 861, 863). Glucagon promotes gluconeogenesis not only from amino acids, but also from non-protein sources, such as lactate and pyruvate (179, 180, 193, 194, 197, 198, 655, 683, 863). Indeed, in the presence of lactate, gluconeogenesis may account for two-thirds of the total glucose produced by the isolated perfused rat liver, while glycogenolysis accounts for only one-third (683). Gluconeogenesis from lactate and pyruvate requires bypassing of the energy barriers which make reversal of the glycolytic pathway impossible (179). Glucagon may accomplish this task in three possible ways: 1. by an action on the glycolytic pathway, resulting in a greatly increased ratio of glucose 6-phosphate to fructose disphosphate [although this action is considered specific, its precise enzymatic mechanism has not been elucidated as yet; (682-684)]; 2. by increasing the activity of phosphoenolpyruvate carboxykinase (179, 193-197, 253, 419, 420, 579, 715); 3. by increasing the activity of pyruvate carboxylase indirectly. This enzyme has an absolute requirement for acetyl CoA made available in increased supply by glucagon-stimulated lipolysis (245, 755, 756,

826, 827, 837, 859, 862, 863, 864). These effects of glucagon stand in sharp contrast to the anabolic effect of insulin which increases protein synthesis (739, 740, 871), lipogenesis and the esterification of free fatty acids and decreases the concentration of cyclic AMP (195, 245, 315, 420, 576, 646) and the activity of phosphoenolpyruvate carboxykinase. Thus, glucagon would increase and insulin would decrease the availability of amino acids, pyruvate, lactate and other glucose precursors to the liver, while, at the same time, glucagon would stimulate and insulin would suppress the induction of the enzymes necessary for the production of new glucose molecules.

Enhanced protein catabolism may be related to the adverse effect of glucagon on the growth of animals (667) and of transplantable tumors (376, 668), on the development of inflammatory reactions (434), on placental weight and on fetal development (138, 340, 785) and to the ability of glucagon to act as an inducer of cellular autophagy and as a modifier of hepatic lysosomes (150).

4. Effects of glucagon on oxygen consumption and basal metabolic rate

Metabolic balance studies have demonstrated that the failure of glucagon-treated animals to gain weight could not be accounted for completely by the increased nitrogen excretion and by the reduction of food intake (see Section VII, 6). Therefore, basal metabolic rates were determined and found to be increased as much as 50 per cent within one hour of glucagon administration (142, 315, 525). This calorogenic effect appears to be mediated, at least in part, through an action of glucagon on the thyroid and the adrenals (142, 468). The mechanism of this phenomenon is not clear: adrenal mediation cannot be attributed entirely to epinephrine, for glucagon and epinephrine do not behave identically in this respect. For example, the calorogenic response to glucagon in the rat decreases after cold adaptation while the response to epinephrine increases (338). Furthermore, glucagon injections increase the apparent metabolic rate (amount of exhaled CO₂) of rats, but not that of mice (525). Attempts to demonstrate a direct effect of glucagon on oxygen consumption have given contradictory results: according to one group of investigators, glucagon suppresses the respiration of rat liver slices (91), while according to others, it stimulates not only that of liver slices, but that of adipose tissue as well (302, 765). In unanesthetized dogs, 1 mg of glucagon administered intravenously increases the oxygen saturation of portal blood, but this may be due to the opening of arteriovenous shunts, rather than to changes in oxygen uptake (373, 374).

5. Effects of glucagon on plasma electrolytes and on renal function

Glucagon causes a marked, but fleeting, increase in serum potassium followed by a mild, but more prolonged, hypokalemia (182, 183, 221, 251, 543, 869). The first is probably due to potassium release from the liver and can be

counteracted by insulin (220, 746), the second may be due in part to insulin secretion, in part to increased urinary excretion of potassium (141, 186, 220, 513, 614). Potassium release from the liver is generally believed to be a concomitant of glycogenolysis; however, suitable doses of cyclic AMP can cause

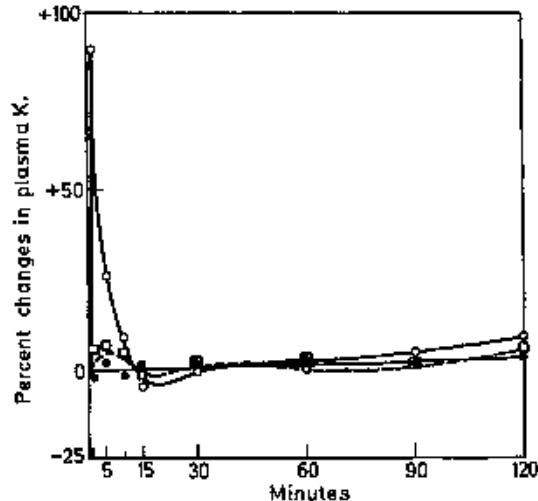


Fig. 8. Effect of glucagon on plasma potassium in normal dogs (251). ●—● Saline, ○—○ Glucagon, □—□ Glucagon + DHE.

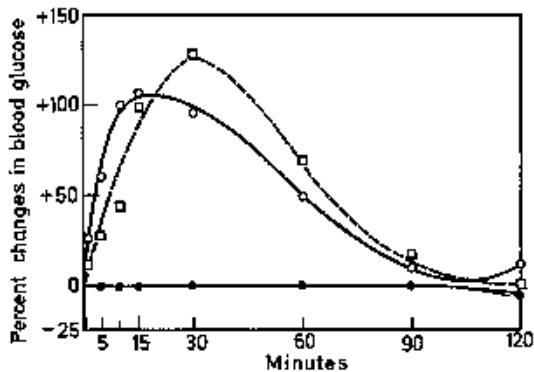


Fig. 9. Effect of glucagon on blood glucose in normal dogs (251). ●—● Saline, ○—○ Glucagon, □—□ Glucagon + DHE.

hyperglycemia without hyperkalemia, at least in cats (184), while glucagon-induced hyperkalemia appears to precede phosphorylase activation and hyperglycemia (220) and occurs also after liver glycogen has been depleted by repeated injections of glucagon and hyperglycemia no longer can be obtained (181, 182). The hyperglycemic and the hyperkalemic response to glucagon may be separated also by means of dibenamine or dihydroergotamine which block the rise in serum potassium, but not the rise in serum glucose (183, 251) (Fig. 8 and Fig. 9). Thus, the hypothesis that different receptors may be involved in these two responses, originally suggested for epinephrine (764), may apply also to glucagon. It is interesting to note here that potassium ions share with

glucagon the ability to stimulate insulin release (287, 288) and lipolysis (60). Glucagon causes a consistent decrease in serum inorganic phosphate and an increase in its urinary excretion independent of the hyperglycemic response (26, 130, 151, 154, 227, 232, 543). These glucagon-induced changes in serum and urinary phosphate levels have been demonstrated also in diabetic patients and in alloxan-diabetic and depancreatized dogs, even though, under these conditions, a glucose load no longer causes the normal decrease in serum phosphate. For these reasons, it is believed that the effect of glucagon on phosphate does not depend upon its ability to stimulate insulin secretion (151, 154). Studies on the distribution of ^{32}P , after injections of labeled orthophosphate into rats, demonstrated that glucagon causes a decrease in the radiophosphorus content of the femur, epididymal fat and carcass and an increase in that of the heart and the liver, but not of the hepatic phospholipids (782), suggesting an increased phosphorus turnover (152, 153). The administration of glucagon is followed by an increased excretion of calcium, sodium, chloride, iodine, uric acid, bicarbonate and water (26, 91, 141, 156, 186, 513, 614), probably because glucagon increases effective renal plasma flow and filtration rate, although it may also act directly upon the renal tubule to decrease ion reabsorption (91, 186, 513, 614). The diuretic effect of glucagon cannot be fully suppressed by the administration of posterior pituitary extracts (91, 141), indicating that the increased urinary volume is, at least in part, the result of osmotic diuresis secondary to increased electrolyte excretion. The recent observation that cyclic AMP increases the permeability of the isolated toad bladder to water (568) suggests that glucagon may affect also the permeability of the nephron. Single injections, as well as intravenous infusions of glucagon, produce a prompt and marked fall in serum calcium (575, 576). Thus, glucagon causes hypophosphatemia, hypocalcemia, enhanced urinary excretion of calcium and phosphate, a series of changes similar to those observed in clinical and experimental pancreatitis, where they are accompanied by high serum glucagon levels and significant A cell hyperplasia. These findings suggest that an overproduction of glucagon by the inflamed pancreas may be responsible, at least in part, for the electrolyte disturbances and that glucagon-induced hypocalcemia may be the cause of the hyperparathyroidism associated with chronic pancreatitis (573—576).

6. Effects of glucagon on the gastrointestinal system

Several recent publications have confirmed the facts that glucagon is a general depressant of gastrointestinal activity. For example glucagon, injected intravenously, inhibits gastrointestinal motility in unanesthetized dogs (544), reduces the gastric secretion of rats with occlusion of the pylorus (51), reduces the concentration of hydrochloric acid in mixed gastric content of patients with peptic ulcer (18) and inhibits meat- or gastrin-induced HCl

secretion in innervated and denervated gastric pouches (316). It is possible that these inhibitory effects of glucagon may be secondary to the shunting of blood away from the gastrointestinal mucosa (373, 374). On the other hand, the effect of glucagon on histamine-induced gastric secretion is uncertain, as inhibition (455), stimulation (109) or no effect at all (316) have been reported. This controversy may have been partially resolved by the observation that the effect of glucagon on histamine-stimulated secretion depends upon the relative doses of glucagon and histamine, upon the manner in which glucagon is given and upon the presence or absence of vagal innervation (455). It is interesting to note that a similar uncertainty exists regarding the effects of insulin, which may stimulate (334, 335, 399) or inhibit (178, 377) gastric secretion. Perhaps the problem could be solved by injecting insulin or glucagon into depancreatized animals in which the compensatory secretion of one or the other hormone cannot occur (615). It has been stated that some of the effects of glucagon on the digestive system may depend upon the central nervous system (590) and that glucagon may be a factor in the central "glucostatic" regulation of hunger (500, 501, 703, 757, 758), perhaps increasing glucose concentration or modifying the arterio-venous difference in the hypothalamic blood. However, the fact that the inhibitory effects of glucagon on gastrointestinal activity do not require an intact vagal innervation (174, 503) and the lack of proportionality between these inhibitory effects and the degree of peripheral hyperglycemia do not support this view. Furthermore, glucagon inhibition may be greater than that produced by glucose itself and may reach its peak two to four hours after glucagon injection, at a time when peripheral blood glucose concentration has already returned to pretreatment levels (18, 500). It is, therefore, tempting to conclude that glucagon may have a direct effect on the smooth muscle and the glands of the gastrointestinal system. Glucagon causes marked hyperglycemia in young rats, but has no apparent effect on their food intake (339) although, in most other cases, glucagon causes anorexia, nausea and a significant depression of appetite (233, 590). Thus, the increasing glucagon secretion observed in starvation lasting 48 hours or more (424, 803), may be a means of protection from the painful sensation of hunger. It has been reported that glucagon causes a two- to three-fold increase in the bile flow of dogs and a marked increase in the biliary content of taurocholic acid and of sodium, chloride and bicarbonate ions (533).

7. Actions of glucagon, of cyclic AMP and of phosphorylase on other tissues and functions

Human and mouse skin contain both phosphorylase *a* and *b* and the enzyme systems for their interconversion (519); glucagon reduces the glycogen content of skin slices (619), but the action of glucagon on phosphorylase activity in this tissue has not been investigated. Glucagon increases

fructose and glucose uptake by bovine spermatozoa (497), reduces the amount of hexoses bound to erythrocyte stroma after it had been increased by insulin (636), increases phosphorylase activity in the yolk sac of the chick embryo (283) and the rate of disappearance of alcohol from the blood of dogs (114, 545). Glucagon has no effect on the glycogen content of the avian glycogen body (311, 724), on spontaneous glycolysis in preserved human blood (440), on the blood flow, oxygen consumption and substrate uptake by human brain *in vivo* (275) and on the metabolism of rat kidney mitochondria *in vitro* (441). On the other hand, many substances, other than glucagon, are known to modify hepatic phosphorylase activity. Among them are the catecholamines which stimulate adenyl cyclase not only in the liver, but also in the heart, skeletal muscle, intestinal smooth muscle, lung, spleen, cerebellum, parotid gland, avian erythrocytes and in a variety of other tissues (77, 78, 473, 538, 556, 557, 621, 764); and glycogen, which causes an approximately six-fold increase in the V_{max} of phosphorylase α for glucose 1-phosphate, with no apparent change in K_m (832). Similarly, fasting (63, 200) and the electrical stimulation of the splanchnic nerve (712, 713) increase hepatic phosphorylase activity. The widespread occurrence of cyclic AMP as a biological mediator has been pointed out (764). The following list, probably incomplete, enumerates the known effects of cyclic AMP: this nucleotide activates phosphorylase in the adrenal cortex to an extent comparable to that of ACTH and mediates the effect of ACTH on 11β -hydroxylation (131, 276, 640, 643, 890); mediates the effects of the catecholamines on brain tissue (401, 620) and on the pineal gland (844), of parathyroid hormone on the kidney (110), of vasopressin on water flow and sodium transport in the epithelial membranes of the bladder and the renal tubules of the toad (568, 569) and of oxytocin on the uterus (447); in addition, cyclic AMP activates phosphorylase in the liver fluke *Fasciola hepatica* (487, 488); mediates the effect of melanophore-stimulating hormone (54), the lipolytic effect of serotonin in adipose tissue (49) and the effect of glucagon on insulin secretion (429, 481, 760, 761). An increase in cyclic AMP and/or phosphorylase activation appear to be a necessary step for the stimulation of corpora lutea by pituitary and chorionic gonadotropin or anterior pituitary extracts (493, 494, 744, 855), for the stimulation of the thyroid gland by the anterior pituitary (762), for the glycogenolytic effect of corpora cardiaca extracts in the cockroach (745), for the central nervous system and the gastric effects of histamine (307, 380), for the effect of stress on the anterior pituitary (366), for some of the effects of puromycin (336), fasting (296) and ether anesthesia (408), and, possibly, for the regulation of pinocytosis in mouse macrophages (117). On the other hand, a decrease in cyclic AMP and/or a depression of phosphorylase activity may mediate the inhibitory effects of acetylcholine on the myocardium (538); the effects of uridine diphosphoglucose (477), of glucose 6-phosphate and of deoxyglucose 6-phosphate (242)

Table 3. Effect of hormones and of other agents on tissue cyclase and/or phosphorylase.
S = stimulation or increase; I = inhibition or decrease

Agent	Tissue	Effect
Glucagon	Liver, heart, adipose tissue, spermatozoa, skin, leucocytes, pancreatic B cells	S
Epinephrine; Norepinephrine	Liver, heart, skeletal muscle, intestinal smooth muscle, adipose tissue, central nervous system, lung, spleen, pineal gland, parotid gland, avian erythrocytes	S
ACTH	Adrenals, adipose tissue	S
Thyroid-stimulating hormone	Thyroid	S
Pituitary gonadotropins	Ovary	S
Parathyroid hormone	Kidney	S
Oxytocin	Uterus	S
Vasopressin	Toad bladder, frog skin, adipose tissue	S
Melanophore-stimulating hormone	Frog skin	S
Glycogen	Liver	S
Fasting	Liver	S
Stress	Anterior pituitary	S
Splanchnic nerve stimulation	Liver	S
Ether anesthesia	Liver	S
Puromycin	Liver	S
Histamine	Central nervous system, gastric mucosa	S
Serotonin	Fasciola hepatica, adipose tissue	S
Corpus cardiacum	Insects	S
Insulin	Liver, adipose tissue	I
Prostaglandins	Adipose tissue	I
Acetylcholine	Heart	I
Glucose 6-phosphate	Liver	I
Deoxyglucose 6-phosphate	Liver	I
Uridine diphosphoglucose	Liver	I

on the liver; of prostaglandin on adipose tissue; and the effects of insulin on liver and adipose tissue (59, 85, 194, 372, 379, 695, 747—749) (Table 3). Thus, glucagon shares the property of stimulating the reactivation of phosphorylase with at least 15 other hormones and neurohormones, albeit with some important differences in tissue specificity. The final physiologic results depend upon the function of each tissue and the presence of other enzyme systems. In the liver, phosphorylase activation leads to glycogenolysis and, in the presence of glucose 6-phosphatase, to the formation of free glucose and hyperglycemia. In skeletal muscle and other tissues, having no glucose 6-phosphatase, glycogenolysis gives rise to pyruvate and lactate. In adipose tissue, the activation of lipase leads to hydrolysis of triglycerides and liberation of glycerol and free fatty acids. In bladder and kidney, the flow of water and the transport of sodium are increased. In the adrenals and pancreas, the increased activity of the pentose phosphate shunt increases the amount of reduced coenzyme II (NADPH) available for the synthesis of steroid hormones and of insulin. Elsewhere, the melanophores relax, the uterus contracts and the corpus

luteum synthesizes progesterone. Thus, it has been suggested that the hormones act as "first messengers" which have as their targets the adenylyl cyclase systems of specific tissues, cyclic AMP would then act as intracellular "second messenger" (beta-adrenergic receptor?) affecting different metabolic functions depending upon the enzymatic profile of the cell. The specificity of hormone-tissue interactions has not been adequately explained. It may be determined by the molecular configuration of the hormone, by the specificity of the adenylyl cyclase system, or by the metabolic capabilities of the cell (83, 762, 764). Another factor may be an intrinsic difference between tissue phosphorylases, as suggested by the fact that muscle and liver phosphorylase differ in their response to adenosine 5'-phosphate (622) and in their apparent K_m for glucose 1-phosphate (474). Still other factors may be a difference between complexes which glycogen forms with phosphorylase to constitute metabolically active subcellular particles (699, 716), or the relative affinity of the hormone for the receptor site and for the hormone-sensitive system in the cell. For example, liver adenylyl cyclase is more sensitive to glucagon than to epinephrine, both in broken cell preparations, where it leads to a greater accumulation of cyclic AMP, and in the perfused organ. In the latter, glucagon, at a concentration of less than 4 $\mu\text{g}/\text{ml}$ of perfusing fluid, causes a level of phosphorylase activity several-fold greater than that produced by a concentration of epinephrine of 100 $\mu\text{g}/\text{ml}$ (480, 734).

VIII. Syndromes due to deficiency or excess of glucagon

1. Glucagon deficiency

No pure glucagon-deficiency syndrome can be produced by surgical means because the removal of all possible sources of glucagon or glucagon-like materials would require the simultaneous removal of the pancreas and of a large portion of the gastrointestinal tract (171, 227, 232, 261, 395) (see Section V). Obviously, the major digestive and metabolic consequences of this destructive intervention (332) would overshadow the desired experimental results and, furthermore, hyperglycemic hormones of pituitary and adrenal origin would continue to be secreted. Selective and permanent destruction of the A cells in a manner comparable to the destruction of the B cells by alloxan has been attempted repeatedly by means of X-ray irradiation or the administration of toxic substances, including some of the orally active antidiabetic drugs, of cobalt chloride and, more recently, of phenol red and of decamethylenediguanidine dihydrochloride (synthalin A). Although, in some cases, these agents caused a reduction of the number of A cells and in the amount of glucagon extractable from the pancreas, complete and permanent destruction of the A cells and total disappearance of glucagon, in general, has not been achieved (17, 41, 227, 232, 291, 458, 823). In the chicken, subcutaneous injections of synthalin A (10 mg/kg) caused a significant, but temporary hyperglycemia, followed by severe hypo-

glycemic convulsions and death. A marked hydropic degranulation was found in the A cells of birds killed in the convulsive state, suggesting that the destruction of the A cells had been sufficient to liberate significant amounts of preformed glucagon (early hyperglycemic phase) and to produce an effective glucagon insufficiency (31). The similarity of this effect with the effect of alloxan on the B cells is obvious. Clinical glucagon insufficiency may have existed in some patients with hypoplasia of the A cells accompanied by hypoglycemia (50, 163, 291, 508), but this was not the case in other patients in whom serum glucagon measurements were done (796). Certain animals which are naturally either very poor or very rich in glucagon appear to be especially suited for the investigation of this problem (see Section IV). Among them are certain species of teleost fish, urodele amphibians and carnivorous birds whose pancreatic islets contain few, if any, A cells and little or no extractable glucagon. In these animals, which have relatively low fasting blood sugar, pancreatectomy causes insulin deficiency and hyperglycemia (189, 227, 232, 272, 392, 583, 628, 887, 891). In contrast, in lizards and ducks, which have a pancreas rich in A cells and in glucagon and a relatively high fasting blood sugar, removal of the pancreas causes hypoglycemia followed sometimes, but not always, by hyperglycemia (344, 524, 582). Indeed, some of these animals appear to be in double jeopardy and to suffer from an insufficiency of glucagon, revealed by a profound and often fatal hypoglycemia in the fasting state and an insufficiency of insulin revealed by postprandial hyperglycemia and a decreased tolerance for glucose (517). Other suitable animals for these experiments appear to be the tunny (607) and the chicken which have two types of islets: an "alpha" type composed mainly of A cells and a "beta" type composed mainly of B cells. In the chicken, the beta islets are scattered throughout the pancreas, but the alpha islets are confined to the third and splenic lobes which may be removed selectively. This form of partial pancreatectomy causes profound hypoglycemia with convulsions and death within 12 to 36 hours and is attributed to glucagon insufficiency (520). Profound fasting hypoglycemia has been noted also in the depancreatized monkey (108, 118), suggesting that glucagon insufficiency exists in all depancreatized animals and that it may be a significant factor in decreasing their tolerance to fasting (728). An insufficient secretion of glucagon may be responsible also for the accumulation of liver glycogen occurring in patients with hepatomegaly associated with certain types of diabetes and of glycogen storage disease (227, 348, 349, 558, 854) (see Section X, 1).

2. Glucagon excess

Until recently, no clear-cut syndromes of glucagon excess were known, although several cases of so-called A cell tumors of the pancreas accompanied by hyperglycemia and glucosuria have been reported (100, 772). In most cases, these were diffuse neoplasms with extensive destruction of the pancreas and/or

metastases to the liver, which alone could have accounted for the observed metabolic derangements. In other cases, these tumors were similar to those found in patients without diabetes or decreased tolerance for glucose (20, 61, 281, 331, 484). A major difficulty lies in the fact that, in general, the designation of the cell type was based on staining methods which do not differentiate between A₂ cells, the makers of glucagon, and the A₁ or D cells, which produce gastrin or a gastrin-like secretagogue. Three recent communications report the first well-documented cases of glucagon-containing tumors. The first paper describes an undifferentiated bronchogenic carcinoma with metastases to the liver in a patient who had no endocrinologic disturbances, although the tumor contained significant amounts of both insulin and glucagon (811). The second paper narrates the story of a 42-year old diabetic woman suffering from a metastatic islet-cell tumor composed of histochemically and histologically demonstrated A cells and containing 14 µg of glucagon per gram of tumor. This is a very considerable amount of glucagon, if one considers that the total glucagon content of human pancreas averages less than 25 µg. The ability of the tumor to secrete glucagon in biologically active amounts was demonstrated by the high fasting plasma glucagon concentration (17 to 56 mµg/ml compared to normal values of less than 2 mµg/ml) which fell markedly after a glucose load, and by the existence of secondary hyperinsulinemia and of glucagon unresponsiveness (504). The third paper describes the case of a 26-year old diabetic woman suffering from a very large A cell tumor accompanied by high levels of serum glucagon-like activity. The tumor was detected clinically about 6 months before diabetes became manifest, suggesting that the development of diabetes may have been influenced by the tumor (886). Another possible form of primary glucagon hypersecretion with secondary hyperinsulinism is the obese-hyperglycemic syndrome of mice, which is characterized by high levels of hepatic phosphorylase, accelerated turnover of liver glycogen, hyperglycemia, insulin resistance and increased lipogenesis (708, 873). Recently, a transplantable islet-cell tumor of the hamster has been described. The tumor contains both A and B cells, appears to produce significant amounts of glucagon and insulin and, hopefully, may become a useful tool for investigating hormone secretion *in vivo* and *in vitro* (234, 286, 727).

3. The diabetogenic effect of glucagon

Glycogenolysis, hyperglycemia, negative nitrogen balance, fat mobilization and ketogenesis are glucagon effects similar to those occurring in fasting and in diabetes and have provided the stimulus for experiments designed to produce metaglucagon diabetes. The results of these experiments have been disappointing, as they have produced only moderate losses of carbohydrate tolerance, transient hyperglycemia and glycosuria with normal fasting blood sugar, atrophy of the A cells, hypertrophy and degranulation of the B cells and other

mild and reversible changes in islet structure (3, 99, 822, 885). Although these effects could be enhanced by the simultaneous administration of cortisone, ACTH or growth hormone (232, 759), an apparently true metaglucagon diabetes with reduction of islet tissue, glycogen infiltration and hydropic degeneration of the B cells could be produced only by the intensive treatment of rabbits with glucagon from birth to the age of 5 months. Unfortunately, no further contributions to this interesting problem have appeared in the literature. Overproduction of glucagon may be a factor in modifying the response to alloxan and the character of experimental diabetes in certain strains of rats (69) and of diabetes in man. This may be due in part to a direct effect of glucagon on the B cells, in part to insulin antagonism and to glucagon-induced hyperglycemia, in part to increased production of free fatty acids and ketone bodies with inhibition of glucose utilization (19). As stated above, glucagon-producing tumors of the pancreas may contribute to the pathogenesis of diabetes, although the above-mentioned patient suffering from a glucagon-producing bronchogenic carcinoma did not have detectable metabolic disturbances (811). True metaglucagon diabetes should be characterized by increased glucose production, normal or slightly decreased glucose utilization and by increased levels of plasma insulin: a hypothetical syndrome similar to prediabetes or mild adult-onset diabetes. Although this similarity is intriguing, measurements of serum glucagon in 14 cases of prediabetes have given inconclusive results (424). Secondary glucagon overproduction may contribute to the hyperglycemic rebound which follows single episodes of insulin hypoglycemia and sometimes complicates the management of diabetes and may be a factor in the progressive deterioration of carbohydrate tolerance which sometimes accompanies chronic insulin overdosage or hyperinsulinism. Serum glucagon levels have been found normal in a few cases of acromegaly, slightly elevated in a few cases of pheochromocytoma, not measurable in one case of TURNER syndrome and in one depancreatized patient (424), but significantly elevated in patients with chronic pancreatitis and hyperparathyroidism (572). The possibility that glucagon may be related to the ZOLLINGER-ELLISON syndrome has been ruled out by the realization that the pancreatic tumors associated with the disease do not secrete glucagon, but a gastrin-like hormone and, in some cases, insulin (80, 353, 707, 893, 894).

IX. The physiologic role of glucagon

The evidence reviewed in the preceding pages strongly suggests that glucagon is a hormone essential for the regulation of glycogenolysis, gluconeogenesis and lipolysis and is involved not only in the adjustment of blood glucose, amino acids and free fatty acids levels, but also in the intermediary metabolism of carbohydrate, protein and fat. In the intact organism, glucagon causes a series of responses, some of which are due to a direct effect on hepatic phos-

phorylase and adipose tissue lipase, others to direct or indirect effects on gluconeogenetic enzymes, still others to the indirect effects of hormone-induced hyperglycemia and/or lipolysis (Fig. 10). One may discuss the physiologic role of glucagon by assuming that a state of glucocytopenia exists, perhaps as the result of fasting, exercise, excessive insulin administration or circadian rhythms (417). Under these conditions, insulin production would decrease as a result of fasting and hypoglycemia or because an excess of circulating insulin inhibits further insulin secretion (88, 241). At the same time, hypoglycemia would cause an immediate increase in glucagon secretion and the concentration

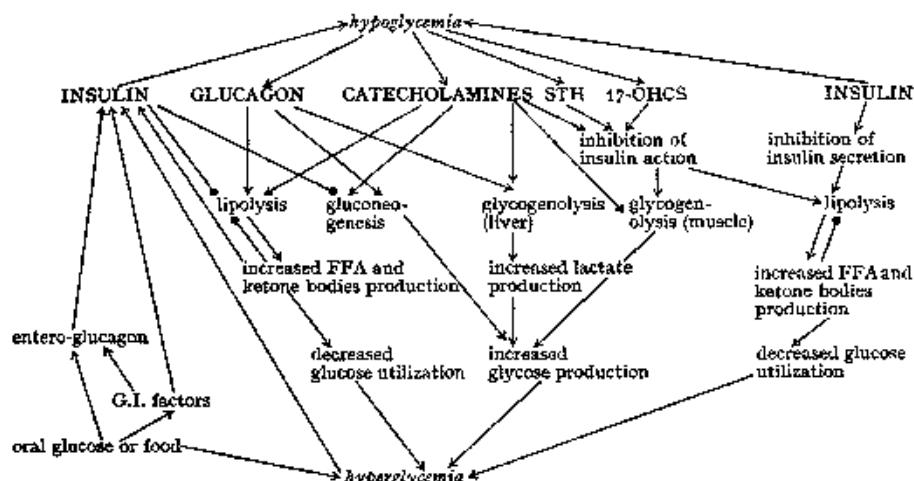


Fig. 10. The hormonal control of blood glucose concentration. \rightarrow stimulation or increase; $\rightarrow\!\!\!\rightarrow$ inhibition or decrease. STH = Somatotropin = growth hormone; 17-OHCS = 17-Hydroxycorticosteroids

of blood glucose would be restored to normal by the rapid increase in hepatic glycogenolysis and gluconeogenesis. In addition, the secretion of glucagon and the relative insufficiency of insulin would raise the level of blood sugar by promoting lipolysis, increasing the concentration of serum free fatty acids and ketone bodies and thus inhibiting glucose utilization. In this action, glucagon would be aided by a simultaneous increase in the secretion of hormones such as growth hormone, adrenal corticoids and epinephrine which tend to increase glucose production and fat mobilization and to inhibit insulin secretion, insulin action and glucose utilization by muscle and adipose tissue (5, 132, 265, 266, 407, 439, 469, 518, 528, 542, 609, 610, 611, 658, 659, 676, 680, 729, 743, 754, 831, 839, 852, 895). Thus, the dwindling supplies of glucose would be diverted from the liver and the peripheral tissues to the essential function of the brain (88, 617, 618, 624, 795, 799, 800). Conversely, if a state of hyperglycemia or glucose abundance exists, perhaps as a result of food intake, inhibition of glucose utilization (37, 595, 709) or excessive glucagon administration, insulin would be secreted, glucagon and growth hormone secretion would be inhibited (232, 412, 799, 800, 806, 807), glycogenolysis, gluconeogenesis and lipolysis would be suppressed, glucose utilization by muscle would be stimulated and

the concentration of glucose in the blood would be restored to normal. Under these circumstances insulin secretion would be stimulated not only by the elevated levels of blood glucose and amino acids, but by glucagon itself and by secretin, gastrin and pancreozymin secreted in response to the ingestion of food and to portal hyperglycemia (34, 68, 92, 93, 103, 134, 169-173, 225, 226, 262, 287, 289, 386, 395, 423, 444, 629, 761, 779, 786, 801, 808, 815, 870).

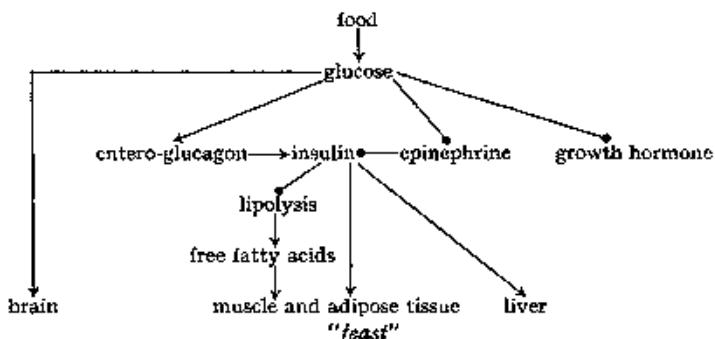


Fig. 11. Role of glucagon during glucose abundance. → stimulation or increase; —● inhibition or decrease

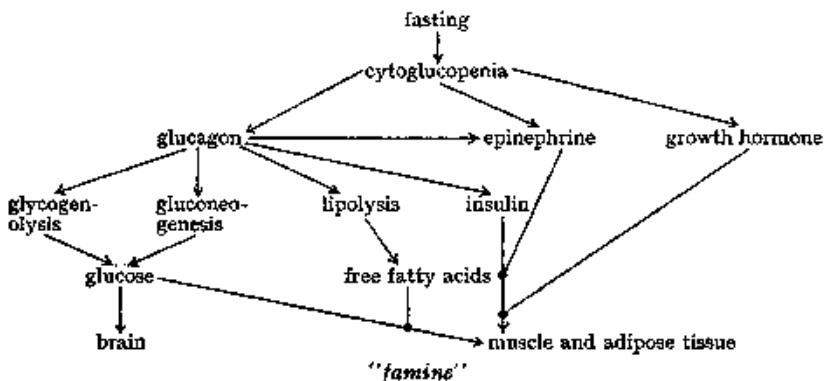


Fig. 12. Role of glucagon during glucose lack. → stimulation or increase; —● inhibition or decrease

One may conclude that, when glucose is scarce and blood sugar is low, glucagon, epinephrine and other hormones cause a breakdown of liver glycogen, increase gluconeogenesis, mobilize free fatty acids and suppress glucose utilization by muscle and adipose tissue, whereas when glucose is in abundant supply, insulin suppresses glycogenolysis, gluconeogenesis and fatty acid mobilization and contributes to the removal of glucose by increasing its utilization and storage (Fig. 11 and Fig. 12). The relative roles of glucagon and epinephrine in this regulation have not been fully clarified. It should be pointed out that, while there is little doubt that the secretory response of the adrenal medulla to hypoglycemia occurs very rapidly and that epinephrine is an effective blood sugar-raising hormone, glucagon has several physiologic "advantages" over epinephrine. For example, glucagon is secreted into the portal system and reaches its target organ directly, in contrast to epinephrine which, although

released in large amounts from the adrenal glands during hypoglycemia, is largely inactivated and what remains reaches the liver only after having been diluted in the general circulation. Thus, during insulin coma the concentration of epinephrine rises only from 0.2 to 1.4 µg/l of plasma (265), a concentration believed to be insufficient to cause effective glycogenolysis (728, 734). Glucagon has more than one hundred times the effectiveness of epinephrine as a glycogenolytic agent, although only one seventh the effectiveness as a lipolytic agent (199, 302, 734). Glucagon stimulates insulin secretion (111, 290, 852) while epinephrine inhibits it. When secreted in effective amounts, epinephrine causes tachycardia, tremor, sweating and other unpleasant side-effects, whereas glucagon, in physiologic doses, can correct hypoglycemia without untoward signs or symptoms. Finally, although epinephrine, like glucagon, causes hepatic glycogenolysis, it acts preferentially on muscle, resulting in the formation of lactic acid which must be converted to liver glycogen before reappearing in the blood as glucose. Thus, glucagon, because of its potency, its site of production, its mode of action and the lack of side-effects, seems ideally suited for the maintenance of a normal blood glucose concentration in the post-absorptive state and for the proper distribution of the available glucose between the sites of utilization and the sites of storage. A comparison of some effects of glucagon and insulin is presented in Table 4 [see also (449)]. As pointed out in the preceding pages, some of these opposing effects of the two hormones may be explained by their action on several enzyme systems. Thus, glucagon, directly or indirectly, stimulates phosphorylase, lipase, phosphoenolpyruvate carboxykinase (PEPCK) and pyruvic carboxylase (PC) and inhibits phosphofructokinase (PFK), glucokinase and glycogen synthetase activities, while insulin, directly or indirectly, stimulates, among others, the activities of glucokinase, glycogen synthetase, PFK, PEPCK and PC and inhibits the activities of phosphorylase and lipase. Indeed, many effects of glucagon resemble those of fasting and of insulin lack and, at least as far as fatty acid mobilization is concerned, of muscular exercise and cold exposure (4, 74, 107, 194, 267, 273, 297, 298, 360, 529, 549, 551, 555, 593, 634, 716, 723, 826-828, 830, 836, 857, 859). The conclusion that many *effects* of glucagon are opposite to those of insulin seems inescapable, although the mode of action of the two hormones may not be directly related. In view of the preceding considerations, it is suggested that, in the intact animal, glucagon may have the following *physiologic functions*: 1) maintenance of a normal blood glucose concentration in the post-absorptive state and, possibly, its restoration following insulin hypoglycemia; 2) apportionment of glucose supplies between liver, peripheral tissues and central nervous system during "feast" and "famine"; 3) stimulation of lipolysis and transfer of free fatty acid and of ketone bodies from liver and adipose tissue to heart and muscle during starvation; 4) stimulation of insulin secretion; 5) regulation of protein catabolism; 6) regulation of appetite.

Table 4. Comparison of some effects of glucagon and insulin

	Glucagon	Insulin
Blood glucose	S	I
Liver glycogen	I	S
Hepatic glucose production	S	I
Glucose utilization	I	S
Gluconeogenesis and urea production	S	I
Nitrogen balance	I	S
Fatty acid synthesis	I	S
Net triglyceride breakdown	S	I
Ketone body production	S	I
Hepatic K ⁺ release	S	I
Gastrointestinal activity	I	S
Hunger and food intake	I	S
Cyclic AMP formation	S	I
Phosphorylase activity	S	I
Glucokinase activity	I	S
Glycogen synthetase activity	I	S
Phosphoenolpyruvate carboxykinase activity	S	I
Glucose 6-phosphatase activity	S	I

S = stimulation or increase; I = inhibition or decrease.

and food intake; 7) regulation of the renal excretion of electrolytes; and, possibly, 8) regulation of plasma potassium, calcium and phosphorus concentration and of hepatic and renal blood flow.

A review of the physiologic functions of glucagon would not be complete without mentioning several observations which contradict statements made in the preceding pages and do not appear to fit the proposed hypotheses. For example, it has been reported that glucagon injections stimulate the synthesis of glycogen by the mouse diaphragm *in vivo* (115) and the uptake of glucose by the eviscerated, nephrectomized and functionally hepatectomized rabbit; that glucagon stimulates the uptake and oxidation of glucose and acetate by the perfused rat and toad heart (410, 584) and by bovine spermatozoa (498), that glucagon inhibits the rate of acetate and glucose incorporation into lipids (270, 565), corrects the depression of plasma lipid arachidonic acid caused by pancreatectomy (97), enhances the effect of insulin on glucose uptake by rat diaphragm and epididymal fat pad *in vitro* (2) and has other "insulin-like" effects *in vitro* and *in vivo* (302, 432, 442, 456, 812, 872). It has also been reported that β -hydroxybutyrate and deoxyglucose not only do not stimulate, but either inhibit or have no effect on insulin secretion by slices of rabbit pancreas (122, 250, 386, 649, 650) and that 19-nortestosterone derivatives inhibit the hyperglycemic response to glucagon (776). There are no satisfactory explanations for many of these findings, although it is tempting to dismiss them by blaming insulin impurities in glucagon preparations or compensatory insulin secretion.

X. The use of glucagon in diagnosis and therapy

1. Diagnostic procedures

The response to a test dose of glucagon (20 µg/kg intravenously) may be used in the differential diagnosis of the glycogen storage diseases and offers a clue to their characteristic enzymatic defect. Thus, in type I, glucagon causes a rise in lactate but not in glucose because, although glycogenolysis can proceed normally to glucose 6-phosphate, the lack of glucose 6-phosphatase prevents the formation of free glucose; in type II, characterized by deficiency of acid maltase, glucagon causes hyperglycemia because there are no deficiencies in the pathway of glycogenolysis via the phosphorylase system; in type III, characterized by a deficiency of debranching enzyme, glucagon causes hyperglycemia only after a meal, when the outer branches of the glycogen molecule upon which phosphorylase acts, are sufficiently long; in type IV, the deficiency of branching enzyme results in long outer branches and, therefore, glucagon causes hyperglycemia; in type V, the deficiency in muscle phosphorylase does not interfere with hepatic glycogenolysis and glucagon again causes a rise in serum glucose; in types VI and IX, characterized by a deficiency of hepatic phosphorylase and hepatic phosphorylase kinase, respectively, glucagon causes little or no hyperglycemia; in types VII and X, hepatic glycogenolysis is normal and glucagon should cause a normal rise in blood glucose, although the deficiencies of phosphoglucomutase and of phosphofructokinase in muscle should interfere with the rise in serum lactate; in type VIII, the decreased hepatic phosphorylase activity appears to be due to a defect of enzyme activation rather than to lack of enzyme and, since glucagon reverses this defect, hyperglycemia should occur; finally, in type XI, hepatic glycogenolysis is normal, but the deficiency of synthetase reduces the amount of glycogen available and glucagon hyperglycemia is reduced (33, 71, 133, 219, 229, 230, 232, 328, 332, 345, 347—351, 453, 467, 558, 564, 733, 770, 780) (Table 5). Of great interest are recent studies demonstrating that muscle phosphorylase *b* kinase in mice is located on the X chromosome and is inherited following classical mendelian laws. Strains of mice lacking this enzyme have a muscle glycogen content of 1.2 %, while in strains of mice with the enzyme the muscle glycogen content is only 0.5 % (471). A low hepatic glycogen content is probably responsible also for the reduced hyperglycemic response to glucagon observed in liver and renal disease, in severe diabetes, in prematurity and in adrenal cortical insufficiency (95, 116, 126, 215—217, 230, 232, 359, 411). In general, glucagon response is normal in patients with hypoglycemia due to extra-pancreatic tumors (490, 824) and decreased in some patients with insulinoma and functional hypoglycemia (227, 229, 230, 232, 496, 752). Thus, the hyperglycemic response to glucagon may be used as a harmless, if perhaps not always a very useful, diagnostic procedure (75, 76, 126, 229, 230, 553, 752). On the other hand, the pressor response to the intravenous injection of 0.5 to 1.0 mg

Table 5. Classification of glycogen storage

Type		Organs involved	Glycogen structure
I	Hepatomegalic glycogenesis	Liver, kidney	Normal
II	Cardiomegalic glycogenesis	Heart, skel. muscle, liver kidney	Normal
III	Limit dextrinosis	Skel. muscle, liver, kidney, heart	Limit dextrine (Short outer branches)
IV	Amylopectinosis	All organs (Reticulo-endothelial system)	Long outer and inner branches
V	Phosphorylase deficiency	Muscle	Normal
VI	Phosphorylase deficiency	Liver	Normal
VII	Multiple enzyme deficiency	Muscle	Normal
VIII	Phosphorylase deficiency	Liver	Normal
IX	Phosphorylase deficiency	Liver	Normal
X	Phosphofructokinase deficiency	Muscle	Normal
XI	Glycogen storage deficiency	Liver	Normal

of crystalline glucagon, which forms the basis of a recently introduced provocative test for pheochromocytoma, may be dramatic and is potentially dangerous (425, 437, 438, 705). The serum insulin response to glucagon has been used for measuring insulinogenic reserve in normal, prediabetic and diabetic subjects (34, 664). Finally, an elevated serum glucagon concentration appears to be a useful diagnostic aid in most cases of hyperparathyroidism, whether associated with pancreatitis or not (572).

2. Therapeutic uses

Glucagon has been used in the treatment of the sequelae of overdosage of insulin or sulfonylurea drugs, for the smooth termination of insulin shock in psychiatric patients, in the treatment of idiopathic hypoglycemia, of hypo-

disease. Response to glucagon and epinephrine

Enzyme deficiency or excess (E)	Comments
Glucose 6-phosphatase	Glucagon and epinephrine cause rise in lactate, but not in glucose. Fatty liver. Hyperlipemia
Alpha-1,4-glucosidase (Acid maltase)	Glucagon and epinephrine cause rise in glucose and lactate. All tests normal
Amylo-1,6-glucosidase (Debranching enzyme)	Glucagon and epinephrine cause hyperglycemia only after a meal when outer branches are "long". Muscle glycogen > 4% diagnostic
Amylo-(1-4 → 1-6) transglucosidase (Branching enzyme)	Glucagon and epinephrine cause hyperglycemia. Polysaccharide gives bluish color with iodine. Hepatic fibrosis
Phosphorylase (Muscle)	Glucagon and epinephrine cause greater rise in glucose than in lactate. Can perform only "aerobic" work. No rise in lactate after muscle work. Myoglobinuria
Phosphorylase (Liver)	Glucagon and epinephrine cause little or no rise in glucose
Phosphoglucomutase and other enzymes	Glucagon and epinephrine should cause greater rise in glucose than in lactate
Activation of phosphorylase in adequate	Glucagon and epinephrine restore enzymatic activity. Cause hyperglycemia
Phosphorylase kinase	Glucagon and epinephrine do not cause hyperglycemia. Epinephrine causes a rise in lactate. Phosphorylase may be activated by substituting an activating system <i>in vitro</i>
Phosphofructokinase; glycogen synthetase (E); UDPG-pyrophosphorylase (E)	Glucagon and epinephrine should cause greater rise in glucose than in lactate
UDPG-glycogen transglucosylase (E)	Mental retardation. Hypoglycemia. Low liver glycogen (0.5% in fed state)

glycemia due to islet-cell and other tumors, of hypoglycemia due to leucine sensitivity and of neonatal hypoglycemia associated with visceromegaly and other defects (94, 95, 98, 120, 143, 149, 163, 192, 237, 246, 402, 414, 460, 489, 535, 547, 548, 639, 654, 658, 685, 714, 726, 865). The effect of glucagon on hypoglycemia due to ethanol ingestion is uncertain (218, 239). The effects of crystalline glucagon are of rapid onset and relatively short duration and lasting hyperglycemia can be achieved only with repeated injections. On the other hand, zinc glucagon is a relatively insoluble long-acting complex and one intramuscular or subcutaneous injection of 0.2 to 0.5 mg/kg (up to a total dose of 2 to 10 mg) appears sufficient to cover a 24-hour period (414, 467, 657, 841). When using glucagon for the correction of hypoglycemia one must remember that the restoration of blood glucose to satisfactory levels occurs at the expense

of liver glycogen and of tissue protein and that the beneficial effects may be temporary unless they are followed by the administration of a carbohydrate meal, as soon as this can be given safely. Only in this manner can one prevent a relapse into insulin hypoglycemia and stop the breakdown of protein.

Glucagon has been used as a palliative measure in the treatment of v. GIERKE's disease, where it may cause a temporary decrease in the size of the liver, sometimes accompanied by decreased tenderness and clinical improvement (257, 594, 733). Intramuscular injections of glucagon before each meal result in a significant depression of food intake and have been used as an effective aid in weight reduction (588, 692). The use of glucagon in the therapy of rheumatoid arthritis and related disorders (232) apparently has been abandoned. Glucagon injected directly into the lesion appears to have a harmful effect in acne vulgaris (293), while intradermal injections of glucagon may alleviate insulin allergy (411).

XI. Conclusions

The extensive studies reviewed in these pages lead to the following tentative conclusions. 1) Glucagon is a well-characterized peptide secreted by the A₂ cells of the islets of LANGERHANS. 2) The *mode of action* of glucagon consists in the stimulation of hepatic phosphorylase and of adipose tissue lipase. 3) Glucagon has at least four targets: the liver, the B cell, the adipose tissue and the myocardium. 4) Glucagon injections have numerous *effects* which have been ascertained beyond reasonable doubt. Among these are a stimulation of hepatic glycogenolysis and gluconeogenesis, an increase in blood glucose and free fatty acid concentration and in protein catabolism, a stimulation of insulin secretion and of the renal excretion of electrolytes and an inhibition of gastrointestinal activities. 5) The *physiologic role* of glucagon has not been fully elucidated, but the following hypothesis appears justified. Glucagon is a potent hormone, secreted in response to hypoglycemia and forms an important link in the regulation of blood glucose levels. This homeostatic function, although essential for the brain and the well-being of the organism, may be only a reflection of a more fundamental role of glucagon in the readjustment of metabolic pathways during "feast" and "famine". It should be pointed out that glucagon has been found in measurable amounts only in the pancreas and in the blood of the pancreatic vein, whereas its concentration in the systemic blood is very low and sometimes not detectable. For this reason, a full understanding of the physiologic actions of glucagon in organs other than the liver and the B cell must await the discovery of assay methods more sensitive than those now available. Similarly, the role of glucagon in the pathogenesis of diabetes and other diseases and its possible usefulness in diagnosis and therapy require further elucidation.

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Biochemie, Pharmakologie und Toxikologie der Inhaltsstoffe von Hymenopterengiften

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Mit 14 Abbildungen

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I. Allgemeine Bemerkungen*

Bienen, Wespen und Hornissen zählen zu den aktiv giftigen Tieren. Im Gegensatz zu manchen Fischen und Amphibien bringen sie ihr Gift dem potentiellen Gegner während einer Angriffshandlung bei. Dazu benötigen sie einen spezifischen Stechapparat, der sich vom reproduktiven System ableitet. Der Giftapparat der Schlangen ist dagegen dem Verdauungstrakt zuzuordnen. Eine strenge Korrelation zu der Zusammensetzung und Wirkungsweise läßt sich daraus zwar nicht ableiten; doch ist klar, daß für die Hymenopteren nur schnell wirkende Gifte sinnvoll sein können, weil sie zur Abwehr eines unmittelbaren Angriffs eingesetzt werden. Schlangengifte haben demgegenüber eine polyvalente Funktion. Ihre Beziehung zum Verdauungssystem äußert sich im reichlichen Gehalt der Viperidengifte an Proteasen, Esterasen und Nucleotidasen. Diese Schlangengifte haben also im Gegensatz zu den Hymenopterengiften gleichzeitig digestiven und aggressiven Charakter. Bei den Elapiden-giften treten die genannten Enzyme zurück; statt dessen finden sich basische Polypeptide, so daß ihre Zusammensetzung rein formal derjenigen der Hymenopteren entspricht.

Keiner der bisher bekannten Inhaltsstoffe der Gifte gehört einer grundsätzlich neuen Stoffklasse an, fast alle können von Aminosäuren abgeleitet werden. Man findet Proteine im engeren Sinne; zu ihnen sind auch die gift-

* Zusammenfassende Darstellungen aus den letzten Jahren: NEUMANN u. HABERMANN (1960); BEARD (1963); MELDRUM (1965); HABERMANN (1968a und b).

Tabelle 1. Pharmakologisch bzw. biochemisch aktive Bestandteile verschiedener tierischer Gifte

	Biene	Wespe	Hornisse	Elapiden	Crotaliden	Viperiden
Biogene Amine	Histamin	Histamin Serotonin	Histamin Serotonin Acetylcholin	—	—	—
Polypeptide und Proteine (nicht enzymatisch)	Melittin Apamin MCD-Peptid	Wespenkinin	Hornissenkinin	Neurotoxin Cardiotoxin Hämolyein	Crotactin Crotamin	?
Enzyme	Phospholipase A	Phospholipase A Phospholipase B Hyaluronidase	Phospholipase A Phospholipase B (?) Hyaluronidase Cholinesterase	Phospholipase A Phospholipase B (?) Hyaluronidase Hyaluronidase zahlreiche weitere Enzyme	Phospholipase A Hyaluronidase zahlreiche weitere Enzyme	Phospholipase A Hyaluronidase

eigenen Enzyme zu zählen. Daneben sind Polypeptide vertreten, etwa Crotamin im *Crotalus-terribilis*-Gift; Cobratoxin; Melittin und Apamin in Hymenopterengiften; schließlich Kintine in Wespen- und Hornissengift. Dazu kommen die ebenfalls von den Aminosäuren abzuleitenden biogenen Amine, wie Histamin und Serotonin. Die einzige Ausnahme stellt das im Hornissengift reichlich vorhandene Acetylcholin dar. Als Substanzgruppe sind also die Insekten- wie auch die Schlangengifte relativ einstönnig; so vermisst man Bestandteile aus der Gruppe der Alkaloide, wie sie bei Amphibien vorkommen, Steroide, die man bei Holothurien gefunden hat, neuartige Toxine, etwa vom Typ des Tetrodotoxins. In Tabelle 1 ist die Zusammensetzung der Hymenopterengifte und im Vergleich dazu die der Schlangengifte wiedergegeben.

Die zunehmende Zahl von Enzymen, die zwischen 1940 und 1950 in Schlangengiften gefunden wurde, verführte zu der Annahme, daß man in ihnen die für die Toxicität entscheidenden Faktoren zu sehen hätte. Darin wurde man bestärkt, als man Phospholipase C mit dem Haupttoxin (α -Toxin) des Gasbrandgiftes identifiziert hatte. Am Beispiel des relativ einfach zusammengesetzten Bienengiftes konnte demgegenüber der Unterschied zwischen nicht-enzymatischen Bestandteilen mit starker Toxicität und Enzymen mit relativ geringer Giftigkeit demonstriert werden. In der Folge wurden auch das Neurotoxin von Cobragift (Übersicht bei CONDREA und DE VRIES, 1965) und Crotactin (W. P. NEUMANN und HABERMANN, 1955), das Haupttoxin des

Klapperschlangengiftes, von allen bisher bekannten Enzymqualitäten getrennt. Es gibt also in den genannten Giften „Toxine“ im klassischen Sinne, d. h. ohne enzymatische Eigenschaften, neben Enzymen. Die Toxicität der Enzyme ist in sehr unterschiedlichem Maß an der Gesamtgiftigkeit beteiligt, bei den Viperiden stärker als bei den Elapiden oder den hier zu diskutierenden Hymenopterengiften. Die Entwicklung der Enzyme zu toxischen Agentien beruht wohl auf der Weiterbildung ihrer bei den Schlangengiften primär digestiven Funktionen, bei den Insekten auf der Fortführung von Funktionen, die normalerweise den Kontakt der Geschlechtszellen erleichtern. In beiden Fällen handelt es sich um den biochemischen Ausdruck der Evolution.

Bienengift ist das erste und nach wie vor klassische Modell, an dem die Kooperation von Toxinen und Enzymen demonstriert werden konnte und bei dem dieses Zusammenwirken besonders transparent ist, wie im folgenden gezeigt wird (Tabelle 1).

II. Bienengift

A. Produktion, Extraktion und allgemeine Eigenschaften des Gesamtgiftes

Die für die Giftproduktion offenbar wichtigste Quelle ist die sog. „saure“ Drüse (Abb. 1). Sie ist relativ groß und steht mit dem Giftsack in direktem Zusammenhang. Die kleinere „alkalische“ Drüse dient wahrscheinlich — wegen des hohen Fettgehalts ihres Sekrets und der Mündung zwischen die „lancets“ — als Schmiervorrichtung für die Mechanik des Stechapparats. Das Gift selbst ist schwach sauer. Hinweise auf eine entscheidende Rolle der „sauren“ Drüse sind bei HAYDACK (1951) zusammengestellt. Besonders bemerkenswert sind die Resultate von BRAUN, der bereits 1933 Extrakte der beiden Drüsarten an der menschlichen Haut verglich: nur die Inhaltsstoffe der „sauren“ Drüse erwiesen sich als wirksam. Die Bedeutung älterer Experimente dürfte damit — zumindest was die Lokotoxicität betrifft — eingeschränkt sein; man hatte früher auf Grund von vorwiegend qualitativen Toxicitätsversuchen an Fliegen angenommen, daß die Produkte der sauren und alkalischen Drüse sich gegenseitig ergänzen. Eine Zuordnung der heute bekannten Giftkomponenten zu den beiden Drüsen ist aber bisher nicht erfolgt; es ist nach wie vor denkbar, daß Giftbestandteile mit geringer Toxicität aus der alkalischen Drüse stammen.

Die saure Giftdrüse besteht aus zwei Lagen von Zellen. In der äußeren, aus sehr großen Einheiten zusammengesetzten Schicht wird offenbar das Gift produziert und in die Sekretkanälchen abgegeben, die im Cytoplasma entspringen und darin einen vielfach gewundenen Verlauf nehmen. Ihr Ende ist ampullenartig erweitert. Die dem Lumen näherte Schicht ist aus Zellen mit sehr viel kleineren Kernen zusammengesetzt. Man vermutet, daß sie das Chitin bilden, welches das Drüsenumen auskleidet. Die Giftblase ist mit den genannten kleinkernigen Zellen ausgekleidet; das sekretorische Epithel endet am Eingang der Giftblase (AURKUM und KNEITZ, 1959) (Abb. 2).

Die Giftproduktion hängt stark vom Lebensalter der Bienen ab. Bei den Puppen läßt sich histologisch kein Giftsekret nachweisen. Zum Zeitpunkt des Schlüpfens sind bereits kleine Giftmengen vorhanden, die bei Reizung abgegeben werden können und

schmerzerzeugende Substanzen enthalten. Zwischen dem 2. und 10. Tag steigt die Giftproduktion langsam an, zwischen dem 10. und 16. Tag sehr schnell. Die Giftblase füllt sich prall. Zwischen dem 20. und 30. Tag, also nach Erreichen des Wächteramtes, nimmt der Durchmesser der Giftdrüse stark ab; auch das histologische Bild weist auf Involution hin. Dementsprechend ist eine Regeneration abgegebenen Giftes bei Sommerbienen nur in den ersten 20 Lebenstagen möglich. Winterbienen, die im Herbst geschlüpft sind, können allerdings auch im Frühjahr noch etwas Gift sezernieren (AUTRUM und KNEITZ, 1959; MÜLLER, 1938). MÜLLER bestimmte das Giftgewicht in Abhängigkeit vom Lebensalter und fand bei 2 Tage alten Bienen 0,014 mg Trockengift, bei 6tägigen Bienen 0,05 mg, bei 11tägigen 0,07 mg, bei 15tägigen 0,1 mg, bei Winterbienen 0,12 mg. Das Trocken gewicht machte etwa $\frac{1}{3}$ des Feuchtgewichts aus. Analoge Zahlen wurden von LAUTER

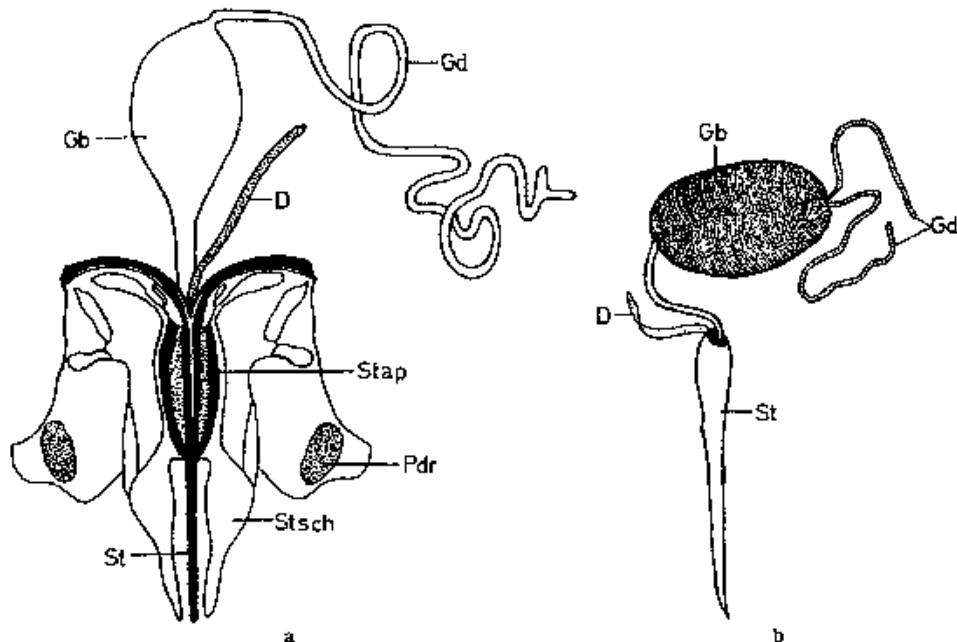


Abb. 1 a u. b. Stechapparate von *Apis mellifica* (a) und *Vespa germanica* (b) (nach MASCHWITZ, 1964). D Dufoursche Drüse; Gd Giftdrüse; Gb Giftblase; St Stachel; Stap Stachelrinnenpolster; Stsch Stachelscheide; Pdr Koschewnikowsche Plattendrüse

und VRLA (1939) erhalten. Über die jahreszeitlichen Schwankungen der Giftmengen sind die Ansichten geteilt (Übersicht bei HAYDACK, 1951). Sicher ist, daß die Art der Ernährung der Bienen, vor allem der Zusatz von Pollen, sich stark auswirkt (LAUTER und VRLA, 1939; MÜLLER, 1938). Die Giftproduktion ist auf die soziale Funktion der Biene abgestimmt; mit dem 16.—20. Tag benötigt sie das Gift beim Wächteramt.

Die Art der Gewinnung hängt davon ab, welche Menge und welcher Reinheitsgrad benötigt wird. Das sauberste Material erhält man, wenn man die Tiere durch eine Gummi- oder Kunststoff-Membran stechen läßt. Benutzt man Gummi, so bleibt der Stechapparat nach Entfernen der Biene zurück und pumpt sich selbsttätig leer. Bei der Benutzung mancher Kunststoffmembranen kann die Biene den Stachel ohne Verletzung zurückziehen, also wiederholt Gift spenden. In jedem Fall befindet sich auf der „sauberen“ Seite der Membran das sirupöse, leicht gelbliche Sekret, auf der „schmutzigen“, d.h. den Bienen zugewendeten Seite Kot und Leibeshöhlenflüssigkeit. Durch geeignet

angebrachte elektrische oder mechanische Reizvorrichtungen läßt sich das beschriebene Verfahren in technischem Maßstab einsetzen. Zur Analyse der Sekrete einzelner Bienen kann man sie mit dem Abdomen auf ein Filterpapier pressen. Der ausgefahrene Stachel verfängt sich in den Papierfasern und wird bei Entfernen der Biene samt dem intraabdominal liegenden Giftapparat

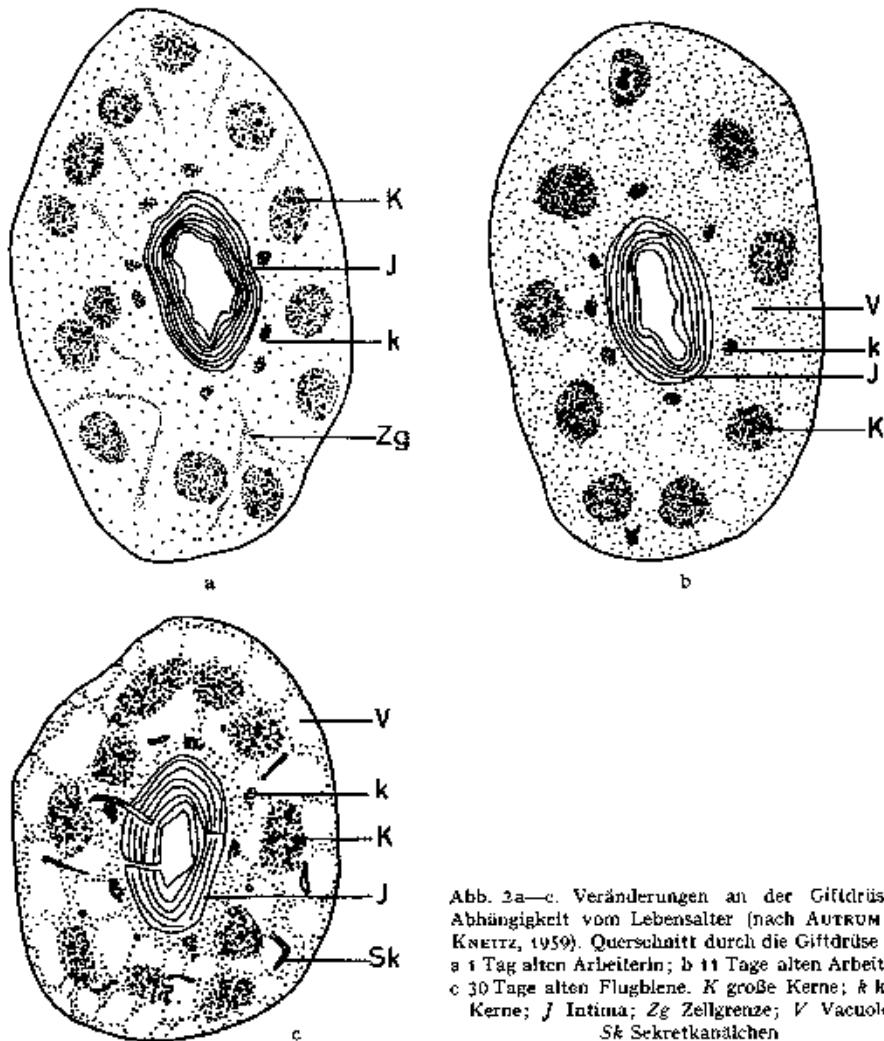


Abb. 2a—c. Veränderungen an der Giftdrüse in Abhängigkeit vom Lebensalter (nach AUTRUM und KNETZ, 1959). Querschnitt durch die Giftdrüse einer a 1 Tag alten Arbeiterin; b 11 Tage alten Arbeiterin; c 30 Tage alten Flugblene. K große Kerne; k kleine Kerne; J Intima; Zg Zellgrenze; V Vacuolen; Sk Sekretkanälchen

herausgerissen. Die autonom arbeitende Giftblase pumpt ihren Inhalt ins Papier; er kann anschließend z.B. der Papierelektrophorese unterworfen werden (NEUMANN und HABERMANN, 1954a). Geringe Verunreinigungen mit Leibeshöhlenflüssigkeit müssen dabei in Rechnung gestellt werden.

Gelegentlich ist man daran interessiert, die Tiere mehrmals zu entgiften. Hierzu wurde ein Rahmen angegeben, der mit elektrisch aufladbaren Drähten und darunter liegendem Nylon-Taft bespannt ist. Er läßt sich unmittelbar unter die Bienenstöcke schieben. In diesem Gewebe verfangen sich die Widerhaken nur ausnahmsweise (BENTON

und STEWART, 1963). Eine Art „elektrischer Stuhl“ für einzelne Insekten lässt ebenfalls die Tiere überleben (O'CONNOR et al., 1963).

Wesentlich weniger rein waren früher verwendete Giftpräparate, die durch Extraktion der gesamten Stechapparate gewonnen wurden; das Verfahren ist heute praktisch verlassen.

Frisches Bienengift ist eine leicht trübe, schwach gelbliche, honigähnlich riechende Flüssigkeit, die mit Wasser beliebig mischbar ist. Als Dichte wird 1,1313 angegeben (HAYDACK, 1951), worin sich der sehr hohe (ca. 30 %) Gehalt an Feststoffen ausdrückt. Bei mikroskopischer Betrachtung fallen zahlreiche stärker lichtbrechende Kugelchen auf, die vielleicht lipoide Natur sind; nähere Untersuchungen scheinen nicht vorzuliegen. Die meisten, vor allem die älteren Angaben über den Gehalt von Gesamtgift an Mg, Ca, Cu (HAHN und LEDITSCHKE, 1936) oder an lipoiden Verbindungen bzw. deren Spaltstücken (FLURY, 1920) bedürften einer Nachkontrolle, da oft mit Gesamtextrakten von Stechapparaten gearbeitet wurde. Sorgfältige Analysen von rohem bzw. über das Pikrat vorgereinigtem Gesamtgift stammen von FISCHER und NEUMANN (1953). Stark positiv war die Reaktion nach MOLISCH auf Hexosen, während die nach TOLLENS auf Pentosen negativ ausfiel. Aschegehalt zwischen 3 und 4 %. Phosphor: 0,42 %. Sulfat war nicht nachweisbar, Chlorid nur in Spuren; Ca⁺⁺ 0,26 %; Mg⁺⁺ 0,49 %. Na⁺ und K⁺ wurden (spektroskopisch) nicht identifiziert. Wasserdampfflüchtige Säure (z.B. Ameisensäure) wurde nicht gefunden. 75 Gew.-% des Rohgutes waren mit Pikrinsäure fällbar.

Angaben über die Bruttozusammensetzung eines Vielkomponenten-Systems wie des Bienengiftes sind nur von beschränktem Wert; wichtiger ist die Kenntnis der einzelnen, besonders der pharmakologisch bzw. biochemisch aktiven Bestandteile. Eine klare Trennung der in Frage kommenden Substanzen war Vorbedingung für ihre Charakterisierung; so gelang beides erst, als das Rüstzeug der modernen Protein- und Peptidchemie zur Verfügung stand. Anschließend konnte man oft die sorgfältigen Beobachtungen der älteren Autoren, besonders von LANGER (1897), HAHN u. Mitarb. (1936, 1937), TETSCH und WOLFF (1936) in das moderne Schema einbauen.

B. Trennverfahren

Heute stehen zwei sehr verschiedene, sich gegenseitig ergänzende Prinzipien zur Trennung der Giftkomponenten zur Verfügung.

a) Man kann sich die unterschiedliche Ladung der Giftbestandteile zunutze machen. Auf dieser Basis gelang erstmals die elektrophoretische Aufteilung der Polypeptide bzw. Proteine in drei Fraktionen, die damals F0, FI und FII genannt wurden. FII enthielt die beiden Enzyme Phospholipase A und Hyaluronidase; FI bestand, wie wir heute wissen, im wesentlichen aus Melittin mit Beimischungen des in sehr viel geringerer Menge im Bienengift vorhandenen Apamins. Auch F0 war nach gegenwärtigen Kenntissen uneinheitlich. Zwar erlaubte die Elektrophorese nur eine Gruppentrennung, doch gelang mit ihr, wie einleitend bereits erwähnt, die erstmalige Unterscheidung der beiden Haupt-

typen von Wirkstoffen: der Toxine und der Enzyme. Sie ließ sich auch auf die Trennung der Giftsekrete einzelner Bienen anwenden. Dabei erkannte man neben den Polypeptiden bzw. Proteinen den schneller wandernden Histamin-Fleck, eine noch schneller kathodisch laufende ninhydrinpositive Komponente, die möglicherweise der Leibeshöhlenflüssigkeit entstammt, und schließlich mindestens eine ninhydrinpositive und eine ninhydrinnegative saure Substanz, die beide bisher noch nicht charakterisiert wurden (NEUMANN *et al.*, 1952; NEUMANN und HABERMANN, 1954a). Auch die Trennung an Ionenaustauschern beruht wenigstens zum Teil auf der unterschiedlichen Ladung der Giftkomponenten. So gelang die Differenzierung von Hyaluronidase und Phospholipase A an Amberlite CG 50 (HABERMANN und W. P. NEUMANN, 1957); F0, Phospholipase A und Apamin ließen sich von Melittin an Carboxymethyl-Cellulose trennen (HABERMANN und REIZ, 1965a).

b) Die komplette Scheidung aller bekannten Bestandteile wurde erst durch Anwendung eines zweiten Prinzips erreicht: der Gelfiltration an Sephadex

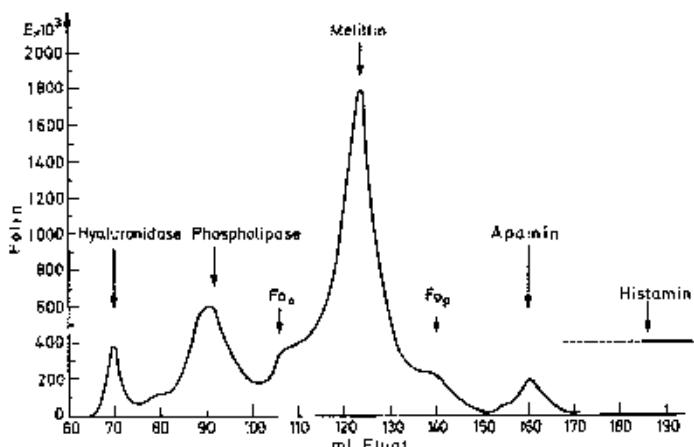


Abb. 3. Trennung von Bienengift an Sephadex G 50. Ordinate: Extinktion der Folinischen Reaktion ($\text{O}—\text{O}$). Abszisse: ml Eluat. Laufmittel: 0,1 M Ammoniumformiatpuffer pH 4,5

G 50, bei der sich eine Sortierung nach der Molekülgröße ergibt. Zuerst erscheint Hyaluronidase als größtes Protein im Eluat, gefolgt von Phospholipase A. Melittin und Apamin schließen sich an, am Schluß tritt Histamin, zusammen mit anderem niedermolekularem Material, aus der Säule (Abb. 3.) F0-Komponenten werden im unmittelbaren Bereich des Melittingipfels eluiert; sie lassen sich von ihm durch anschließende Chromatographie an Carboxymethylcellulose trennen. Eine derartige Chromatographie ist auch zur Nachreinigung der übrigen Giftbestandteile empfehlenswert (HABERMANN und REIZ, 1964, 1965a).

Bisher wurden also folgende pharmakologisch aktive Bestandteile näher charakterisiert: Histamin, Melittin, F0-Komponenten (MCD-Peptid), Apamin, Hyaluronidase, Phospholipase A.

Lipoidlösliche Substanzen aus Bienengift sind noch nicht mit seinen pharmakologischen Wirkungen in Zusammenhang gebracht worden. TERSCH und WOLFF (1936) extrahierten mit Äther einen stark duftenden Stoff; mit Chloroform erhielten sie einen sterinartigen, in länglichen Nadeln ($F 120^\circ$) kristallisierenden Anteil. Es könnte sich dabei um die Inhaltsstoffe der mikroskopisch sichtbaren Lipidkügelchen (s. S. 226) handeln.

C. Biochemie und Pharmakologie der einzelnen Giftkomponenten

1. Histamin

Histamin besitzt im Rahmen dieser Übersicht nur insoweit Interesse, als es zu den pharmakologischen Gifteffekten beiträgt. Man muß dabei allerdings unterscheiden, ob es aus dem Gift selbst stammt oder durch dessen Komponenten aus dem Gewebe freigelegt wird. Die Hemmbarkeit einer Giftwirkung durch ein spezifisches Antihistaminicum bedeutet also nur, daß das biogene Amin dabei eine Rolle spielte, nicht aber, daß das gifteigene Histamin verantwortlich war. Auf die Mediatorfunktion körpereigener Stoffe bei der Giftwirkung wird später (s. S. 239) eingegangen werden. Die pharmakologische Bedeutung gifteigenen Histamins tritt gegenüber derjenigen anderer Giftbestandteile weit zurück. Bisher kann kein Effekt selektiv oder überwiegend darauf zurückgeführt werden. Seine Menge im Trockengift wird verschieden angegeben: Wir selbst fanden bei Prüfung schonend gewonnenen Giftes am Katzenblutdruck einen Histamingehalt von höchstens 0,1 %. Nur dieser Teil der Blutdrucksenkung war durch das Antihistaminicum Avil hemmbar (SALZMANN, 1953). REINERT (1936), der mit Pikrinsäurefällung arbeitete, und SCHACHTER und THAIN (1954), die Extrakte des Giftapparates am isolierten Meerschweinchenileum prüften, errechneten zwischen 0,8 und 1,5 % Histamin. Die meisten biologischen Testobjekte sprechen aber auch auf andere Giftkomponenten, z.B. Melittin und Phospholipase, an. Ein quantitatives chemisches Bestimmungsverfahren wurde bisher noch nicht auf Bienengift angewandt. Die Reinerschen Befunde konnten von ACKERMANN und MAUER (1944) nicht reproduziert werden; am isolierten Meerschweinchenileum konnten sie erst dann Histamineffekte nachweisen, wenn sie zuvor die höhernukleären Giftbestandteile durch Trichloressigsäurefällung entfernt hatten. Beides steht im Einklang mit eigenen Befunden.

Besonders schonend gewonnenes (NEUMANN und HABERMANN, 1954a) und auch technisches (HABERMANN und REIZ, 1965a) Bienengift enthält weitere niedermolekulare, ninhydrinpositive, bisher nicht identifizierte Verbindungen. Glattmuskuläre Aktivität läßt sich bisher nur derjenigen papierchromatographischen bzw. papierelektrophoretischen Fraktion zuordnen, welche dem Histamin entspricht.

2. Melittin

Melittin ist nach Menge und pharmakologischer Aktivität das Haupttoxin des Bienengiftes. Photometrische Auswertung von Papierelektrophogrammen ergab als Verteilung der Peptide bzw. Proteine auf die einzelnen Fraktionen F0:FI:FII = 1:16:5 (NEUMANN und HABERMANN, 1954a). Keine dieser elektrophoretischen Fraktionen kann jedoch nach heutiger Kenntnis als einheitlich bezeichnet werden (s. S. 226). Bei Gelfiltration anderer Giftchargen wurden 12,0—15,6 % des Gesamtgewichts in der Phospholipasefraktion wiedergefunden, 50,4—57,8 % in der Melittinfaktion, dagegen nur 1,9 bis 2,3 % in der Apaminfraktion (HABERMANN und REIZ, 1965a) und etwa der gleiche Prozentsatz als MCD-Peptid (HABERMANN, unveröffentlicht).

a) Chemische Eigenschaften

Melittin (HABERMANN, 1954a) zählt zu den bisher nicht zahlreichen Naturstoffen, deren Wirkungsmechanismen sich unmittelbar aus ihren chemischen Eigenschaften ableiten lassen. Seine Chemie, Biochemie und Pharmakologie stellen eine logisch befriedigende Einheit dar. Die entscheidenden Konstruktionsmerkmale des Melittinmoleküls wurden auf Grund pharmakologischer Befunde postuliert.

Melittin ist ein Polypeptid ohne enzymatische Eigenschaften. Die Existenz eines derartigen gifteigenen Wirkstoffes läßt sich anhand der heutigen Kenntnisse bereits aus älteren Arbeiten erschließen; doch konnte seine Charakterisierung erst mit Hilfe der in den letzten 20 Jahren verfeinerten Verfahren der Protein- und Peptidchemie gelingen. Zunächst wurde durch Papierelektrophorese, wie bereits erwähnt, eine noch nicht völlig reine, aber ganz überwiegend aus Melittin bestehende „Fraktion I“ kleinpräparativ erhalten (NEUMANN *et al.*, 1952; NEUMANN und HABERMANN, 1954a). Die dieser Fraktion damals zugesprochenen pharmakologischen und biochemischen Eigenschaften beziehen sich auch auf das reine Melittin. Auf Anregung von W. NEUMANN und HABERMANN suchten dann FISCHER und W. P. NEUMANN (1953) nach einem einfacheren Weg zur Isolierung von „Fraktion I“; sie konnten sie an bestimmten Cellulose-Arten von allen anderen Giftbestandteilen trennen. Ihr Produkt, das für die meisten pharmakologischen und biochemischen Untersuchungen verwendet wurde (s. unten), war frei von Schwefel und damit auch von Apamin und MCD-Peptid. Das Verfahren von FISCHER und W. P. NEUMANN ist insofern kritisch, als Melittin bei zu geringer Säulenbelastung überhaupt nicht, bei zu großer Säulenbelastung stark verunreinigt im Eluat erscheint. Das Trennvermögen hängt stark vom Carboxylgehalt der verwendeten Cellulose ab. Man machte sich also unbewußt — vor der Einführung substituierter Cellulosen — einen Ionenaustauschereffekt zunutze (W. P. NEUMANN, unveröffentlicht). — Quantitativ wie qualitativ leistungsfähiger und zuverlässiger ist die Kombination von Gelfiltration an Sephadex G 50 mit Chromatographie an Carboxymethyl-Cellulose. Sie ist auch wirtschaftlicher, weil sie die gleichzeitige Gewinnung aller bekannten Giftbestandteile erlaubt (HABERMANN und REIZ, 1965a). Eine einfache Chromatographie an Carboxymethyl-Cellulose kann bereits reines Melittin liefern (KREIL, 1965), weil das Peptid wesentlich stärker als alle Begleitstoffe an saure Ionenaustauscher gebunden wird.

Melittin ist gut wasserlöslich, läßt sich als Pikrat fällen und mit salzaurem Aceton ins Hydrochlorid überführen. Beim Erhitzen im Neutralen oder Sauren trübt sich die Lösung nicht. Es ist — zumindest was sein hämolytisches Vermögen betrifft — über einen weiten pH-Bereich (pH 2,2—8) relativ thermostabil. Sein UV-Spektrum weist auf Tryptophangehalt hin. UV-Bestrahlung zerstört Spektrum und pharmakologische Aktivität (HABERMANN, 1955a, 1958a). Durch Cellophanmembranen dialysiert Melittin äußerst langsam; die

Tabelle 2. Aminosäureanalyse von Melittin, Apamin und MCD-Peptid (HABERMANN und REIZ, 1965 b; HABERMANN, unveröffentlicht)

	MCD-Peptid (72 Std Hydrolyse)	Melittin (72 Std Hydrolyse)		Apamin (22 ^{1/2} Std Hydrolyse)			
	Molver- hältnis	µMol je mg	Molver- hältnis	µgN je mg	µMol je mg	Molver- hältnis	µgN je mg
Lys	4.75	0.868	2.86	24.3	0.383	0.96	10.7
His	2.12	...	—	—	0.362	0.91	15.2
Arg	1.89	0.591	1.95	33.1	0.806	2.02	45.2
Thr		0.482	1.59 ^b	6.7	0.385	0.97	5.4
Ser	(0.15)	0.193	0.64 ^b	2.7	(0.040)	(0.01)	0.6
Glu		0.688	2.26	9.6	1.270	3.18	17.8
Pro	1.15	0.304	1.00	4.3	0.389	0.98	5.4
Gly	1.10	0.860	2.84	12.0	—	—	—
Ala	—	0.575	1.90	8.1	1.154	2.90	16.2
Val	0.95	0.689	2.28	9.6	—	—	—
Ileu	4.04	0.847	2.79	11.9	—	—	—
Leu	—	1.140	3.70	15.6	0.417	1.05	5.8
Asp	2.05	—	—	—	0.395	0.99	5.5
1/2 Cys	3.56	...	—	—	1.570	3.94	22.0
Met	—	—	—	—	—	—	—
NH ₃		1.1	3.7 ^d	15.4	2.1	5.1 ^d	29.4
Try ^a			1.11	8.7	—	—	—
Aminoacyle (Gewichts- prozent)		85.9			85.7		
Aminoacyle/Mol	22	26		18			
Minimales MW	2593	2840		2036			
% HCl		6.3		6.8			
% Stickstoff		16.30		18.24			
% Glührück- stand		< 1		1.4			
Ausbeute ^c		99.4		98.3			

^a Aus UV-Absorption.^b Mit der Hydrolysezeit zunehmende Verluste.^c Prozent des eingesetzten Stickstoffs.^d Nicht zur Errechnung des Amid-Stickstoffs verwertbar, da zum Teil durch Verunreinigung mit Ammonchlorid bedingt.

Befunde von HAHN und LEDITSCHKE (1937) lassen sich heute so deuten, daß Melittin und Phospholipase A durch Dialysiermembranen wesentlich stärker zurückgehalten werden als das kleinere Apaminmolekül.

Melittin besteht aus 26 Aminoacylen, deren Relation aus Tabelle 2 hervorgeht. Entsprechend seinem im Alkalischen liegenden Isoelektrischen Punkt stehen fünf basische Aminosäuren (3 Lysine, 2 Arginine) zwei sauren Bausteinen gegenüber (HABERMANN und REIZ, 1965 b); überdies sind, wie die Strukturaufklärung zeigt, sämtliche Carboxylgruppen amidartig verschlossen. Schwefelhaltige Aminosäuren fehlen, ebenso Tyrosin, Phenylalanin und Histi-

din. Die gleiche Aminosäurezusammensetzung wurde später auch von KREIL (1965) mitgeteilt. Das aus der Bausteinanalyse errechnete minimale Molekulargewicht von 2840 entspricht dem tatsächlichen. Das ließ sich auf chemischem Wege nachweisen, und zwar durch quantitative Bestimmung des N-terminalen Glycins nach Überführung in das PTH-Derivat. Physikalische Verfahren hatten anomale Werte für die Molekülgröße geliefert, die erst nachträglich, nach der kompletten Strukturaufklärung, verstanden wurden. Man sollte z.B. auf Grund der Bausteinanalyse annehmen, daß das Peptid gut dialysabel sei, was aber nicht der Fall ist. Bei Gelfiltration von Gesamtgift an Sephadex G 50 erscheint die Melittinfaktion als relativ breiter Gipfel, der in Abhängigkeit von Belastung und Beimischung ein einziges Maximum oder deren zwei aufweisen kann. Die Eluierbarkeit ist außerdem konzentrationsabhängig. An mit Proteinen bzw. Peptiden bekannten Molekulargewichts vorgeeichten Säulen verhält sich Melittin wie eine Substanz mit einem apparenten Molekulargewicht um 12000, wenn man große Mengen aufträgt; kleinere Mengen werden stärker retiniert (HABERMANN und REIZ, 1965 b). Legt man das auf rein chemischem Wege ermittelte Molekulargewicht als richtig zugrunde und bedenkt man, daß Quervernetzungen über SS-Brücken entfallen und auch die OH-Gruppen der Serine und Threonine sowie die ϵ -Aminogruppen der Lysine frei sind, so bleibt nur der Schluß, daß Melittin nicht molekulardispers, sondern in Form von Micellen vorliegt. Das bei Gelfiltration ermittelte scheinbare Molekulargewicht gibt demnach ein „Micellar-Gewicht“ an, wobei eine Micelle aus mindestens vier Melittinmolekülen zu bestehen hätte.

Auch die von FISCHER und NEUMANN (1961) beschriebene Inhomogenität des Melittins bei Tiselius-Elektrophorese möchten wir aus der unterschiedlichen Größe der Einzelmicellen erklären, ebenso die von den Versuchsbedingungen abhängigen Anomalien bei Gelfiltration im Verband von Gesamtgift (HABERMANN und REIZ, 1965 a, b).

Hinweise auf die Micellarstruktur von Melittinlösungen wurden schon früher erhalten. Melittin erniedrigt die Oberflächenspannung wäßriger Lösungen sehr viel stärker als andere Peptide oder Proteine; bezüglich der erforderlichen Dosen entspricht es etwa dem Lysolecithin oder Digitonin (HABERMANN, 1958b; HABERMANN und REIZ, 1965 b). Da nun Melittin stark oberflächenaktiv, stark basisch und (wohl aus beiden Gründen) sehr stark an saure Gruppen, wie Ionenaustauscher (HABERMANN, 1954a) und sogar Sephadex G 25 (HABERMANN und REIZ, 1965a) absorbierbar ist, bezeichneten wir es als Invertseife auf Peptidbasis (HABERMANN und JENTSCH, 1966). Die Strukturaufklärung (HABERMANN und JENTSCH, 1966, 1967) zeigte, daß die Invertseifenstruktur sich in der Aminosäure-Folge wiederspiegelt (Abb. 4). Es läßt sich bei dieser Schreibweise besonders gut erkennen, daß die basischen, hydrophilen Aminosäuren (bis auf ein Lysin) nahe am C-terminalen Ende aufgereiht sind, während sich zum N-Terminus hin ein stark hydrophober Kettenanteil erstreckt.

Nun ist aus der Proteinchemie bekannt und aus physikochemischen Gründen zu fordern, daß sich die hydrophilen Aminosäuren an der Proteinoberfläche anordnen, die hydrophoben aber im Innern globulärer Proteine. Daraus geht hervor, daß — je kleiner eine Peptidkette ist — desto größer das Verhältnis der polaren zu den nichtpolaren Aminosäuren sein muß, wenn eine geordnete globuläre Struktur gewährleistet sein soll. Beim Melittin reichen die vorhandenen, exzentrisch angeordneten hydrophilen Aminosäuren sicher nicht zu einer kompletten Umhüllung; es werden sich die Moleküle zu Micellen in der bei Seifen bekannten Weise zusammenlegen müssen, um in den thermodynamisch wahrscheinlichsten Zustand zu gelangen.

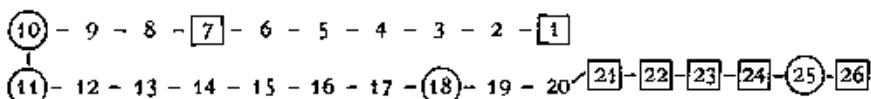


Abb. 4. Anordnung der hydrophoben, hydrophilen neutralen und basischen Aminosäuren im Melittin.
□ hydrophile basische Reste; ○ hydrophile neutrale Reste

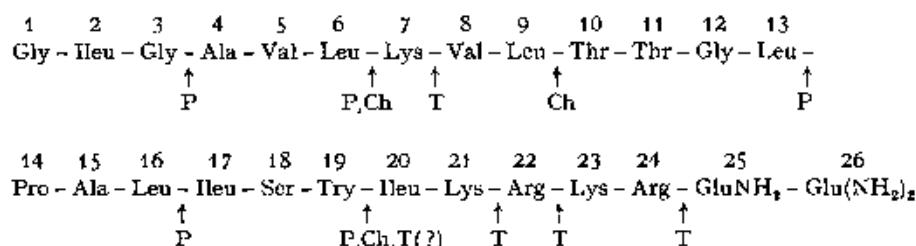


Abb. 5. Primärstruktur des Melittins und Angriffspunkte der Enzyme (nach HABERMANN und JENTSCH, 1966; chymotryptische Angriffspunkte nach HABERMANN und EGGS (unveröffentlicht)). T Trypsin; Ch Chymotrypsin; P Pepsin

Für die Strukturaufklärung des Melittins reichten die klassischen Verfahren der Peptidanalytik aus. Mittels Edman-Abbaus wurde Glycin als einzige N-terminale Aminosäure identifiziert und quantitativ bestimmt (HABERMANN und REIZ, 1965 b), ferner die Sequenz bis in Position 9 ermittelt. Die Carboxylseite des Peptids erwies sich als resistent gegen Carboxypeptidase A und B; auch nach Hydrazinolyse fand man keine freie Aminosäure. Beides deutet auf einen amidartigen Verschluß hin. Tryptische, peptische und chymotryptische Spaltstücke des Melittins wurden durch Kombination von Ionenaustausch an phosphorylierter Cellulose und Papierchromatographie getrennt und ihre Struktur durch Bausteinanalyse, Abbau nach EDMAN, Hydrazinolyse sowie mit Carboxypeptidase A und weiteren Enzymen bestimmt. Kombination der Fragmente lieferte den Aufbau des Gesamtpeptids (HABERMANN und JENTSCH, 1966, 1967; EGGS, 1958) (Abb. 5).

Melittin ist zum Teil N-substituiert. Das zeigte sich, als neben dem tryptischen, schwach basischen N-terminalen Heptapeptid gly-ileu-gly-al-a-val-leu ein neutrales Analogon gefunden wurde, das beim 1. Schritt des Edman-Abbaus keine PTH-Aminosäure lieferte. Der damals mit x bezeichnete Substituent (HABERMANN und JENTSCH 1966,

1967) wurde inzwischen als Formylrest identifiziert (KREIL und KREIL-KISS, 1967). Etwa 10% des gesamten Melittins sind in dieser Weise N-terminal blockiert. Dadurch wird der Invertseifen-Charakter des Melittins weiter verstärkt. — In multicellulären Organismen wurden bisher keine N-terminal formylierten Peptide gefunden.

b) Biochemische und pharmakologische Wirkungen

Gerade beim Melittin zeigt sich besonders deutlich, daß biochemische und pharmakologische Effekte nicht zu trennen sind; beruhen doch beide auf den physikochemischen Phänomenen der Oberflächenaktivität und der Basizität, die sich auf die Aminosäuresequenz zurückführen lassen. Es ist eine Frage der Definition, ob man die Hämolyse als pharmakologische, die Beeinflussung der Mitochondrienfunktion dagegen als biochemische Wirkung einstuft. Nach wie vor stellt das hämolytische Vermögen des Melittins das beste Modell zum Verständnis seines Wirkungsmechanismus dar, so daß mit seiner Besprechung begonnen werden soll.

a) *Hämolyse*. Schädigt man das Melittin-Molekül durch UV-Bestrahlung, so ändert sich nicht nur das typische, auf dem Tryptophangehalt beruhende Spektrum, sondern auch sein hämolytisches Vermögen nimmt dabei ab (HABERMANN, 1955a, 1958a). Man sollte daraus jedoch nicht den Schluß ziehen, daß die Intaktheit des Tryptophanrestes für das hämolytische Vermögen unerlässlich sei. Substituiert man am Pyrrolring mit 2-Hydroxy-5-Nitro-Benzylbromid, so nimmt die hämolytische Aktivität zu. Durch sichtbares Licht wird der Pyrrolring des Tryptophans zerstört, was sich durch die Veränderung des UV-Spektrums und den negativen Ausfall der Ehrlichschen Reaktion manifestiert. Das hämolytische Vermögen sinkt jedoch erst dann in beträchtlichem Maße ab, wenn nicht nur der Pyrrolring, sondern weitere bei 220 m μ absorbierende Molekülregionen geschädigt werden (HABERMANN, unveröffentlicht). Die hämolytische Potenz von Melittinfragmenten und eines Melittin-Analogen wird S. 250 diskutiert.

Melittin gehört zu den „direkt“ wirkenden Lysinen, d.h. es vermag gewaschene Erythrocyten zu hämolsieren. Ihm läßt sich Phospholipase A als zweites, jedoch „indirekt“ wirkendes Hämolysin aus Bienengift gegenüberstellen; dieses Enzym führt bekanntlich extracelluläres Lecithin in Lysolecithin über, das seinerseits die Hämolyse bewirkt. Die Wirkungsweise des Melittins läßt sich am besten verstehen, wenn man sie mit derjenigen des Lysolecithins und des Digitonins konfrontiert (HABERMANN, 1955a, 1958a).

Die Dosis-Wirkungs-Kurve ist für Lysolecithin steiler als für Melittin; die Hämolyse durch Bienengesamtgift zeichnet sich durch eine extrem steile Dosis-Wirkungs-Beziehung aus (Abb. 6). Zusatz von gereinigter Phospholipase A verstärkt die Melittinhämolyse erheblich; es ist also mit sehr großer Wahrscheinlichkeit anzunehmen, daß die andersartige Kurve des Gesamtgifts aus der Kombination von Melittin und Phospholipase A resultiert (Abb. 7). Phospholipase A greift nicht das Lecithin intakter roter Blutzellen an (mit Aus-

nahme der Korpuskeln des Meerschweinchens); durch Melittin vorgeschädigte Erythrocyten sind dagegen für das Enzym zugänglich. Das dabei entstehende Lysolecithin bringt erneut Zellen zur Hämolyse und verschafft dem Enzym weiteres Substrat, wodurch die Reaktion lawinenartig anschwillt. Dazu kommt

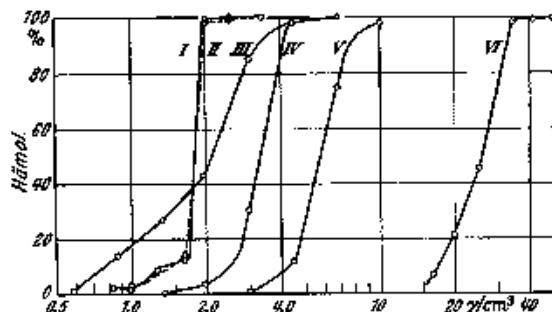


Abb. 6. Vergleich der Dosis-Wirkungs-Kurven verschiedener Hämolytica (HABERMANN, 1958a). Ordinate: % Hämolyse (gewaschene menschliche Erythrocyten). Abszisse: μg Hämolsin/ml Ansatz. I natives, II gereinigtes Bienengift; III Melittin; IV Quartamon; V Zephirol; VI Naja-nigricollis-Gift. Man beachte die auffallend steile Dosis-Wirkungs-Kurve von Gesamtgift im Vergleich zur sehr flachen Beziehung beim Melittin.

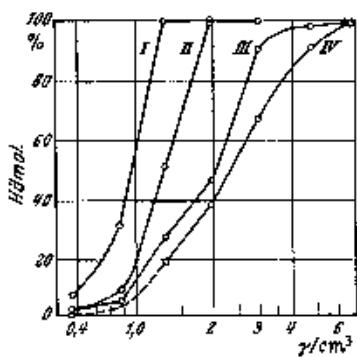


Abb. 7

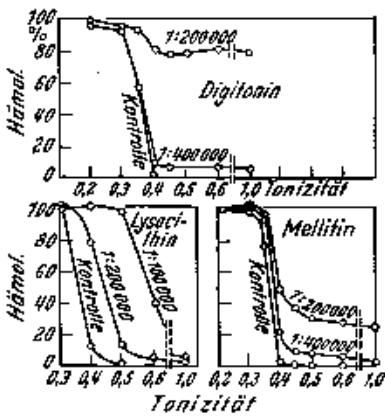


Abb. 8

Abb. 8. Osmotische Resistenz von mit oberflächenaktiven Hämolyseinen vorbehandelten Erythrocyten (HABERMANN, 1958a). Ordinate: % Hämolyse. Abszisse: Tonizität im sekundären Ansatz. Beschriftung der Hämolysekurven: Lysinkonzentration im primären Ansatz

möglicherweise ein zweiter kombinatorischer Effekt von Melittin und Phospholipase A: das Polypeptid begünstigt, wie andere oberflächenaktive Substanzen, die enzymatische Spaltung von gereinigtem Lecithin (HABERMANN, 1957b).

Die Geschwindigkeit der Melittinhämolyse steigt mit der Temperatur, der Endwert ist bei 37°C bereits binnen weniger Minuten nahezu erreicht; er ist im Bereich zwischen 20 und 40°C relativ wenig temperaturabhängig (HABERMANN, 1958a). Im Gegensatz dazu nimmt das hämolytische Vermögen von Lysolecithin (COLLIER, 1952) und Digitonin mit sinkender Temperatur

zu, wenn auch der Ablauf der Hämolyse langsamer wird. Die Ursache für diesen Unterschied ist nicht bekannt; vielleicht ist wichtig, daß Digitonin und Lysolecithin mit Lipoiden der Zellwand reagieren, während Melittin Bindungen mit sauren Bestandteilen eingeht. Eine andere Deutung der größeren Lysolecithinempfindlichkeit bei tiefer Temperatur wird S. 270 diskutiert; sie läßt allerdings außer acht, daß die Digitoninempfindlichkeit sich in analoger Weise ändert. Der — etwa im Verhältnis zu der enzymatischen Hämolyse durch α - und β -Toxin von Gasbrandgift — relativ schnelle Ablauf der Melittinhämolyse spricht für eine in kurzer Zeit komplettete Reaktion mit der Zellwand. Das hämolytische Vermögen nimmt mit dem pH-Wert zwischen 5,4 und 9 zunächst steil, dann langsam zu; der Befund kann dahingehend gedeutet werden, daß Melittin als freie Base maximal wirksam ist, ähnlich wie etwa lokalanaesthetische oder antibiotische Eigenschaften zahlreicher Verbindungen mit dem Dissoziationsgrad abnehmen (HABERMANN, 1958a). Das pH-Optimum der Lysolecithinhämolyse liegt demgegenüber bei pH 6 (WILBUR und COLLIER, 1943; COLLIER, 1952).

Bei der Prüfung der osmotischen Einflüsse wurden scheinbar widersprüchliche Resultate erhalten. Das hämolytische Vermögen von Melittin sinkt mit steigender Tonizität des Inkubationsmediums; dieses Phänomen ist bei Lysolecithin schwächer, bei Digitonin nicht eindeutig ausgeprägt. Inkubiert man dagegen zunächst in isotonem Milieu und prüft dann die osmotische Resistenz der Erythrocyten, so findet man nach Digitonin keine, nach Melittin nur eine schwache, nach Lysolecithin dagegen eine ausgeprägte Resistenzminderung. Eine Deutung der Relationen zwischen osmotischem Druck und hämolytischem Vermögen könnte davon ausgehen, daß die Reaktion zwischen Melittin und der Erythrocytenwand, ähnlich wie die Bindung von Proteinen und Peptiden an Ionenaustauschern, durch Erniedrigung der Ionenstärke begünstigt wird; die der Bindung folgende Schädigung der Zellwand ist dagegen weitgehend von der Tonizität des Mediums unabhängig. Die Melittinhämolyse hat also im Sinne WILBRANDT's (1942) eine starke „nicht-osmotische“ Komponente. Die Lysolecithinhämolyse gehört dem „osmotischen“ Typ an (COLLIER, 1952) (Abb. 8).

Bei der Hämolyse wird Melittin (wie auch Lysolecithin oder Digitonin) „verbraucht“; die drei Hämolytica lysieren — läßt man sie in submaximalen Konzentrationen auf mehrere, sukzessiv zugegebene Zellmengen einwirken — nur die erste Portion, nicht die späteren. Lysolecithin und Digitonin werden an intakte menschliche Zellen adsorbiert und dadurch inaktiviert, wie Versuche mit Variation der Zellkonzentration zeigen. Beim Melittin ist dieser Effekt weniger eindeutig; doch steigt die durch eine gegebene Hämolysinkonzentration freigesetzte Hämoglobinmenge nur relativ gering an, wenn man die Zellkonzentration erhöht. Nun ist die Dosis-Wirkungs-Beziehung des Melittins außerordentlich flach. Es ist also die Annahme naheliegend, daß die

einzelnen Erythrocyten gegen Melittin sehr unterschiedlich empfindlich sind und infolgedessen bei Erhöhung der Zellzahl zwei Prozesse einander entgegenlaufen: Die Zunahme der absoluten Menge besonders empfindlicher Erythrocyten und die Inaktivierung des Melittins durch Bindung an relativ resistente Zellen. — Durch das gewaschene Sediment osmotisch hämolysierter Erythrocyten wird die Melittinhämolyse deutlich gehemmt; das Überstehende ist in dieser Hinsicht ohne eindeutigen Einfluß. Die Lysolecithinhämolyse wird durch das Sediment, wahrscheinlich seinen Cholesterinanteil (s. S. 237), ebenfalls inhibiert; die durch Gesamtgift hervorgerufene Hämolyse wird dagegen erheblich gefördert, wohl durch Überführung des zelleigenen Lecithins in Lysolecithin (HABERMANN, 1958a).

Das hämolytische Vermögen eignet sich besonders gut dazu, um Inhibitoren des Melittins und Lysolecithins zu studieren. Tests mit komplexen Systemen sind in dieser Hinsicht mehrdeutig; Antihistaminica und Serotoninantagonisten hemmen z.B. nicht den direkten Melittineffekt an der Haut, sondern die durch das Peptid freigesetzten körpereigenen Wirkstoffe (s. S. 240). Die Melittinhämolyse kann durch Faktoren beeinflußt werden, die an Erythrocyten angreifen; hierzu zählt die Variation des osmotischen Drucks oder der Einfluß von Schwermetallsalzen. Sie läßt sich aber auch durch Reaktionspartner des Melittins abschwächen. Entsprechend der basischen Natur des Polypeptids kommen hierfür vor allem höhernukleare, saure Substanzen in Frage. Wie Protaminsulfat, so reagiert auch Melittin mit Heparin und Heparinoiden (z.B. Thrombocid); seine Wirksamkeit nimmt dabei ab. Dies gilt nicht nur für sein hämolytisches Vermögen (HABERMANN, 1958a), sondern auch für alle übrigen untersuchten Effekte, etwa die Kontraktur von Herz- und Skelettmuskulatur des Frosches und die Auslösung von Schmerz und Entzündung bei lokaler Applikation am Kaninchenauge (SCHLÖGL, 1953). Lecithin, das im neutralen pH-Bereich anionisch vorliegt, dürfte nach dem gleichen Prinzip hemmen. Worauf die kräftige Inaktivierung durch Blutplasma beruht, wäre noch zu klären. Neben Lecithin und anderen sauren Lipoiden — Ganglioside hemmen z.B. ebenfalls erheblich (HABERMANN, unveröffentlicht) — kämen plasmaeigene saure Glykoproteine als Reaktionspartner des Melittins in Frage. Zerkleinertes Gewebe, z.B. Froschmuskulatur, inhibiert die Melittinhämolyse wie auch seine Muskelwirkung und seine Reizwirkung am Kaninchenauge. Die Natur der dabei eintretenden Bindung ist noch unbekannt; wahrscheinlich spielen auch hier saure Komponenten eine Rolle (SCHLÖGL, 1953). Dafür wäre die außerordentlich feste, praktisch irreversible Bindung des Melittins an manche saure Ionenaustauscher, z.B. Amberlite CG 50 (HABERMANN und W. P. NEUMANN, 1957), ein Modell.

Das Inhibitorspektrum von Melittin differiert, wie nicht anders zu erwarten, grundsätzlich von dem des Lysolecithins und des Digitonins. Diese beiden Hämolytica werden — im Gegensatz zu Melittin — durch Cholesterinzusatz

gehemmt; mit Digitonin bildet sich der bekannte, spezifische Komplex, während Lysolecithin infolge seiner Eigenschaften als Lösungsvermittler unspezifisch mit Cholesterin reagiert. So ist wohl auch die Hemmbarkeit von Lysolecithin durch Lecithinzusatz zu verstehen, mit dem sich Digitonin bekanntlich nicht verbindet (HABERMANN, 1958a).

Andere Hemmstoffe der Melittinhämolyse setzen die Reaktionsfähigkeit der Erythrocytenwand nicht nur für Melittin, sondern auch für weitere Hämolytica herab. So hemmt 0,001 M Zinkchlorid die Melittin- und Lysolecithinhämolyse sehr stark, während 0,0001 M Zinkchlorid eher etwas fördert. Ähnliches mag für Citrat gelten, das ebenfalls Melittinhämolyse (HABERMANN,

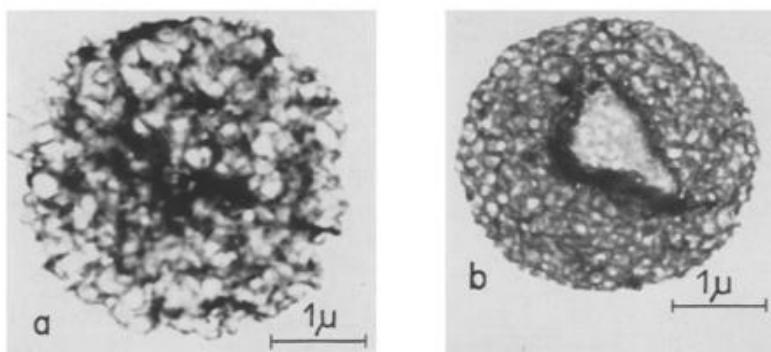


Abb. 9. a Melittinhämolyse (1:10000) eines menschlichen Erythrocyten (HABERMANN und MÖLBERT, 1954). b Stroma eines durch Hypotonie hämolysierten Erythrocyten, der nach dreimaliger Waschung mit Wasser in eine BienenGiftlösung 1:10000 gebracht wurde

1958a) und Lysolecithinhämolyse beeinflußt. Für letztere wurde bereits eine Änderung des Erythrocytenvolumens als Ursache der Citratwirkung diskutiert (WILBUR u. COLLIER, 1943). Auch bei der Citratwirkung auf die Melittinhämolyse ist die Endkonzentration des Anions im erythrocytenhaltigen Ansatz und nicht bei der Vorinkubation mit Melittin entscheidend.

Eine Sonderstellung nimmt die Hemmung der Melittinhämolyse durch Thioglykolsäure ein. Melittin ist schwefelfrei; Cystein, KCN, Sulfid haben keinen Einfluß. Eine Reaktion mit Disulfidbrücken des Hämolsins entfällt also, desgleichen eine unspezifische Veränderung der Zellwand; denn die Lysolecithin- oder Digitoninhämolyse wird nicht modifiziert. Nachdem bei der Mischung von Thioglykolsäure mit Melittin eine deutliche Trübung eintritt, ähnlich wie bei Vereinigung mit dem sulfurierten Polysaccharid Thrombocid, nehmen wir einen analogen Inaktivierungsmechanismus durch Bindung zwischen dem basischen Hämolin und der Säure an (HABERMANN, 1958a).

Das licht- und elektronenmikroskopische Bild (Abb. 9) der durch Melittin bzw. Lysolecithin hämolysierten Zellen paßt vorzüglich zu den bereits aufgeführten kinetischen bzw. biochemischen Daten. Behandelt man Erythrocyten oder durch osmotische Hämolyse gewonnene Schatten mit hohen Melittinkonzentrationen (um 0,1 mg/ml), so schrumpfen sie stark; ihre Hülle wird starr und bekommt eine netzartige Struktur. Dünnschnitte zeigen, daß ihr Inneres leer ist. Insgesamt erscheint die Zellwand stark denaturiert. Ganz

anders sind die Veränderungen nach Lysolecithin: Die Schatten werden — entsprechend den solubilisierenden Fähigkeiten dieses Hämolsins — überaus zart. Bei ihrer Präparation aus konzentrierteren ($> 0,1 \text{ mg/ml}$) Lysolecithin-Lösungen lösen sie sich auf. Es sind nur noch unbedeutende Reste unter dem Elektronenmikroskop sichtbar (HABERMANN und MÖLBERT, 1954).

Unter dem Einfluß verschiedener Hämolytica kommt es zu prolytischem Kaliumverlust, d.h. der prozentuale Austritt des niedermolekularen Kaliums geht dem des hochmolekularen Hämoglobins voraus. Der prolytische Kaliumverlust nimmt in der Reihe ab: Melittin $>$ Lysolecithin $>$ Digitonin. Abhängigkeit der Hämolyse vom osmotischen Milieu, Hämolysetyp und prolytischer Kaliumverlust gehen also nicht parallel (Tabelle 3). Beziehungen zwischen

Tabelle 3. Fehlende Beziehung zwischen Hämolysetyp, prolytischem Kaliumverlust und Abhängigkeit vom osmotischen Milieu (Nach HABERMANN, 1955a, 1958a)

	Melittin	Lysolecithin	Digitonin
Hämolyse-Typ*	nicht osmotisch	osmotisch	nicht osmotisch
Abhängigkeit vom osmotischen Milieu	deutlich	sehr gering	keine
Prolytischer Kaliumverlust	stark	gering	angedeutet
Hemmbarkeit durch Lecithin	deutlich	deutlich	keine
Hemmbarkeit durch Cholesterin	keine	stark	stark

* In Anlehnung an die Methodik von WILBRANDT (1942) bestimmt.

hämolytischem Vermögen und Stoffwechsel-Beeinflussung wurden nicht gefunden. Melittin und Lysolecithin hemmen z.B. nicht die Erythrocyten-Cholinesterase; die Glucose-Oxydation in Gegenwart von Methylenblau wird erst in lytischen Konzentrationen beeinflußt (HABERMANN, 1958a).

Die Melittinhämolyse ließ sich an dem Modell der Lipid-Sphärule imitieren (SESSA, WEISSMANN *et al.*, 1968), die man aus Ovolecithin (70 %) und Cholesterin (10 %) unter Zusatz von 20 % Dicetylphosphat (anionisch) oder Stearylamin (kationisch) herstellen kann. Melittin erhöhte die Permeabilität beider Präparationen beträchtlich in Konzentrationen, wie sie auch zur Hämolyse benötigt werden. Die äußere Lipidhülle zeigte elektronenoptisch „beading“ und schließlich Kraterbildung. Melittin kann also mit protein- und mucoid-freien Lipidmembranen reagieren.

Man könnte nach der massiven Erythrocytenschädigung *in vitro* annehmen, daß Melittin *in vivo* ebenfalls hämolsiert und vielleicht gerade dadurch den Tod der Versuchstiere hervorruft. Dies ist jedoch — zum mindest bei Kaninchen — nicht der Fall. In subletalen Dosen (1—2 mg/kg) sieht man einen deutlichen Anstieg des Hämatokrits, der, da er nicht von einer Zunahme des Hämoglobins begleitet ist, auf eine Zellschwellung hinweist. Das Plasmakalium bleibt normal, das extracelluläre Hämoglobin an der Grenze der Meßbarkeit. Bei einem durch Melittin (4 mg/kg) binnen 15 min

getöteten Tier war allerdings eine geringe Hämolyse eingetreten, die sich in einer Erhöhung des Plasmakaliums von 22 auf 33 mg-% und einer Zunahme des extracellulären Hämoglobins auf 1 g-% äußerte; doch ist nicht sicher, ob diese Verschiebungen bereits intravital oder erst beim Abzentrifugieren des Plasmas eingetreten waren (STOCKEBRAND, 1965).

β) *Freilegung pharmakologisch aktiver Substanzen.* Melittin ist ein universell zellschädigendes Agens, ebenso Lysolecithin. So verwundert es nicht, daß beide Wirkstoffe cellulär fixierte pharmakologisch aktive Substanzen freizulegen vermögen. Der *Kaliumverlust* bei der Hämolyse war bereits ein Beispiel in diesem Sinne. Isolierte Skelettmuskulatur des Frosches gibt ebenfalls Kalium unter dem Einfluß von Melittin ins Nährmedium ab (HABERMANN, unveröffentlicht); damit dürfte die Erniedrigung ihres Ruhepotentials (HEYDENREICH, 1957) im Zusammenhang stehen. Erregung mit anschließender Schädigung isolierter Organe ist eine regelmäßige Folge der Einwirkung von Melittin; beides wird an Skelet- und Herzmuskulatur, am Meerschweincheneileum, am Phrenicus-Zwerchfell-Präparat, am isoliert durchströmten Ganglion cervicale superius beobachtet. Man ist versucht, diese Erscheinungen auf allmählichen Schwund des Ruhepotentials durch Erhöhung der Natriumpermeabilität zu beziehen; doch wären zur Sicherung der Hypothese weitere Versuche erforderlich. Das gleiche gilt für die wahrscheinliche, aber noch nicht gesicherte Beziehung zwischen Kaliumverlust und Schmerzerzeugung. — In vivo wurde das Membranpotential der Froschmuskulatur selbst durch extreme Bienengiftdosen (100 mg/kg) nicht erniedrigt.

Melittin zerstört Mastzellen im isolierten Mesenterialstück der Ratte (HABERMANN, unveröffentlicht; KACHLER, 1958). Aus dem isolierten Rattenzwerchfell setzt es, ebenso wie Phospholipase A und eine Reihe weiterer Komponenten tierischer Gifte, histaminähnliche Substanz frei (STRIEBECK, 1958). Gewaschene Peritonealmastzellen der Ratte geben ihr *Histamin* unter dem Einfluß von Melittin (nicht von Phospholipase A!) ab. Lysolecithin schädigt sie ebenfalls. Dinitrophenol hemmt zwar die Histaminfreisetzung durch Compound 48/80, nicht aber die durch Melittin oder Lysolecithin. Ein derartiger Hemmeffekt ist in Anbetracht des auf dem Tensidcharakter beruhenden „direkten“ Angriffs der beiden Lysine an der Zellwand auch nicht zu erwarten. — Der Effekt von Phospholipase A ist offenbar indirekter Art. Nur wenn extracelluläres Substrat zur Verfügung steht, z.B. in Stücken isolierter Rattenhaut, kann sie Mastzellen zerstören (ROTHSCHILD, 1965). Analog wie bei der Hämolyse ist also ein kombinierter Effekt von Melittin und Phospholipase A zu erwarten: Melittin stellt unter anderem durch Mastzellen-Zerstörung Substrat für das Enzym bereit, das dann wiederum in das cytolytische Lysolecithin transformiert werden könnte. — Die *in vitro*-Freisetzung von *Serotonin* läßt sich an Thrombocyten-Präparaten studieren. Auch hier ist Melittin im Vergleich zu Phospholipase A hochwirksam (HABERMANN und

SPRINGER, 1958; MARKWARDT u. Mitarb., 1966). — FREDHOLM (1966) hat inzwischen die Uvnässche Hypothese revidiert, nach der Phospholipase die Mastzellen zerstören sollte. Bei Gelfiltration des von dieser Arbeitsgruppe verwendeten Präparates aus Bienengift erschien zunächst Phospholipase, dann erst der mastzellzerstörende Faktor im Eluat. Sein Molekulargewicht wird auf

Tabelle 4. Einfluß von Histamin- und Serotonin-Antagonisten auf die Erhöhung der Capillarpermeabilität der Rattenhaut für Evans-Blau. (Nach ROTHSCHILD, 1965)

Permeabilitätserhöhendes Agens	ohne Vorbehandlung	nach Vorbehandlung mit		
		Diphenhydratamin (25 mg/kg)	BOL 148 (2 mg/kg)	Diphenhydramin + BOL 148
Phospholipase A (1 µg)	++++	+++	+ + +	—
	++ + +	++	++	—
	+ + +	+ ·	· ·	· ·
	+ + - +			—
Melittin (1 µg)	+++	++	++	—
	+++	+	+	—
	+ + + +	++	++	—
	+ + +			—
Compound 48/80 (1 µg)	+ + + +			—
	+ + +	+++	++	—
	+ + + +	+	++	+
	+ + +	++		—
Kochsalzlösung (0,05 ml)	±	±	—	—
	—	—	—	—
	+	—		—
	—			—

Blaufärbung (— +)

1000--5000 geschätzt; doch soll er nicht mit Melittin identisch sein, da er weder Thrombocyten noch Erythrocyten lysiert und durch Dinitrophenol hemmbar ist. Wie S. 252 näher diskutiert, handelt es sich um eine der F₀-Komponenten, die als zweites „direkt“ mastzelldegranulierendes Prinzip (MCD-Peptid) anzusprechen ist.

Offenbar spielt die Freisetzung von Histamin und Serotonin auch *in vivo* eine wesentliche Rolle. ROTHSCHILD (1965) applizierte Melittin bzw. Phospholipase A in kleinen Mengen (1 µg) bei Ratten intracutan und prüfte den lokalen Austritt von zirkulierendem Evans-Blau. Beide Pharmaka erhöhten die Gefäßdurchlässigkeit. Vorherige Gaben des Antihistaminicums Diphenhydramin¹ (25 mg/kg) bzw. des Serotonin-Antagonisten Bromlysergsäurediäethylamid (2 mg/kg) minderten die melittin- bzw. enzymbedingte Gefäßdurchlässigkeit; Kombination der beiden Antagonisten hob sie auf

¹ 2-Benzhydryloxy-N,N-dimethyläethylamin.

(Tabelle 4). Dabei ist allerdings in Rechnung zu stellen, daß nur kleine Dosen der Giftkomponenten appliziert wurden. Höhere Dosen dürften auch auf direktem Wege die Gefäße schädigen.

Der lebhafte Schmerz nach einem Bienenstich ist nur zum Teil durch freigesetztes oder im Gift vorhandenes Histamin bedingt. **GESKE** und **JUNG** (1950) konnten durch vorhergehende intravenöse Injektion von Neoantergan² zwar die Hauttemperatur in der Stichregion senken, nicht aber die übrigen Lokalsymptome beeinflussen. Andererseits besteht bei den Betroffenen kein Zweifel, daß der Stichschmerz durch lokale Applikation eines antihistaminhaltigen Präparates gemindert werden kann.

Ob Melittin, MCD-Peptid, Phospholipase A einzeln oder in Kombination für die Histaminfreisetzung aus isoliert durchströmten Organen (Lunge von Meerschweinchen und Hund; Leber des Hundes) (**FELDBERG** und **KELLAWAY**, 1937) sowie für die Adrenalinfreisetzung (**FELDBERG**, 1940) verantwortlich sind, müßte vergleichend untersucht werden. Melittin wie auch Phospholipase A lassen aus dem isolierten Zwerchfell histaminähnliche Substanzen austreten (**STRIEBECK**, 1958) und sind zur Mastzellzerstörung in Gewebsstücken imstande, z. B. im isolierten Rattenmesenterium (**KACHLER**, 1958; **BREITHAUPP**, 1968).

Beim Cobragift scheinen analoge Verhältnisse vorzuliegen wie beim Bienengift. Man kann einen dialysablen Anteil, der basische Peptide, z. B. Neurotoxin, enthält, von einem hochmolekularen Anteil unterscheiden, dem auch die Phospholipase-Aktivität zukommt. Beide Fraktionen setzen *in vivo* und *in vitro* Histamin frei; doch ist der hochmolekulare Anteil der wirksamere (**MAY et al.**, 1967).

Erwartungsgemäß werden auch Leukocyten durch Melittin angegriffen. Noch 10^{-7} M Peptidlösung degranuliert Peritoneal-Leukocyten unter Verlust ihrer lysosomalen Struktur (**SESSA et al.**, 1968).

γ) Glatte Muskulatur. Bei der Wirkung von Melittin auf glatte Muskulatur können drei verschiedene Prozesse unterschieden werden. Im Prinzip wurden sie bereits von **FELDBERG** und **KELLAWAY** (1937) für Gesamtgift beschrieben.

a) Sehr kleine Dosen scheinen nicht direkt, sondern durch Aktivierung organeigener Mechanismen zu stimulieren. Atropin kann z. B. die melittinbedingte Kontraktion des isolierten Meerschweinchenileums abschwächen.

b) Kontraktionen nach höheren Dosen sind dagegen atropinresistent; sie können durch Papaverin oder Novocain unterbrochen werden (**ABEL**, 1956). Antihistaminica sind in dieser Hinsicht nicht eindeutig wirksam. Höhere Dosen schädigen das Organ; die Kontraktion löst sich nur langsam.

c) Im Endzustand ist das Organ gegen eine Wiederholung der Melittindosis und auch gegen andere Stimulatoren, etwa Histamin oder Acetylcholin, resistent.

Stets ist der Ablauf der Melittinkontraktion langsamer als etwa eine Erregung durch Histamin. Man könnte also Melittin zu den „slow reacting substances“ zählen; doch fehlt bei „typischen“ SRS, etwa Bradykinin oder Substanz P, die schädigende Komponente. Phospholipase A ist noch stärker tachyphylaktogen als Melittin (**HABERMANN**, 1957a).

² N,N-Dimethyl-N'-(p-methoxybenzyl)-N'-(2-Pyridyl)-äthyldiamin.

Einige Nebenbeobachtungen sprechen für einen glattmuskulären Angriff auch *in vivo*. So bringt intravenös injiziertes Bienengift — wahrscheinlich durch seinen Melittingehalt — die Nickhaut der Katze zur Kontraktion; der Effekt tritt auch nach Durchschneidung des Halssympathicus ein und wird durch vorherige Applikation des Antihistaminicums Avil⁸ nicht abgeschwächt, sondern eher verstärkt. Außerdem sensibilisiert intravenös gegebenes Bienengift die Nickhaut für den elektrischen Reiz (v. BRUCHHAUSEN, 1955). Es wäre aber noch zu prüfen, ob die genannten Effekte von Gesamtgift tatsächlich dem Melittin und nicht etwa einem noch unbekannten, ebenfalls muskulotropen Bestandteil zukommen.

Tabelle 5. Schwellenkonzentration für Erzeugung von Potentialabfall und Sekundärkontraktur am isolierten Froschsartorius. (Nach HEYDENREICH, 1957)

	Potentialdifferenz	Sekundärkontraktur
Bienen-Nativgift	1:40000 bis 1:80000	1:40000
Gereinigtes Bienengift	1:160000	1:80000
Melittin	weniger als 1:160000	1:80000
Naja-tripudians-Gift	1:80000 bis 1:160000	1:80000 bis 1:160000
Naja-haje-Gift	1:160000 bis 1:320000	1:80000
Naja-nigriceps-Gift	1:160000 bis 1:320000	1:80000
Crotalus-terribilis-Gift	1:5000	1:2500 bis 1:5000
Vipera-ammodutes-Gift	1:5000 bis 1:10000	ohne Effekt bis 1:2500
Lysocithin	zwischen 1:10000 und 1:20000	1:5000 bis 1:10000
Histamin	ohne Effekt bis 1:1000	ohne Effekt bis 1:1000
Dinitrophenol	1:10000 bis 1:20000	1:2000 bis 1:5000
Digitonin	um 1:160000	1:160000

δ) *Quergestreifte Muskulatur und deren Synapsen.* Wie bereits erwähnt, verlieren isolierte Skelettmuskeln des Frosches unter dem Einfluß von Melittin oder Lysocithin Kalium; gleichzeitig wird das Ruhepotential vermindert, der Muskel verkürzt sich (HEYDENREICH, 1957). Die Erhöhung der Permeabilität drückt sich in einer Gewichtszunahme isolierter Muskulatur durch Wasseraufnahme aus (LAMPARTER, 1954); schließlich sinkt unter dem Einfluß von Melittin der Gehalt an anorganischem und organischem Phosphat im isolierten Froschsartorius (HEYDENREICH, 1957). Man kann die aufgeführten biochemischen und elektrischen Veränderungen als Analogien zu den bei der Hämolyse ablaufenden Reaktionen betrachten. Es ist einleuchtend, daß es auf dieser Basis zur Kontraktur kommt (Tabelle 5).

Die Melittin-Kontraktur ist von der durch Gesamtgift bedingten qualitativ nicht zu unterscheiden; die übrigen Giftkomponenten, wie Apamin und Phospholipase, wirken in dieser Hinsicht, wenn überhaupt, sehr viel schwächer. Bei geeigneter Dosierung verläuft die Kontraktur in zwei Phasen. Einer initialen, relativ schnellen Verkürzung folgt ein Minimum, diesem ein sehr langsam

* 1-Phenyl-1-(2-pyridyl)-3-dimethylaminopropan.

ablaufendes zweites Maximum (Abb. 10). Tachyphylaxie und Empfindlichkeitsminderung gegen andere Reize sind ausgeprägt (RÖTHEL, 1953). Die Bienengiftkontraktur lässt sich nur bei Anwendung starker Verdünnungen (1:500000) durch Auswaschen wenigstens teilweise aufheben; Melittin haftet relativ stark an der Muskulatur und wird aus seinen Lösungen durch Schütteln mit zerkleinerten Muskeln entfernt; dabei schwindet, wie zu erwarten, nicht nur die Fähigkeit zur Auslösung der Muskelkontraktion, sondern auch hämolytisches Vermögen und lokale Reizwirkung (SCHLÖGL, 1953). Novocain setzt die Kontrakteureigung herab, hebt sie aber nicht auf. Auch anodische Polarisation ist wirkungslos. Das Aktionspotential sinkt; es restituiert sich

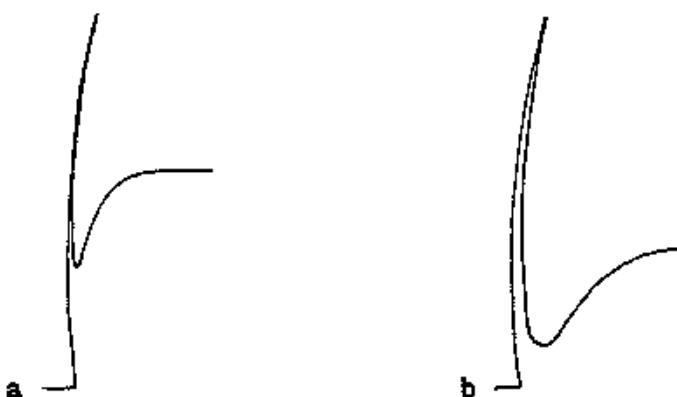


Abb. 10. Zweiphasige Kontraktion des Froschsartorius nach gereinigtem Gesamtgift (a) bzw. Melittin (b)
1:20000 (nach HEYDENREICH, 1957)

bei der Erschlaffung nach Bienengiftkontraktion nicht, sondern fällt weiter ab. Es handelt sich also um eine Schädigungskontraktur (HOFMANN, 1952a). Am isolierten Rattenzwerchfell wird die neuromuskuläre Erregungsleitung früher geschädigt als die Kontraktilität; doch sind gleichzeitig Zeichen von Kontraktur zu erkennen (HOFMANN, 1952b). An Kaltblüterpräparaten erfolgt die Schädigung beider Systeme nahezu, aber nicht völlig gleichzeitig (HOFMANN, 1952a; RÖTHEL, 1953). Zur Auslösung der Sekundärkontraktur werden im allgemeinen etwas höhere Giftkonzentrationen benötigt als zur Erzeugung des Potentialabfalls. Die am Froschsartorius erforderlichen Schwellenkonzentrationen sind in Tabelle 5 (HEYDENREICH, 1957) zusammengestellt. — Auch die curareähnliche Wirkung wird durch Melittin, nicht durch phospholipasehaltige, melittinfreie Fraktionen hervorgerufen (RÖTHEL, 1953). Wegen ihres endgültigen Charakters kann die Melittin-Lähmung nicht mit der nach Curare oder Dekamethonium verglichen werden, obgleich sie der letzteren hinsichtlich der Kontrakteureigung ähnelt. Sehr wahrscheinlich ist sie — wie die Muskelkontraktion — Ausdruck einer Schädigung der Grenzflächen. Die Nervenleitung wird weder im N. phrenicus der Ratte (HOFMANN, 1952b) noch im N. ischiadicus des Frosches (RÖTHEL, 1953) durch stark kontraktionsauslösende Bienengiftdosen beeinflusst. Dagegen sind

freie Axone des Tintenfisches sehr empfindlich gegen Bienengesamtgift (ROSENBERG und PODLESKY, 1963). Es ist denkbar, daß das umgebende Bindegewebe den Nerven vor der Bienengiftwirkung schützt, aber auch, daß bei der Präparation freier Axone diese vorgeschädigt und dadurch für eine der Giftkomponenten leichter zugänglich werden.

In vivo-Effekte auf das willkür-motorische System der Katze sind unbedeutend. So wird die indirekte Erregbarkeit des M. tibialis durch subletale bis letale Bienengiftdosen nicht eindeutig verändert; auch das Katzenzwerchfell behält unter diesen Bedingungen seine Erregbarkeit (v. BRUCHHAUSEN, 1955). Das Vergiftungsbild bei Mäusen liefert keinen Anhalt für einen curareähnlichen Angriff.

Der neuromuskuläre Block nach Bienengift läßt sich nicht mit dem durch Cobragift bedingten vergleichen, wie dies früher (HOFMANN, 1952b) versucht wurde. Zwar ist das Wirkungsbild nach Gesamtgift demjenigen von Bienengift ähnlich; auch ist die Cobragift-Phospholipase offenbar unwirksam. Die Kontrakturerzeugung beruht jedoch auf einem stark basischen Faktor, dem Cardiotoxin. Das schwächer basische Cobragift-Neurotoxin hat ähnliche elektrophysiologische Veränderungen wie *d*-Tubocurarin zur Folge. Es mindert das Endplattenpotential, nicht dagegen die terminalen neuralen Potentiale, die Aktionspotentiale der Muskulatur oder die Ruhepotentiale. Die spontanen Miniatur-Endplattenpotentiale werden stark vermindert, desgleichen die antidrome Nervenaktivität nach Physostigmin. Die Minderung der Acetylcholinfreisetzung durch Cobragift beruht dagegen — wie die Verkleinerung der terminalen neuralen Potentiale — auf dem depolarisierenden Cardiotoxin (CHANG und LEE, 1966).

e) *Ganglionäre und zentrale Synapsen*. Wie wir am Beispiel der glatten und quergestreiften Muskulatur gesehen haben, werden erregbare Systeme durch Melittin zunächst stimuliert, dann gelähmt. Analoge Prozesse laufen an den Synapsen des zentralen Nervensystems und der vegetativen Ganglien ab. Bereits 1—2 µg Melittin, in die Durchströmungsflüssigkeit des isoliert perfundierten Ganglion cervicale superius der Katze injiziert, sensibilisieren das Präparat für Acetylcholin bzw. präganglionäre elektrische Reizung. Höhere Dosen (um 20 µg) erzeugen eine langdauernde (bis zu 17 min) Erregung, die von Tachyphylaxie und auch verminderter Erregbarkeit bzw. Unerregbarkeit des Ganglions gegen den elektrischen Reiz gefolgt ist. Die Nickhaut reagiert dagegen noch auf postganglionäre Impulse. Es sei hier auf den grundsätzlichen Unterschied gegenüber Lyssolecithin hingewiesen. Diese Substanz macht — allerdings in relativ hohen Dosen (100—200 µg) — das Ganglion weniger empfindlich gegen den Acetylcholinreiz, während der elektrische Reiz in wesentlich geringerem Maße beeinflußt wird. Die Hemmung ist reversibel (SEIFERT, 1958). Das Beispiel zeigt besonders deutlich, daß neben der Erniedrigung der Grenzflächenspannung — dieses Phänomen ist beiden Substanzen gemeinsam — noch weitere Moleküleigenschaften die spezifische pharmakologische Wirkung mitbedingen. Dabei wäre in erster Linie an Basizität und Adsorbierbarkeit des Melittins zu denken. Das basische, aber kaum oberflächenaktive Protaminsulfat ist, wenn überhaupt, sehr viel schwächer ganglionär wirksam als Melittin. SEIFERT (1958) stellte folgende Aktivitätsreihe am Halsganglion auf: Melittin > gereinigtes Bienengift > *Naja naja*, *Naja nigricollis* >

Crotalus terrificus > *Vipera ammodytes*. Das zuletzt genannte Gift sensibilisiert lediglich. Da es reichlich Phospholipase A enthält, kann dieses Enzym schon deshalb keine entscheidende Rolle bei der Ganglienblockade spielen. In vivo ließ sich bisher keine Ganglioplegie durch Melittin nachweisen (v. BRUCHHAUSEN, 1955); Injektion subletaler Dosen des Gesamtgiftes sensibilisiert die Nickhaut selbst, läßt aber die elektrische Erregbarkeit des Ganglion cervicale superius der Katze unbeeinflußt.

Es ist anzunehmen, daß das Verhalten des isolierten Halsganglions ein verhältnismäßig einfaches Modell der unter Melittin am *zentralen* Nervensystem eintretenden Veränderungen liefert. Einstweilen ist darüber noch sehr wenig bekannt. Ältere Untersuchungen mit systemisch appliziertem Gesamtgift weisen auf gesteigerte Krampfbereitschaft bei Meerschweinchen hin (GERLICH, 1950), die einem Elektroschock unterworfen wurden. Bei Katzen mit intaktem Zentralnervensystem wird der gleichseitige Extensorreflex durch Gesamtgift abgeschwächt, nach vorhergehender Rückenmarksdurchschneidung dagegen verstärkt (v. BRUCHHAUSEN, 1955). Die zugrunde liegenden Versuchsanordnungen geben den angeführten Experimenten nur orientierenden Charakter; vor allem wurde mit Gesamtgift gearbeitet, das mit dem Apamin (s. unten) einen selektiv zentralnervös wirksamen Stoff enthält.

Aus einigen Experimenten von ROSSBACH (1955) geht jedoch zweifelsfrei ein zentraler Angriff von Melittin hervor. Bringt man die Substanz in Mengen von 50—200 µg in den IV. Ventrikel der anaesthetisierten Katze, so ändert sich der Blutdruck akut. Senkung, Steigerung und auch biphasische Effekte treten auf. Die Atmung wird irreversibel gelähmt, der homolaterale Flexorreflex erheblich abgeschwächt. Auffallend ist der schnelle Eintritt und die starke Variabilität der Effekte; auch Tachyphylaxie wird beobachtet. In der nur einige Minuten währenden Latenzzeit dürfte die Substanz kaum in die Tiefe des Hirnstamms eingedrungen sein. Es ist vielmehr zu vermuten, daß mindestens ein Teil der Effekte durch Erregung oberflächlich gelegener sensibler Strukturen zustande kam. — Analoge Resultate wurden auch bei Injektion von Gesamtgift in die Arteria carotis erhalten; es wäre jedoch noch festzustellen, ob Melittin dabei das ursächliche Agens war. Die akute Melittinvergiftung weist keine Symptome auf, die einen primären Angriff am Zentralnervensystem wahrscheinlich machen; der Atemstillstand geht jedoch dem Kreislaufzusammenbruch voraus.

ζ) *Kreislauf*. Blutgefäße: Injiziert man beim Kaninchen (LAMPARTER, 1954; HABERMANN, 1955 b) oder Ratten (HABERMANN, unveröffentlicht) Melittin intracutan, so tritt zirkulierender Farbstoff im Bereich der Quaddel aus den Gefäßen. Melittin entspricht also in dieser Hinsicht basischen Polypeptiden anderer Herkunft, z.B. aus Kalbsthymus (FRIMMER und HEGNER, 1963). Wie S. 240 bereits näher ausgeführt, sind dabei mehrere Faktoren beteiligt: Mastzellerstörung und damit Freisetzung von Histamin und Serotonin, in

höheren Dosen aber auch eine direkte Gefäßwirkung. WALDVOGEL und FRIMMER (1967) prüften Melittin am Scrotalpräparat der Ratte; nach intravenöser Tusche-Injektion ist hier eine Differenzierung des Angriffspunkts im Gefäßverlauf möglich. Melittin erhöht in den untersuchten Dosen nur die Permeabilität der Venolen, während Lysolecithin (wie Phospholipase A) auch die Capillaren schädigt. Sicher ist die Störung der Blut-Gewebsschranke durch Melittin wesentlich für die lokale Reaktion nach Bienengift.

Ob auch bei systemischer Applikation von Melittin derartige Permeabilitätseffekte auftreten, ist zweifelhaft. Zwar steigt nach Melittininjektion (1—4 mg/kg) bei Kaninchen der Hämatokrit an, doch ohne gleichzeitige Erhöhung der Zellzahl. In erster Linie werden also die Erythrocyten stärker durchlässig, so daß sie schwelen; für transcapilläre Flüssigkeitsverluste besteht einstweilen kein Anhalt (SROCKEBRAND, 1965).

Die Gefäßweite isolierter Organe wird durch Melittin modifiziert. Das Läwen-Trendelenburgsche Froschpräparat reagiert auf Melittin (Verdünnungen bis herab zu 1:10⁶) mit einer oft biphasischen Vasoconstriction, während das isoliert durchströmte Kaninchenohr je nach Reaktionslage und Melittinmenge mit einer Verengerung oder Erweiterung seiner Gefäße antworten kann (SILBER, 1953). Eine Beteiligung von Histamin ist dabei wenig wahrscheinlich, weil das Froschpräparat gegen dieses Amin recht unempfindlich ist und Histamin an den untersuchten Kaninchenohren nur gefäßverengend wirkte.

Das isolierte Froschherz ist gegenüber Melittin hochempfindlich (NEUMANN und HABERMANN, 1954a). Auf kleine Konzentrationen (10 µg/ml und weniger) reagiert es mit einer Verstärkung der Systole; höhere Konzentrationen bringen es in Kontraktur und schädigen auch Reizbildungs- und Reizleitungssystem, was sich in Änderung der Schlagfrequenz, Extrasystolen und Blockerscheinungen äußert.

Die peripheren Kreislauffeffekte des Melittins sind also recht komplex. Dazu kommt ein eventueller zentraler Angriff, der einstweilen aber nur bei lokaler Einbringung in den IV. Ventrikel zweifelsfrei nachgewiesen wurde (s. S. 245). Dennoch lassen sich einige typische Kreislaufreaktionen auf Melittin herausstellen:

a) Injiziert man bei narkotisierten Katzen einige mg/kg Melittin oder Bienen gesamtgift schnell intravenös, so sinkt der Blutdruck abrupt ab; das Tier wird bradykard und bekommt Extrasystolen; die Atmung sistiert für einige Züge. Nach einigen Minuten erholt sich das Tier und zeigt deutliche Tachyphylaxie bei Wiederholung des Versuchs. Durchschneiden des Vagus hebt die Reaktion auf (HABERMANN, unveröffentlicht).

b) Applikation ca. 10fach kleinerer Melittindosen oder Wiederholung der genannten höheren Melittindosen ergibt eine viel weniger dramatische Antwort: Eine kurzdauernde Blutdrucksenkung, der eine länger dauernde Steigerung folgen kann. Bei Wiederholung der Melittindosis tritt die Blutdrucksteigerung immer stärker hervor.

c) Setzt man — unter künstlicher Beatmung — extreme Melittindosen ein, so erreicht die Hypertension maximale Werte. Auf ihrer Höhe erscheinen Extrasystolen und Blockzeichen im EKG, schließlich Kammerflattern. Es fällt auf, daß dabei gleichzeitig generalisierte fibrilläre Zuckungen der Skelettmuskulatur auftreten. Die Atemlähmung geht dem finalen Kreislaufzusammenbruch voraus. Mit Bienengesamtgift kann man ein analoges Bild hervorrufen.

Die Blutdrucksenkung ist resistent gegen Atropin und das Antihistaminicum Avil; die Blutdrucksteigerung tritt auch nach Ergotamin, Yohimbin oder Adrenalektomie auf (HABERMANN, 1954a; HACKSTEIN, 1953; SALZMANN, 1953). Gesamtgift mindert auch die depressorische Wirkung einer Reizung des Halsvagus sowie von Acetylcholin-Injektionen bei der Katze; es wäre noch zu prüfen, ob Melittin dafür verantwortlich ist (HACKSTEIN, 1953).

Es bereitet Schwierigkeiten, die älteren Untersuchungen über die Kreislaufwirkungen von Bienengift zu analysieren; denn man hat es dabei mit kombinierten Effekten von mindestens vier Komponenten zu tun: Melittin, Phospholipase A, MCD-Peptid, Histamin. FELDBERG und KELLAWAY (1937) unterschieden erstmals zwischen den Kreislaufeffekten des im Gesamtgift vorhandenen Histamins und denjenigen anderer Komponenten. Bei Verwendung von Rohgift traten die Histamineffekte in den Vordergrund. Benutzten sie dagegen gereinigtes Gift, so wurden wesentlich höhere Dosen benötigt, die dann zu Drucksenkung im großen Kreislauf, Anstieg des Drucks in der A. pulmonalis, Hämo-konzentration, hämorrhagischem Lungenödem und gelegentlich auch Herzversagen führten. — Beim Hund beobachteten FELDBERG und KELLAWAY eine langdauernde Hypotonie im großen Kreislauf ohne Anstieg des Drucks in der Pulmonalarterie. Portal-venendruck und Lymphfluß im ductus thoracicus nahmen zu. Postmortal fielen sub-endocardiale Blutungen sowie hämorrhagische Schleimhautschäden im Duodenum auf.

η) Lokal- und Allgemeintoxicität. Schmerz, Rötung und Schwellung gehören zu den charakteristischen Folgen eines Bienenstichs. Hier seien nur die Befunde erwähnt, die für eine Beteiligung des Melittin sprechen. Im übrigen sind praktisch alle untersuchten Giftkomponenten, wie Histamin, Hyaluronidase, Phospholipase A und einige F0-Komponenten, vor allem das MCD-Peptid, lokal wirksam.

Injiziert man Melittin intracutan in den menschlichen Vorderarm, so schmerzt die Injektionsstelle; sie rötet sich und schwüllt an. Bei subcutaner Applikation höherer Dosen bei Mäusen wird die darüber liegende Haut nekrotisch. Melittin schädigt also wohl nicht nur die Blutgefäße und die Mastzellen (s. S. 239), sondern auch die übrigen Zelltypen des Bindegewebes. Zur Deutung des Wirkungsmechanismus kommt neben dem direkten Angriff des Peptids eine Freilegung von Mediatoren in Frage (s. S. 240); Melittin zerstört überdies lysosomale Strukturen in Leukocyten und isolierte Lysosomen aus Rattenleber (SESSA *et al.*, 1968). Auch durch Applikation in den Bindegautsack des Kaninchens erzielt man dosisabhängige entzündliche Reaktionen. Nach Menge und Wirkungsintensität ist Melittin als das wichtigste lokaltoxische Agens des Bienengiftes zu bezeichnen.

Die Todesursache nach Melittininjektion ist noch unbekannt. Erstaunlich ist die beträchtliche Diskrepanz zwischen der LD 50 bei intravenöser (3,5 mg/kg) und subcutaner Injektion. Nach Versuchen mit Gesamtgift zu schließen, liegt die subcutane LD 50 mindestens 20mal höher. Ob es sich dabei um den Ausdruck adsorptiver Bindung oder schneller enzymatischer Zerstörung handelt oder einfach um die Notwendigkeit eines kurzzeitig sehr hohen Melittinspiegels, wie er nur durch intravenöse Injektion zu erreichen ist, muß einstweilen dahingestellt bleiben. Eine bevorzugte Schädigung bestimmter Organ-systeme läßt sich bisher nicht als Todesursache namhaft machen: Mäuse machen nach intravenöser Injektion einen abnorm ruhigen, dyspnoischen Eindruck. Vor dem Tod werden Zeichen von Hyperexcitation, wie zielloses Umherlaufen, auch vereinzelt Krämpfe, beobachtet, die aber als unspezifische, vielleicht auf Hypoxie beruhende terminale Reaktionen gedeutet werden können (WŁOSZYK, 1954). Wie schon erwähnt, läßt sich beim Kaninchen weder eine generalisierte Erhöhung der Gefäßpermeabilität noch eine intravasale Hämolyse als Todesursache nachweisen.

§) *Modifikationen enzymatischer Reaktionen.* Da das Peptid mit Zellpartikeln reagiert (HABERMANN, 1958b), andererseits für zahlreiche enzymatische Reaktionen die Intaktheit entsprechender Trägerstrukturen erforderlich ist, hat man mit einer Beeinflussung mannigfacher Enzymsysteme zu rechnen. An dieser Stelle sei nur auf drei Angriffspunkte hingewiesen: Blutgerinnung, oxidative bzw. energieliefernde Reaktionen und ATP-Spaltung. Phospholipase A (s. S. 287) inhibiert derartige Systeme ebenfalls, so daß die Melittineffekte im Verband des Gesamtgiftes nicht dominieren.

Melittin stört an mindestens zwei Stellen die *Gerinnung*. In der ersten Phase hemmt es Organthromboplastine, z.B. aus Gehirn und Lunge, die bekanntlich partikelgebunden sind. Melittin ist in dieser Hinsicht ca. 20mal schwächer wirksam als Phospholipase A. Ein zweiter Angriffsplatz liegt am Fibrinogen, das durch Melittin fast ebenso stark gefällt werden kann wie durch Protaminsulfat. Die Annahme eines spezifischen Reaktionsmechanismus ist in beiden Fällen überflüssig. Für die Thromboplastinhemmung genügt der Tensidcharakter; auch Lysolecithin oder Digitonin sind dazu imstande. Die Fibrinogenfällung kann durch Salzbildung des basischen Peptids mit sauren Gruppen erklärt werden (HABERMANN, 1954c). — Melittin änderte bei intravenöser Injektion (2 mg/kg) weder die Blutungszeit noch die Gerinnungszeit von Mäusen (REDELBURGER, 1958). Auch beim Kaninchen lag die Gerinnungszeit bei Applikation einer letalen Melittindosis im Bereich der Norm (STOCKEBRAND, 1965).

Analoge Mechanismen dürften auch der Störung von *Stoffwechselreaktionen* zugrunde liegen. So entkoppelt Melittin die oxidative Phosphorylierung im Homogenat von Rattenleber (HABERMANN, 1954b). Die Bernsteinsäuredehydrierung durch Rattenlebermitochondrien wird gehemmt, was aber nur

dann sichtbar wird, wenn man Triphenyltetrazoliumchlorid als Elektronen-acceptor verwendet. Benutzt man statt dessen Kaliumferricyanid, so wird eine Aktivierung registriert, während Phospholipase A in jedem Falle inhibiert (HABERMANN, 1955a). Melittin ruft gleichzeitig eine Trübung der Suspensionen von Zellpartikeln (HABERMANN, 1958b) und auch von Mitochondrien hervor; die Aktivierung der Succinatdehydrogenierung geht annähernd der Trübungs-zunahme parallel. Es ist bekannt, daß zwischen Succinat und Triphenyltetrazoliumchlorid erheblich mehr Redox-Stufen geschaltet sind als zwischen Succinat und Ferricyanid. Eine oder mehrere, nahe am Succinat liegende Reaktionen werden also durch Melittin erleichtert, andere, ferner liegende gehemmt (HABERMANN, 1955a) (Tabelle 6).

Tabelle 6. Vergleich von Melittin, Protaminsulfat und verschiedenen Schlangengiften im Dehydrogenaseansatz (Ferricyanid- bzw. Formasanverfahren). (HABERMANN, 1955a)

Zusatz (Endkonzentration)		Ferrocyanidbildung (μMol)	Formazanbildung (μMol)
Kontrolle		1,9	0,066
Melittin	1: 40000	5,3	0
	1: 160000	3,1	0
	1: 640000	2,2	0,019
	1: 2560000	—	0,024
Protaminsulfat	1: 1000	—	0,019
	1: 10000	3,3	0,037
	1: 100000	2,5	0,052
Vipera-ammodytes-Gift	1: 2500	0,8	—
	1: 25000	0,7	—
Naja-nigricollis-Gift	1: 2500	0,9	—
	1: 25000	0,9	—
Crotalus-terribilis-Gift	1: 2500	0,5	—
	1: 25000	1,2	—

Eine dritte Analogie zwischen Melittin und Phospholipase A findet sich in seiner Wirksamkeit auf strukturgebundene ATPasen. So wird die ATP-Spaltung durch Skeletmuskel-Grana vom Meerschweinchen in Gegenwart von Melittin um 59 % erhöht. Diese (ouabainresistente) ATPase ist normalerweise durch Ca^{++} aktivierbar; unter Melittin gelingt dies nicht mehr. Das Bienengiftpeptid ist in dieser Hinsicht weitaus wirksamer als alle anderen bisher untersuchten Verbindungen, was wegen der Beziehung zwischen Calciumtransport und Muskelkontraktion besonders interessant sein mag (PORTIUS und REPKE, 1963a). Die $\text{Na}^+ + \text{K}^+ + \text{Mg}^{++}$ -aktivierte (ouabainempfindliche) ATPase der Zellmembran des Herzmuskels wird durch Melittin (1:100000) zu ca. 50 % inaktiviert. Der Effekt ist unspezifisch; denn die Hemmung der Transport-ATPase wird durch Erhöhung der K^+ -Konzentration von 5 auf 85 mM nicht vermindert. Die Begleit-ATPase wird etwas (zu 15 %) aktiviert (REPKE und PORTIUS, 1963).

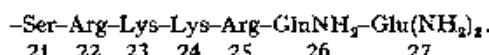
a) Strahlenschutz-Wirkung. SHIPMAN und COLE (1967) applizierten Mäusen subcutan oder intravenös subtoxische Bienengiftmengen und bestrahlten sie 1 Std bis 1 Tag später mit 825 r. Während alle Kontrolltiere starben, hatte Bienengift bei beiden Applikationsarten einen deutlichen radioprotektiven Effekt. Auch eine Polypeptidfraktion, die hauptsächlich aus Melittin bestand, schützte bei subcutaner Injektion (5,4 γ/g) 57 % der Tiere gegen eine normalerweise sicher tödliche Röntgenbestrahlung. Es ist erstaunlich, daß die Strahlenschutzwirkung mindestens 1 Tag lang anhielt. Drei Deutungen kommen in Frage: a) Melittin könnte als „Streß-Substanz“ eine radioprotektive Adaptationssyndrom auslösen. b) Es könnte eine Funktionsänderung des hämatopoietischen Systems hervorrufen. c) Seine antibakteriellen Eigenschaften (s. unten) könnten die Tiere schützen.

x) Antibakterielle Effekte. Die antibakteriellen Wirkungen von Bienengift wurden erstmals von SCHMIDT-LANGE (1941) beschrieben. ORTEL und MARKWARDT (1955) trennten das Gift elektrophoretisch auf und fanden sie mit der Melittinfraktion verknüpft. Systematische Untersuchungen aus neuerer Zeit stammen von FENNELL u. Mitarb. (1967). Melittin hemmte das Wachstum aller geprüften grampositiven und gramnegativen Bakterienarten, auch penicillinresistente Stämme von *Staphylococcus aureus*. Die Melittinfraktion unterschied sich weder in ihrem Hemm-Spektrum noch quantitativ vom Gesamtgift. Die Befunde überraschen nicht, wenn man die Invertseifenstruktur des Melittinmoleküls in Rechnung stellt (s. S. 232). Die verwendeten Melittinkonzentrationen (30 mg/ml) liegen in einem Bereich, der starke Oberflächenaktivität garantiert.

BENTON *et al.* (1963) entwickelten ein bakteriologisches Bestimmungsverfahren für Bienengift, das auf der Wachstumshemmung gegenüber *Bacillus subtilis* beruht. Sie tränken sog. nutrient discs mit Giftlösung und legen sie auf Agarplatten, die mit ca. 1 Mill. Keimen/ml beschickt und 1 Std bei 36—37° C vorinkubiert worden sind. 5 Std nach Giftapplikation wird der Durchmesser des Inhibitor-Holes gemessen (1 Cornell-einheit entspricht 8,0 ± 0,3 mm). Das wirksame Prinzip verträgt Sterilisation unter Überdruck; ob es sich dabei um Melittin handelt, ist nicht geprüft worden.

c) Zur Pharmakologie eines synthetischen Melittinanalogons sowie von Melittinfragmenten

SCHRÖDER u. Mitarb. (1967) synthetisierten ein Peptid, dessen Struktur nur wenig von der oben (s. S. 232) angegebenen abweicht; es endet C-terminal mit



Seine Bruchstücke waren in tryptischen Hydrolysaten in kleiner Menge gefunden, das intakte Peptid (hypothetisch als Melittin II bezeichnet) jedoch nicht von der Hauptfraktion des Melittins (I) abgetrennt worden. Das synthetische Peptid wanderte elektrophoretisch etwa gleich schnell wie das natürliche. Die aktivsten Präparate besaßen bisher 40—50 % der hämolytischen Wirksamkeit

natürlichen Melittins gegenüber gewaschenen Kaninchenerthrocyten. Die Dosis-Wirkungskurve wies annähernd die gleiche Steigung auf wie beim natürlichen Melittin. Wie dieses bewirkte auch synthetisches Melittin eine langsam ablaufende Kontraktion des isolierten Meerschweinchenileums mit Tachyphylaxie. — Ein wichtiger qualitativer Unterschied ergab sich bei der Testung am Kaninchenblutdruck (Abb. 11); die Drucksenkung trat auf das synthetische Peptid hin langsamer ein und hielt länger an. Offenbar liegt ihr ein anderer Mechanismus zugrunde im Vergleich zur Reaktion auf natürliches Melittin.

Eine Reihe von Melittin-Spaltstücken (Tabelle 7) wurde auf hämolytisches Vermögen (Kaninchenerthrocyten in vitro), Erhöhung der Gefäßpermeabilität für intravenös injiziertes Evans-Blau (Kaninchenrücken), Darnawirksam-

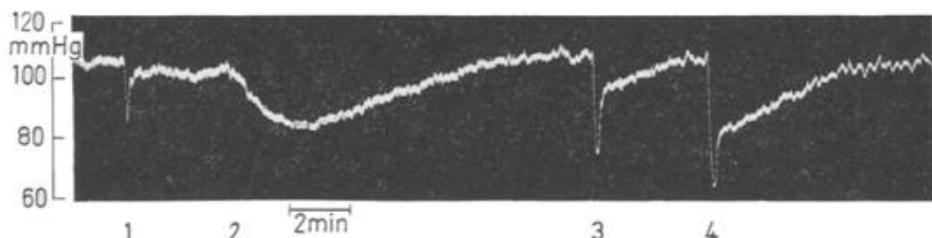


Abb. 11. Vergleich von synthetischem Melittin II und natürlichem Melittin I am Kaninchenblutdruck. Pernocton-Narkose, Atropin-Vorbehandlung. 1 150 µg Melittin I; 2 250 µg Melittin II; 3 500 µg Melittin I; 4 1 mg Melittin I

keit (isoliertes Meerschweinchenileum) und Kreislaufaktivität (Kaninchenblutdruck) geprüft. Die Relationen der hämolytischen Aktivitäten bestätigten die aus der Struktur des natürlichen Melittins abgeleitete „Invertseifen“-Theorie; denn es lysierten nur diejenigen Peptide, bei denen der basische, C-terminale Rest intakt *und* mit einem Teil der hydrophoben Konfiguration verbunden war. Eine Verkürzung um 6 Glieder vom N-Terminus her minderte bereits das hämolytische Vermögen beträchtlich. Die ε-Aminogruppen der Lysine stehen der Hämolyse eher im Wege; denn Butoxycarbonyl-Substitution (BOC) verstärkte die hämolytische Potenz.

Im Gegensatz dazu wird die Capillarpermeabilität auch durch kurze, basische Fragmente erhöht, z.B. durch Peptid 18—27, schwächer durch Peptid 20—27, während 21—26 bzw. 22—27 nicht mehr eindeutig wirkten. BOC-Substitution des Peptids 18—27 minderte die Aktivität nicht, während paradoxerweise die Permeabilitätserhöhung durch Peptid 7—27 nach BOC-Substitution verschwunden war. Die synthetischen Fragmente waren im Permeabilitätstest zum Teil erheblich wirksamer als natürliches Melittin, vielleicht weil sie wegen ihrer geringeren Größe und des fehlenden Invertseifencharakters besser penetrieren können. Natürliches und synthetisches Melittin waren etwa äquiativ; doch scheint die Anfärbung der synthetischen Material enthaltenden Quaddeln diffuser (HABERMANN, unveröffentlicht).

Tabelle 7. Struktur und pharmakologische

Nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1-26	Gly-Ileu-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ileu-Ser-Try-Ileu-Lys-Arg-Lys-Arg-GluNH ₂																								
1-27	Gly-Ileu-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ileu-Ser-Try-Ileu-Ser-Arg-Lys-Lys-Arg-																								
7-27	Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ileu-Ser-Try-Ileu-Ser-Arg-Lys-Lys-Arg-(BOC)(BOC)																								
7-27 (BOC),	(BOC)-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ileu-Ser-Try-Ileu-Ser-Arg-Lys-Lys-Arg-																								
18-27	Ser-Try-Ileu-Ser-Arg-Lys-Lys-Arg-																								
20-27	Ileu-Ser-Arg-Lys-Lys-Arg-																								
20-26	Ileu-Lys-Arg-Lys-Arg-GluNH ₂ -																								
21-26	Ser-Ang-Lys-Lys-Arg-																								
22-27	Arg-Lys-Lys-Arg-(BOC)(BOC)																								
18-27 (BOC),	Ser-Try-Ileu-Ser-Arg-Lys-Lys-Arg-																								
1-14	Gly-Ileu-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro																								
4-14	Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro																								
7-14	Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro																								
1-20	Gly-Ileu-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ileu-Ser-Try-Ileu																								

Bei der Prüfung der Darm- und Kreislaufwirksamkeit der Fragmente mußten relativ hohe Konzentrationen (5 µg/ml bzw. 100 µg/Tier) eingesetzt werden. Immerhin riefen die Peptide 7-27 (BOC) und 22-27 eine kurzdauernde Steigerung des Kaninchenblutdrucks hervor, die wenig dosisabhängig war und langsamer eintrat als z. B. nach Adrenalininjektion. Eine Darmwirksamkeit (langsame Kontraktion) kam ebenfalls nur den beiden genannten Peptiden zu. Wir möchten aber offen lassen, ob nicht eine Begleitsubstanz für die schwachen Effekte in diesen beiden Tests verantwortlich war.

3. F0-Komponenten und MCD-Peptid

Schon bei den ersten elektrophoretischen Trennungen von Bienengift (NEUMANN und HABERMANN, 1954a) trat eine als „F0“ bezeichnete Fraktion auf, die stärker basisch als Melittin war und sich besonders gut bei Elektrophorese im Sauren darstellen ließ. Nach der Färbbarkeit mit Amidoschwarz zu urteilen, machte sie weniger als 5 % der Peptide bzw. Proteine des Gesamtgiftes aus. Bei der später erarbeiteten, verfeinerten Trennung an Sephadex G 50 erschien F0 in zwei Bereichen vor (F0 a) bzw. hinter (F0 p) dem Melittin-Gipfel, aber von diesem teilweise überdeckt. Apamin wandert elektrophoretisch schneller als F0 a, dieses schneller als Melittin (HABERMANN und REIZ, 1965a). Durch anschließende Ionenaustauschchromatographie wurden F0a und F0p in weitere Komponenten zerlegt. Das aktive Agens aus F0p bezeichnen wir als MCD-Peptid (mastzelldegranulierendes Peptid).

MCD-Peptid zerstört Mastzellen in isolierten Mesenterialstücken der Ratte und in Suspension; in diesen Tests ist die Substanz 10- bzw. 100mal wirksamer als Melittin (BREITHAUPT, 1968). Möglicherweise ist MCD-Peptid mit dem von FREDHOLM u. Mitarb. (1966, 1967) beschriebenen, von Melittin und Phospholipase A verschiedenen mastzellzerstörenden Bienengiftfaktor identisch. Da aber Melittin in mindestens zehnmal höherer Konzentration im Gesamtgift

Wirkungen von Melittinfragmenten

26	27	Hämolytische Aktivität (%)	Herstellung	Permeabilitäts-effekte Grenzwert (μg/Injektion)	Darmwirksamkeit in Mengen von 100 μg und darüber	Blutdruck (Kaninchen) in Mengen von 100 μg und darüber
Glu(NH ₂) ₂	100	natürlich	5	+	kurzdauernde Senkung	
GluNH ₂ -Glu(NH ₂) ₂	40–50	synthetisch	5	+	langdauernde Senkung	
GluNH ₂ -Glu(NH ₂) ₂	2	synthetisch	1	0	0	
GluNH ₂ -Glu(NH ₂) ₂	5	synthetisch	> 20	+	Steigerung	
GluNH ₂ -Glu(NH ₂) ₂	< 0,1	synthetisch	1	0	0	
GluNH ₂ -Glu(NH ₂) ₂	< 0,1	synthetisch	5	0	0	
Glu(NH ₂) ₂		natürlich	20	?	?	
GluNH ₂		synthetisch	20	0	0	
GluNH ₂ -Glu(NH ₂) ₂	< 0,1	synthetisch	> 20	+	Steigerung	
GluNH ₂ -Glu(NH ₂) ₂	< 0,1	synthetisch	1	0	0	
GluNH ₂ -Glu(NH ₂) ₂	< 0,1	synthetisch	> 20	0	0	
GluNH ₂ -Glu(NH ₂) ₂	< 0,1	synthetisch	20	0	0	
GluNH ₂ -Glu(NH ₂) ₂	< 0,1	synthetisch	> 20	0	0	

vorliegt, erscheint MCD-Peptid — trotz höherer spezifischer Aktivität — nicht als dominierender Faktor bei der Mastzellzerstörung. Wie andere basische Peptide, so erhöht auch MCD-Peptid die Gefäßpermeabilität der Haut; bei der Ratte ist es ca. 10mal stärker, beim Kaninchen ca. 10mal schwächer wirksam als Melittin. Die Gefäßwirksamkeit beruht wohl nicht allein auf der Mastzellschädigung, weil Bienengiftpeptide (z. B. F0a), die nicht die Mastzellen zerstören, ebenfalls die Permeabilität steigern.

Die Aminosäureanalyse (s. Tabelle 2; S. 30) zeigt, daß MCD-Peptid reichlich basische Aminosäuren sowie Halbcystine enthält, insgesamt 22 Aminosäuren; es unterscheidet sich also von Melittin und Apamin. Sein Verhalten bei Gelfiltration spricht dafür, daß das aus der Aminosäureanalyse zu 2593 errechnete minimale Molekulargewicht dem tatsächlichen entspricht. MCD-Peptid hämolsiert nicht; noch 10 μg senken den Rattenblutdruck mit konsekutiver Tachyphylaxie.

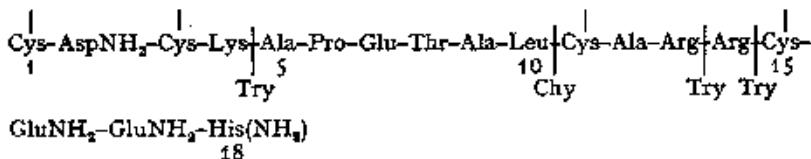
4. Apamin

Eine zentrale Erregung durch Bienengift wurde bereits von LANGER (1897) beobachtet, als er einem Hund ca. 20 mg/kg Bienengesamtgift verabreichte. Später befaßte sich HAHN (1936, 1937) in einer Reihe von Arbeiten mit dem bei Mäusen krampferzeugenden Prinzip; er erkannte bereits, daß es verhältnismäßig leicht dialysabel ist (HAHN und LEDITSCHKE, 1937). Einige Jahre darauf zeigte GERLICH (1950), daß Gesamtgift die Elektrokrampfschwelle bei Meerschweinchen erniedrigt. Bei Toxicitätsprüfungen von Gesamtgift, besonders mit subcutanen Injektionen (bei dieser Applikationsform ist Melittin verhältnismäßig ungiftig) sahen wir wiederholt Krämpfe, hielten sie aber für Sekundäreffekte. Erst bei Gelfiltration von Gesamtgift stellte sich heraus,

daß Bienengift eine spezifisch krampferzeugende Substanz enthält (HABERMANN und REIZ, 1964, 1965a). Sie wird an Sephadex G 50 so stark retiniert, daß sie erst kurz vor den Salzen erscheint; sie dürfte demnach ein verhältnismäßig niedriges Molekulargewicht aufweisen. Das paßt zu der leichten Dialysierbarkeit, wie sie von HAHN beschrieben wurde. Die durch Gelfiltration erhaltene Fraktion wurde durch Ionenaustauschchromatographie an CM-Cellulose nachgereinigt und als einheitlich bei Aminosäureanalyse (Tabelle 2) befunden. Bei der Chromatographie wurden allerdings zwei eng benachbarte, auch elektrophoretisch unterscheidbare Gipfel eluiert, die sich aber weder in ihrer Aminosäure-Zusammensetzung noch bezüglich Wirkungsbild und Letaldosis unterschieden.

Das Peptid enthält 18 Aminosäuren (Tabelle 2) und hat trotz des ausgeglichenen Gehaltes an basischen und sauren Bausteinen seinen I. P. im Alkalischen. Auffallend ist sein Schwefelreichtum. Freie SH-Gruppen fehlen; Reduktion mit Mercaptoäthanol und anschließende Blockade der SH-Gruppen mit Jodacetamid liefert quantitativ das Carboxamid-Derivat von Cystein. Der Schwefel liegt demnach in Form von 2 Disulfidbrücken vor. Dadurch wird die Struktur des relativ kleinen Peptids erheblich versteift. Oxidation mit Perameisensäure oder Reduktion mit anschließender Blockade der SH-Gruppen mindert die pharmakologische Wirksamkeit bis auf nicht mehr meßbare Werte, desgleichen Behandlung des Peptids mit Trypsin. Reoxidation des reduzierten Apamins mit Luft stellt die Toxicität des Ausgangsmaterials zu mindestens 63 % wieder her (HAUX *et al.*, unveröffentlicht).

Die Primärstruktur des Apamins wurde gleichzeitig von zwei unabhängigen Arbeitsgruppen mitgeteilt. SHIPOLINI *et al.* (1967) gingen von nativem Apamin aus, HAUX *et al.* (1967) spalteten zunächst die Disulfidbrücken durch Oxidation zu Cysteinsäure oder durch Reduktion mit anschließender Carboxamidierung. Trennung und Strukturaufklärung tryptischer und chymotryptischer Spaltstücke ergab folgende Sequenz:



Die englische Arbeitsgruppe nimmt einen C-terminalen Verschluß durch eine Amidgruppierung an, während wir diese Frage einstweilen offen lassen. Die Position der Disulfidbrücken ist noch unbekannt.

Im Gegensatz zu Melittin wirkt Apamin weder hämolysierend noch mastzellzerstörend und zeigt bisher auch keinerlei Effekte auf isolierte Organe mit glatter oder quergestreifter Muskulatur. Dagegen erhöht es bei intracutaner Gabe die Permeabilität der Hautgefäße des Kaninchenrückens für zuvor intravenös injiziertes Evans-Blau (HABERMANN, 1963, unveröffentlicht; HABERMANN und REIZ, 1964).

Der bei weitem interessanteste Effekt des Apamins ist seine zentralnervös erregende Wirkung. Es ist das erste bisher bekannte Peptid mit selektivem Angriffspunkt am Zentralnervensystem. Zwar sind in den letzten 20 Jahren weitere neurotoxisch wirkende Peptide aufgefunden worden, die mit Apamin die Basizität und ein relativ niedriges Molekulargewicht gemeinsam haben. Crotamin (aus *Crotalus-terribilis*-Gift) greift aber nach den gegenwärtigen Kenntnissen vorwiegend an der neuromuskulären Synapse an; es erzeugt Spasmen, vor allem der hinteren Extremitäten. Sein Molekulargewicht liegt bei 10000 (GONÇALVES, 1956; MOUSSATCHÉ *et al.*, 1956). Der Angriffspunkt der Scorpamine (aus verschiedenen Skorpionsgiften) ist noch nicht näher bekannt; ihr Molekulargewicht wird ebenfalls um 10000 gefunden (MIRANDA *et al.*, 1964). Zur Gruppe der basischen, relativ niedermolekularen Neurotoxine gehört schließlich der curareähnlich wirkende (CHANG u. LEE, 1966) Faktor aus Cobragift. Er besteht aus 61 Aminosäuren, deren Sequenz weitgehend bekannt ist (EAKER u. PORATH, 1967). Apamin kann demnach als besonders einfach gebauter Prototyp einer Gruppe von Neurotoxinen betrachtet werden.

Injiziert man Apamin Mäusen intravenös, so lassen sich verschiedene Vergiftungsstadien unterscheiden. Während der ersten 15 min verhalten sich die Tiere abnorm ruhig; sie setzen sich in eine Ecke des Käfigs und krallen sich am Boden fest. Als erstes Excitations-Zeichen tritt ein leichtes Mäuseschwanzphänomen auf; die Tiere werden zunehmend unruhiger, wobei die Bewegungen schlecht koordiniert sind. Schließlich häufen sich die klonischen Krämpfe; sie beeinträchtigen die Atmung allmählich so stark, daß das Tier stirbt (LD 50 intravenös um 4 mg/kg, subcutan um 6 mg/kg). Nach subletalen Dosen halten die Excitationserscheinungen bis zu 48 Std an; die Tiere reagieren auf äußere Reize sehr stark, wobei die Abwehrbewegungen relativ gut geordnet sind (HABERMANN, 1963; HABERMANN und REIZ, 1964, 1965 a). — Durchschneidungsversuche zeigen, daß der Angriffspunkt vor allem im zentralen Teil des Nervensystems liegt. Durchtrennt man einen peripheren Nerven, so nimmt die betreffende Muskelgruppe an der Excitation nicht mehr teil. Durchschnidet man das Rückenmark, so tritt nach kleinen Giftdosen nur der craniale Teil des Nervensystems in verstärkte Aktion, nach höheren Dosen aber auch der davon abgetrennte caudale Abschnitt. — Bei Ratten treten die Zeichen der Hyperexcitation gegenüber athetotischen, relativ langsam ablaufenden Bewegungsstörungen zurück. Die LD 50 liegt hier bei 2 mg/kg; die Wirkungsdauer ist bei Überlebenden kürzer (ca. 8 Std) als bei Mäusen.

Verhaltensanomalien, die nicht durch die Motilitätsstörungen bedingt sind, fehlen. Nichtexcitierende Dosen beeinflussen weder die Geschicklichkeit von Mäusen am Drehstab oder an der schießen Ebene noch die typische Schreckreaktion der Goldhamster, wie sie sich durch Anblasen mit Preßluft auslösen läßt, noch ihre Bewegungen im open field. In erster Linie wird also das moto-

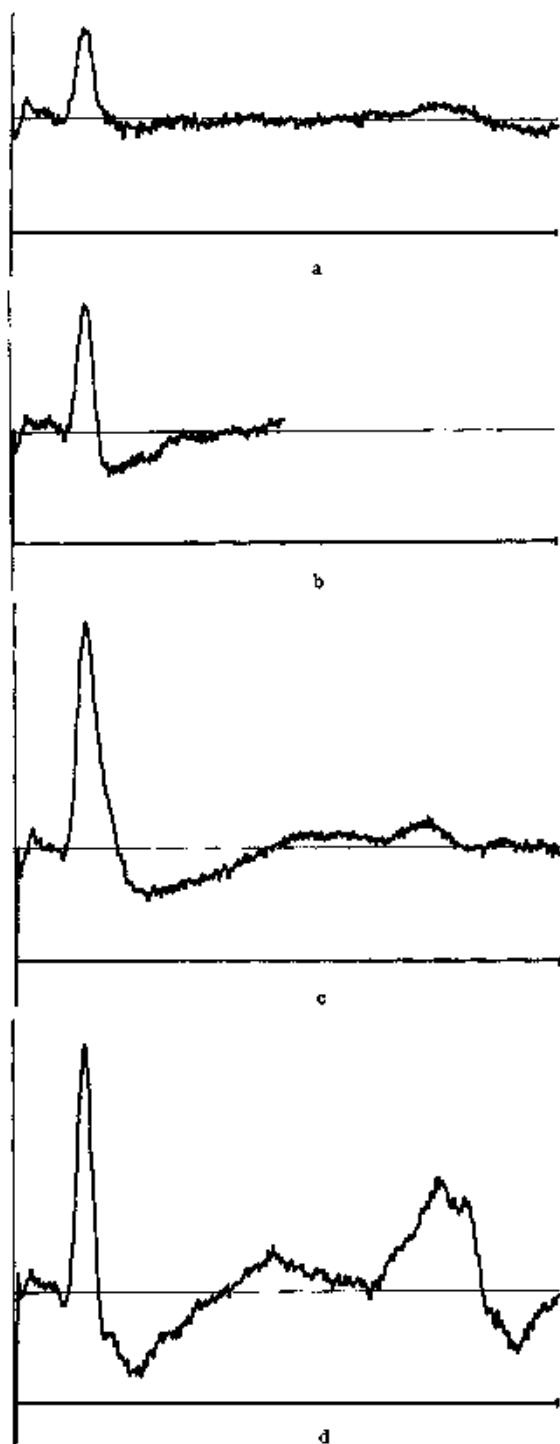


Abb. 12a-d. Veränderung der direkten und indirekten Reflexe im Rückenmark der Katze durch Apamin. Spinalisierte Katze, 2,5 kg. 1,15 mg/kg Apamin intravenös. Reizung des N. tibialis mit Rechteckimpulsen [0,2 msec Breite in 3 sec Folge; bei a und b mit 125 mV; bei c und d mit 183 mV (WELLHÖFER, unveröffentlicht)]. Bei Vergleich von a (= Kontrolle) und b (10 min nach Injektion) erkennt man die Zunahme der Amplitude des monosynaptischen Reflexes, bei Vergleich von c (= Kontrolle) und d (20 min nach Injektion) auch die des polysynaptischen Reflexes. Auswertung von jeweils 25 Reflexantworten mittels Rechner. Zeitachse: 16 msec

rische System betroffen; die athetotische Komponente läßt auf einen Angriff am Hirnstamm schließen (HABERMANN, unveröffentlicht).

Katzen geraten — wie Mäuse — unter Apamin (1 mg/kg) in extreme, mehrere Stunden anhaltende Erregung, die ca. 15 min nach intravenöser Applikation des Peptids beginnt. Wenn man die Hinterwurzeln von Spinalkatzen reizt und die Aktionspotentiale der Vorderwurzeln ableitet, findet man eine erhebliche Verstärkung der monosynaptischen und der polysynaptischen Reflexkomponenten. Die Reizschwelle wird gleichzeitig erniedrigt (WELLHÖNER, unveröffentlicht) (Abb. 12).

5. Hyaluronidase

Zahlreiche bakterielle und tierische Gifte enthalten Hyaluronidasen (Übersicht bei NEUMANN und HABERMANN, 1960). DURAN-REYNALS (1936) beobachtete erstmals spreading-Aktivität von Bienen-Gesamtextrakten. Nachdem man die Relationen zwischen Spreading und Hyaluronidase erkannt hatte, war auch die Genese dieses Gifteffekts klar (CHAIN u. DUTHIE, 1940). Schon Gesamtgift ist annähernd wirkungsgleich mit käuflichen hochgereinigten Präparaten aus Stierhoden. Eine weitere Reinigung gelingt durch Adsorption an den Kationenaustauscher Amberlite CG 50, gefolgt von Elution mit kontinuierlich ansteigender Ionenstärke, wobei besonders auf die präzise Einhaltung des pH-Wertes zu achten ist (HABERMANN, 1957c). — Ein zweiter Weg beginnt mit einer Gelfiltration an Sephadex G 50, wobei Hyaluronidase mit dem Ausschlußvolumen erscheint. Anschließend werden mittels Filtration durch Amberlite die verbliebenen, zum Teil braunen Begleitstoffe entfernt (HABERMANN u. REIZ, 1964, 1965a). Mit beiden Methoden erzielt man eine vierzig- bis fünfzigfache Anreicherung gegenüber dem Ausgangsmaterial. Das Endprodukt ist papierelektrophoretisch zwischen pH 3,0 und 9,0 einheitlich; eine nähere Bestimmung der molekularen Parameter steht noch aus. Dem Enzym kommt auf Grund seines Verhaltens an Sephadex G 50 ein höheres Molekulargewicht (größer als 22000) als den anderen bisher bekannten Wirkstoffen des BienenGiftes zu (STOCKEBRAND, 1965). Es ist auch wesentlich labiler gegen Hitze und Säure. Behandelt man zunächst mit Säure und läßt dann im Neutralen stchen, so kommt eine allmähliche Reaktivierung zustande.

Der Angriffspunkt des Enzyms am Molekül der Hyaluronsäure wurde von BARKER *et al.* (1963) geklärt. Als Hauptprodukte wurden ein Tetra- und ein Hexasaccharid isoliert, die jeweils äquimolare Mengen an N-Acetylglucosamin und Glucuronsäure enthalten. Ihre Sequenz ist $\text{GpAl} \beta 3 \text{GNAc} \beta 4 \text{GpAl} \beta 3 \text{GNAc}$ ($1 \beta 4 \text{GpAl} \beta 3 \text{GNAc}$). Das Bienengiftenzym hydrolysiert also die Polysaccharidkette an den gleichen Stellen wie Testis-Hyaluronidase. Die Wirkungsbedingungen der beiden Enzyme sind aber recht verschieden. So liegt das pH-Optimum des Giftenzyms zwischen pH 4 und 5; es ist wesentlich schärfer als das näher am Neutralpunkt gelegene Aktivitätsmaximum des

Enzyms aus Stierhoden. Wie andere Hyaluronidasen, so ist das Giftenzym durch Plasmabestandteile hemmbar, auch durch Polysaccharidschwefelsäureester (HABERMANN, 1957c). Es bestehen Hinweise darauf, daß die blutgruppenspezifischen Substanzen A und B, Chondroitinsulfat A und C sowie Heparin durch Bienengift gespalten werden können (BARKER *et al.* (1963); auf Grund des beschränkten Reinheitsgrades der eingesetzten Fermentpräparate kann aber nicht ausgeschlossen werden, daß neben Hyaluronidase noch andere polysaccharidspaltende Enzyme im Bienengift vorkommen.

Hyaluronidase begünstigt die Ausbreitung von Wasser und darin gelöstem bzw. dispergiertem Material im Interstitium. Dieser spreading-Effekt ist die einzige bemerkenswerte pharmakologische Wirkung der Bienengifthyaluronidase (HABERMANN, 1957c). Häufig wird angegeben, daß Hyaluronidase auch die Permeabilität der Hautcapillaren erhöhe. Hyaluronidase tritt jedoch bei quantitativer Betrachtung gegenüber anderen Permeabilitätsfaktoren des Bienengifts erheblich zurück. Auch bei partiell gereinigter Stierhodenhyaluronidase wurden Begleitstoffe als Träger der Permeabilitätswirkung erkannt (BENDITT *et al.*, 1951). Vielleicht läßt Hyaluronidase den Konzentrationsgradienten steiler werden, indem sie auf Grund des spreading-Effekts die Weiterdiffusion des durch die Gefäßwand gelangenden Materials im Interstitium erleichtert; so wäre ein indirekter Permeabilitätseffekt gegeben.

6. Phospholipase A

Zahlreiche Wirkstoffe von hohem pharmakologischem oder biochemischem Interesse kommen in tierischen Giften in beträchtlichen Konzentrationen vor, während sie in Gewebsextrakten nur mit Mühe nachweisbar sind. Tierische Gifte sind Fundgruben für Pharmaka mit potentieller physiologischer bzw. pathophysiologischer Bedeutung. So erscheinen, wie in der Einleitung erwähnt, die biogenen Amine Histamin, Serotonin und Acetylcholin in relativ hoher Konzentration in Hymenopterengiften; über ihre sonstige Bedeutung braucht hier nicht diskutiert zu werden. Basische, permeationsfördernde Peptide (Melittin, Apamin, F0-Komponenten) sind im Bienengift reichlich vertreten; inzwischen wurden derartige Verbindungen, denen allerdings die spezifischen neurotoxischen bzw. hämolytischen Eigenschaften fehlen, auch in Organen (s. z. B. FRIMMER u. HEGNER, 1963) gefunden. Hyaluronidase ist als in der belebten Natur außerordentlich verbreiteter spreading factor bekannt geworden. So lag die Vermutung nahe, daß auch der letzte der hier abzuhandelnden Giftfaktoren, die Phospholipase A, über das Bienengift hinaus von Bedeutung sei. Noch im Jahre 1957 lagen im wesentlichen Spekulationen über die Bedeutung körpereigener Phospholipasen vor; damals mußte festgestellt werden, daß unsere Kenntnisse über das Vorkommen von Phospholipase A in normalen oder pathologischen Geweben sehr beschränkt seien (NEUMANN und HABERMANN, 1957). Inzwischen wurde von verschiedenen

Seiten (vgl. Tabelle 8) gezeigt, daß Phospholipase A in zahlreichen Geweben und Körperflüssigkeiten existiert (Übersicht bei CONDREA u. DE VRIES, 1965) und daß ein ständiger Umsatz von Phospholipiden in der Zelle stattfindet, bei dem auch Lysophosphatide entstehen und weiter metabolisiert werden. Infolge des starken Tensidcharakters der Lysophospholipoide könnte also die Permeabilität von Zellgrenzen stoffwechselabhängig gesteuert werden.

Wegen der großen Verbreitung und der zunehmenden Bedeutung von Phospholipasen würde es den Rahmen dieses Reviews sprengen, wollte man diese Enzymgruppe als Ganzes abhandeln. Zudem sind jüngst einige ausführliche Übersichten erschienen, die sich mit ihr beschäftigen (CONDREA und DE VRIES, 1965; MELDRUM, 1965; VAN DEENEN und DE HAAS, 1966). Wenn wir uns weitgehend auf die Bienengiftphospholipase A beschränken, so sprechen dafür mehrere Gründe. Das Gift enthält außer der bei kaum einer Fragestellung störenden Hyaluronidase kein drittes Enzym in größerer Menge. Phospholipase A ist aus Bienengift in sehr gutem Reinheitsgrad darzustellen. Schließlich wurden im Laufe der letzten 15 Jahre gerade mit dem Bienengift-enzym eine Reihe wichtiger pharmakologischer und biochemischer Befunde erhoben, so daß es als Modell für andere Enzyme dieses Typs betrachtet werden kann. Zwar wurde von DOERY und PEARSON (1963) eine Phospholipase B im Bienengift beschrieben, wie sie vor allem im Wespen- und im Hornissen-gift existiert (s. S. 302). Jedoch spielt dieses Enzym, das wir in den uns zur Verfügung stehenden Giftchargen einstweilen nicht gefunden haben, im Bie-nengift sicher eine geringere Rolle als in den genannten anderen Giften.

a) Gewinnung

Der älteste Weg zur Auf trennung von Bienengift, die Elektrophorese, lieferte drei Fraktionen, von denen nur die langsamste (FII) Phospholipase A (neben Hyaluronidase) enthielt. Da eine andere Fraktion (FI) den direkt hämolysierenden, später Melittin ge-nannten Faktor mitführte, war damit bewiesen, daß das Enzym nicht das „Hämolsin“ des Gif tes sein konnte. Auch die Zuordnung der wichtigsten pharmakologischen und bio-chemischen Gifteffekte zu Phospholipase A (z. B. Wirkung auf strukturgebundene Enzym-systeme bzw. Gewebs-Thromboplastin) und Melittin gelang im Anschluß an diese ein-fache Prozedur. Zur Gewinnung größerer Mengen an reinem Enzym sind dagegen andere Verfahren besser geeignet (NEUMANN *et al.*, 1952; NEUMANN und HABERMANN, 1954a).

So kann man das Gift — entweder nach Vorfraktionierung mit Aceton oder besser unmittelbar — durch Amberlite CG 50 filtrieren, wobei die basischen Polypeptide relativ fest adsorbiert werden. Hyaluronidase und Phospholipase A erscheinen im Eluat und lassen sich an Aluminiumoxid-Säulen trennen (HABERMANN und W. P. NEUMANN, 1957). Statt Amberlite CG 50 kann auch eine Chromatographie an CM-Sephadex vorgeschaltet werden (BARKER *et al.*, 1966).

Der dritte und rationellste Weg schließlich führt über die Gelfiltration an Sephadex G 50, wobei das Enzym zwischen Hyaluronidase und Melittin aus der Säule tritt. Es kann dann durch Filtration über Amberlite CG 50 nach-gereinigt werden. Je nach Ausgangsmaterial sind die Endprodukte der Auf-arbeitung 7—13 mal wirksamer, so daß mit einem Phospholipase-Gehalt von

Tabelle 8. Verteilung und Eigenschaften von Phospholipase A in tierischem Material.
(Nach HEGNER, unveröffentlicht)

Vorkommen	Fraktion (intracelluläre Lokalisation)	Substrat (und Position)	Autor
Leber (Ratte)	Mitochondrienfraktion (1200 × g)	bes. Phosphatidyl- äthanolamin,	BJØRNSTAD, 1966a, 1966b
	Mikrosomenfraktion (100000 × g)	kaum Phosphat- idylcholin	
	(Lysosomen ?)	(α'- und β-Position)	
Intestinalschleimhaut (Ratte)	Mitochondrienfraktion	Phosphatidylcholin	EPSTEIN u. SHAPIRO, 1959
	Mikrosomenfraktion Überstand		
Intestinalschleimhaut (Ratte)	Ribosomenfraktion	Phosphatidylcholin	OTTOLENGHI, 1964
Intestinalschleimhaut, (Kaninchen, Ratte, Hund, Kalb)	Mitochondrienfraktion (700—2400 × g)	Phosphatidyl- äthanolamin, Phosphatidylserin starker Phosphat- idylcholin	SCHMIDT <i>et al.</i> , 1957
Ratte: Duodenal- schleimhaut, Colonschleimhaut, Magenschleimhaut, Niere, Leber, Lunge, Herz, Gehirn, Milz	30%ige Ammonsulfat- fällung vom 20000 × g des Homo- genates	Phosphatidylcholin (β-spezifisch α'-fraglich)	ROBERTSON, 1966
Gehirn (Mensch)	Acetonextrakt	Phosphatidylcholin Phosphatidyl- äthanolamin Phosphatidylserin	GALLAI-HAT- CHARD <i>et al.</i> , 1962
Heparinisiertes Plasma (Mensch)		Phosphatidyl- äthanolamin, schwächer Phosphat- idylcholin	VOGEL u. ZIEVE, 1964 VOGEL <i>et al.</i> , 1964
Pankreas (Kind)	Homogenat	Phosphatidyl- äthanolamin Phosphatidylserin, schwächer Phosphat- idylcholin	RIMON u. SHAPIRO, 1959
Pankreas (Mensch)	Homogenat	Phosphatidylcholin	MAGEE <i>et al.</i> , 1962
Pankreas (Mensch)	Homogenat	Phosphatidyl- äthanolamin (β-spezifisch)	VAN DEENEN <i>et al.</i> , 1963
Leber (Ratte)	Mitochondrienfraktion	Phosphatidyl- äthanolamin Phosphatidylcholin	SCHERPHOF u. VAN DEENEN, 1965

(Tabelle 8 Fortsetzung)

Vorkommen	Fraktion (intrazelluläre Lokalisation)	Substrat (und Position)	Autor
Leber (Ratte)	Mitochondrien (Lysosomen?)	Phosphatidylcholin	Rossi <i>et al.</i> , 1965
Leber (Ratte)	Mikrosomenfraktion	Phosphatidyl- äthanolamin, kaum Phosphat- idylcholin	BJØRNSTAD, 1966 b
Leber (Ratte)	Kernfraktion (1000 × g)	Phosphatidyl- äthanolamin $\beta > \alpha'$	SCHERPHOF <i>et al.</i> , 1966
	Mitochondrienfraktion (4500 × g)	$\beta \gg \alpha'$	
	Mikros. Mito. Lysos. (12500 × g)	$\alpha' > \beta$	
	Mikrosomen (100000 × g)	$\alpha' \gg \beta$	
	Überstand	$\alpha' \gg \beta$	
Gehirn (Ratte)	Homogenat 35fache Anreicherung	Phosphatidylcholin (α' -spezifisch)	GATT <i>et al.</i> , 1966
Polymorphkernige Leukocyten (Peritonealexsudat, Kaninchen)	Granulafraktion (8200 × g) Gesamthomogenat Überstand	Phosphatidylcholin	EISBACH <i>et al.</i> , 1963
Duodenalinhalt, Gallensaft, Pankreas- saft, Serum (Mensch) Pankreas (Hund)		Phosphatidyl- cholin Phosphatidyl- äthanolamin	VOGEL u. ZIEVE, 1960 ZIEVE u. VOGEL, 1961
Leber (Ratte)	Mikrosomenfraktion (23000—78000 × g)	α' -(1-Alkenyl)- glycerylphosphoryl- cholin	WARNER u. LANDS, 1961

8—14 % des Trockengiftes zu rechnen ist; die elektrophoretische Fraktion II besteht also im wesentlichen aus diesem Enzym (HABERMANN und REIZ, 1964, 1965 a). Das Endprodukt verhält sich in allen bisher angewandten elektrophoretischen und chromatographischen Tests einheitlich; seine Aminosäureanalyse ist in Tabelle 9 wiedergegeben (STOCKEBRAND, 1965). Nach dem Verhalten bei Gelfiltration zu schließen, liegt sein Molekulargewicht um 19000 (HABERMANN und REIZ, 1965 b). Als einziger N-Terminus wurde Leucin festgestellt (HABERMANN, unveröffentlicht). Der isoelektrische Punkt liegt bei pH 6,6. Das Enzym ist frei von Phosphor (HABERMANN und W. P. NEUMANN, 1957). Es sollte sich vorzüglich für Untersuchungen über Zusammenhänge zwischen Proteinstruktur und enzymatischer Wirkung eignen; denn es hat

nicht nur ein relativ kleines Molekulargewicht, sondern ist auch — zumindest im Säuren — erstaunlich thermostabil. Schließlich ist es bequem zugänglich.

b) Enzymatische Eigenschaften

α) Substrate. Die Spezifität der Phospholipoidspaltung durch das Bienen-giftenzym wurde noch nicht mit modernen Methoden der präparativen und analytischen Lipidchemie geprüft, wie sie bei anderen Phospholipasen, z. B. aus Cobragift, Crotalusgift oder Pankreas erprobt wurden (Übersicht bei CONDREA u. DE VRIES, 1965; MELDRUM, 1965; VAN DEENEN und DE HAAS, 1966).

Tabelle 9. Aminosäure-Zusammensetzung von Phospholipase A. (Nach STOCKEBRAND, 1965)

Aminosäure	μMol	μg
Lys	2,536	370,0
His	1,201	180,5
Arg	0,722	125,4
Asp	1,858	245,8
Thr	1,200	143,1
Ser	1,010	106,1
Glu	0,791	116,5
Pro	0,940	56,4
Gly	1,167	87,6
Ala	0,450	40,1
½ Cys	0,880	106,2
Val	0,563	65,6
Met	0,340	50,8
Ileu	0,380	49,9
Leu	1,035	136,0
Tyr	1,100	199,2
Phe	0,480	79,4
	16,203	2158,6*

* Entspricht einer Ausbeute von 91,7 Gew.-%.

Es besteht jedoch kein Anhalt dafür, daß sich die Phospholipase des Bienen-giftes in wesentlichen Punkten von anderen derartigen Enzymen unterscheidet; sie entspricht ihnen bezüglich relativer Stabilität beim Erhitzen und im Säuren, Aktivierbarkeit durch Lösungsmittler und Calciumionen. Auch ihr pH-Optimum liegt im erwarteten Bereich (pH 7,0—8,5 in Abhängigkeit vom Testsystem). So erscheint es erlaubt, auch die strukturellen Erfordernisse der Enzym-Substratbeziehung auf das hier besprochene Enzym zu übertragen.

Bei den natürlichen Phospholipiden handelt es sich um Glycerin-1,2-Diacyl-3-Phosphorsäure-Verbindungen⁴. Bis vor kurzem nahm man an, daß Phospholipasen vom Typ A daraus stets die dem polaren Anteil des Moleküls benachbarte Fettsäure abspalten; so werden auch die „unphysiologischen“ Glycerin-1,3-Diacyl-2-Phosphorylcholine hydrolysiert. In letzter Zeit mehren sich jedoch Hinweise darauf, daß in verschiedenen Organen (Leber, Milz, Lunge) Enzyme vorkommen, die an der 1-Ester-Position natürlicher Phosphoglyceride angreifen. Man findet nebeneinander 1- und 2-Acylysolecithine in der Rattenleber (VAN DEN BOSCH und VAN DEENEN, 1964, 1965). Man spricht also von Phospholipase A², wenn die Esterbindung in 2-Position hydrolysiert wird. Ihr ist die altbekannte thermostabile Pankreasphospholipase A gleichzusetzen, sicher auch das Enzym aus Crotalus-adamanteus-Gift, dessen Spezifität an verschiedenen isomeren Lysolecithinen eingehend geprüft wurde (DE HAAS und VAN

⁴ Zur Bezeichnung der Positionen mittels Zahlen oder Buchstaben vgl. das Formelbeispiel S. 268.

DEENEN, 1965) (Tabelle 10). Pankreasphospholipase A¹ (DE HAAS, SARDA und ROGER, 1965) und auch die Lecithin und Kolamin-Kephalin spaltende Post-heparin-Phospholipase A¹ (VOGEL und BIERMAN, 1965, 1967) des menschlichen Blutplasmas lassen sich dagegen nicht von begleitender Lipaseaktivität ab trennen; sie sind vielleicht mit den jeweiligen Organlipasen identisch. Die beiden Enzymtypen unterscheiden sich auch in ihrem Inhibitor-Spektrum und ihrer Stabilität (VOGEL u. BIERMAN, 1967). Interessant ist die Spekulation, daß eine Kombination von Phospholipase A¹ und A² zur kompletten Deacylierung der Esterphosphatide führen müßte. Die Phospholipase-Typen der Rattenleber

Tabelle 10. Hydrolyse isomerer Lysolecithins. (Nach DE HAAS und VAN DEENEN, 1965)

	Lysolecithin-Struktur				
	1-Ester	1-OH	1-OH	1-OH	1-OH
	HO-2	Ester-2	2-Ester	2-P-Base	Base-P-2
	3-P-Base	3-P-Base	3-P-Base	3-Ester	3-Ester
	Spaltbar durch				
Phospholipase A ^a	—	+	—	+	—
Phospholipase C ^b	+	+	—	—	+

* Crotalus-adamanteus-Gift; Bienengift-Phospholipase ist weniger spezifisch.

^b B. cereus.

sind nicht gleichmäßig zwischen den Partikelfaktionen verteilt. Mitochondrien enthalten — nach Versuchen mit Phosphatidyläthanolamin — offenbar vorzugsweise Phospholipase A², Mikrosomen Phospholipase A¹ (SCHERPHOF *et al.*, 1966; s. Tabelle 8).

Die sterische Konfiguration der Phospholipoide ist äußerst wichtig; nur L-Lecithine verfallen der Hydrolyse, nicht die entsprechenden D-Formen. Bedeutsam ist ferner, daß der Phosphorsäureanteil mehr als eine Ladung trägt. Sie kann negativ sein, wie bei Phosphatidsäuren, die durch Pankreasphospholipase A und Crotalus-adamanteus-Gift spaltbar sind (RIMON und SHAPIRO, 1950; VAN DEENEN und DE HAAS, 1963). Meist ist die zweite Ladung jedoch positiv, wie bei den „klassischen“ Substraten Lecithin, Serin- und Kolamin-kephalin, Cardiolipin sowie den entsprechenden Plasmalogenen. Inositolphosphatide und Sphingomyeline sind demgegenüber resistent gegen Phospholipase A; Plasmalogene werden langsamer gespalten als die entsprechenden Esterphosphatide, worauf eine Methode zu ihrer Anreicherung (mittels Crotalus-atrox-Gift) beruht. — Neben der sterischen und Positionsspezifität tritt die Bedeutung der Kettenlänge und des Sättigungsgrades der Fettsäuren zurück, wenn sie auch die Spaltungsgeschwindigkeit beeinflussen (Übersichten bei CONDREA und DE VRIES, 1965; VAN DEENEN und DE HAAS, 1966; s. auch S. 265). Glykolderivate können als Substrate für Crotalus-adamanteus-

Gift dienen, ebenso N-substituierte Kolamin-Kephaline (VAN DEENEN und DE HAAS, 1963).

Bienengiftphospholipase verhält sich, was die Spezifität gegenüber den bisher untersuchten Substraten betrifft, wie die aus *Crotalus-adamanteus*-Gift; das zeigt die vergleichende Tabelle 11 von VAN DEENEN und HEEMSKERK (1963). Bei der Hämolyse durch Bienen-Gesamtgift werden Lecithine und Kephaline der Erythrocytenwand gespalten (HEEMSKERK und VAN DEENEN, 1964).

Tabelle 11. *Abbau synthetischer Phosphatide durch Phospholipase A verschiedener Herkunft.*
(Nach VAN DEENEN und HEEMSKERK, 1963)

Emulsionen (10 mg/ml) in Boratpuffer pH 7 wurden mit 0,66 mg/ml Enzympräparation 3 Std bei 37° C inkubiert, dann die freigesetzten Fettsäuren quantitativ gaschromatographisch bestimmt.

Substrat	Hydrolyse (%) durch Phospholipase A aus		
	menschlichem Pankreas	Bienengift	<i>Crotalus-adamanteus</i> -Gift
γ-Stearoyl-β-Lauroyl-Lα-Lecithin	4 ^a	80	45
γ-Oleyl-β-Stearoyl-Lα-Lecithin	10	92	80
γ-Stearoyl-β-Oleyl-Lα-Kolaminkephalin	80	95	85
γ-Oleyl-β-Stearoyl-Lα-Kolaminkephalin	90	99	85

^a Durch Zusatz von Phosphatidsäure wird die Spaltung erheblich aktiviert.

β) *Umsatzbedingungen*. Die Substrate der Phospholipasen unterscheiden sich in einem wichtigen Punkt von denjenigen der meisten anderen Enzyme: sie liegen nicht als echte Lösungen vor, sondern in Form von Micellen oder Suspensionen. Auch die Reaktionsprodukte der Phospholipidspaltung, Lysolecithin und Fettsäuren, bilden Moleküllaggregate und reichern sich infolge ihres Tensidcharakters an den Grenzflächen Wasser/Lipoid an, und damit am Substrat des Enzyms. Die Kinetik der Enzym-Substrat-Reaktion ist daher sehr kompliziert; sie wird durch viele Faktoren modifiziert, deren Bedeutung gerade am Beispiel der Bienengiftphospholipase abgeklärt werden konnte. Wäßrige Lecithinsuspensionen werden durch das Enzym kaum angegriffen (GROSSE und TAUBÖCK, 1942); dagegen stellt eine Verdünnung nativen Eigelbs ein vorzügliches Substrat dar. Man versucht, den günstigen kolloidalen Zustand der Eigelb-Lipoproteine durch entsprechende Zusätze zu Lecithin-Emulsionen zu imitieren. So läßt sich eine beträchtliche Steigerung der Umsatzrate erzielen, wenn man Methanol (bis 40 Vol.-%) zusetzt (NEUMANN u. HABERMANN, 1954b). Die große Stabilität von Phospholipasen des Typs A gegenüber organischen Lösungsmitteln ist auch die Basis des Umsatzes von Phospholipiden in ätherischer Lösung. Das Enzym geht unter Bindung an das Substrat in die organische Phase. Das entstehende Lysolecithin ist ätherunlöslich, so daß seine Konzentration im Reaktionsgemisch niedrig bleibt (HANAHAN,

1952). Nachteil dieses Systems ist seine geringe Variabilität. Zusatz von ca. 9% Äther zur Wasserphase begünstigt den Umsatz von Lecithin durch Phospholipase der Giftschlange *Ancistrodon piscivorus* (MAGEE und THOMPSON, 1960), nicht aber durch Phospholipase A aus menschlichem Pankreas (MAGEE u. Mitarb., 1962). Äther und wohl auch die anderen organischen Lösungsmittel scheinen ihren günstigen Effekt zum Teil durch Verkleinerung der Emulsionen zu erzielen, zum Teil durch Entfernung der Fettsäuren von der Lipid-Wasser-Grenzfläche, wie sich aus der Messung des ζ -Potentials ergibt. Dadurch wird wohl dem Enzym der Zugang zu neuem Substrat erleichtert. In der Beeinflussung des ζ -Potentials könnte einer der Gründe dafür liegen, weshalb die einzelnen Phospholipoidklassen unterschiedlich gegen Phospholipase A empfindlich sind (DAWSON, 1963). So ist auch verständlich, daß z.B. Lysocephalin zusammen mit Lysolecithin entstand, wenn man Eigelb mit dem Gift von *Akistrodon piscivorus* inkubierte, während ein Kephalinpräparat (aus Gehirn) der Phospholipase A des Giftes widerstand (CHARGAFF und COHEN, 1939). Menge und Art der Aktivatoren haben sich nach dem jeweiligen Substrat zu richten. Sie sind — vielleicht außer Calcium — substrat- und nicht enzymspezifisch.

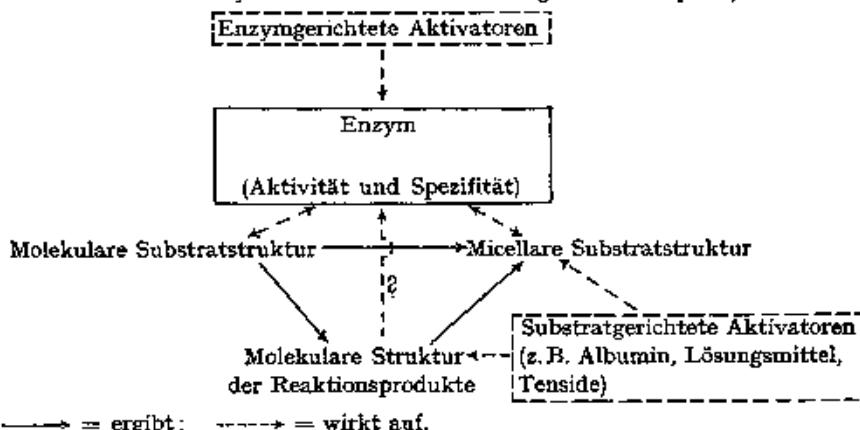
Neben organischen Lösungsmitteln begünstigen oberflächenaktive Substanzen den Umsatz. So läßt sich Bienengiftphospholipase unter Verwendung von Lecithin als Substrat durch Desoxycholsäure, Tween 20 und Lysolecithin aktivieren. Der Einfluß von Desoxycholsäure durchläuft ein Konzentrationsoptimum. Fettsäuren kommen deutlich. Zwei Reaktionen scheinen bei Einsatz von Tensiden einander entgegenzulaufen: Die Zerkleinerung der Micellen erleichtert die enzymatische Angreifbarkeit, während die Beladung der Grenzflächen mit dem Tensid dem Enzym den Zugang verlegen kann. Auch der Einfluß von Proteinen und Peptiden wird von hier aus verständlich. Serumalbumin ist ein kräftiger Aktivator der Bienengiftphospholipase. Daß es bei manchen rohen Enzympräparaten in dieser Hinsicht unbedeutend ist, dürfte mindestens zum Teil daran liegen, daß bereits mit dem Enzympräparat hinreichende Proteinmengen zugeführt werden. Der Effekt ist also im Sinne WILLSTÄTTERs als „ausgleichende Aktivierung“ zu verstehen. Auch Melittin aktiviert Phospholipase A, so daß nicht nur die „direkt“ hämolytische Wirkung des Peptids bei Zusatz von Phospholipase A verstärkt wird (s. S. 233), sondern auch die Phospholipoidspaltung bei Zusatz von Melittin (HABERMANN, 1957b).

Lecithine mit verschiedenen Acylresten bilden bei Dispersion mit Ultraschall in wäßrigen Lösungen Micellen unterschiedlicher Größe und Form aus. Je größer bei annähernd gleichem Micellargewicht (um 10⁶) das Achsenverhältnis ist, desto besser wird das Lipid gespalten. Die Kettenlänge der Acylreste spielt dabei eine untergeordnete Rolle; denn es ergibt sich folgende Reihe der Spaltbarkeit mit Phospholipase A: 1,2-Dibutyryl < 1,2-Dioleyl < 1-butyrtyl,

2-oleyl < 1-oleyl, 2-butyryl (ATTWOOD *et al.*, 1965). Da während der Hydrolyse oberflächenaktive Fettsäuren und Lyssolecithin freiwerden, ferner durch die Spaltung des Substrates selbst eine Änderung der Micellengröße eintritt, läßt sich weder die Beziehung zwischen Substratstruktur und Enzym noch die Relation zwischen Substratstruktur und Micellenform auf einfache Formeln zurückführen. Die Wechselbeziehungen sind in folgendem Schema (Tabelle 12) dargestellt.

Ungeklärt ist einstweilen der Mechanismus der Förderung der Phospholipidspaltung durch Calcium, mit der wohl auch ihre Hemmung durch Calcium bindende Substanzen, wie Fluorid und Citrat zusammenhängt. Die Annahme liegt am nächsten, daß Calcium

Tabelle 12. Enzym-Substrat-Wechselbeziehungen von Phospholipase A



über Kalkseifenbildung die freiwerdenden Fettsäuren abzubinden vermöchte (HANAHAN *et al.*, 1960). Andere Autoren nehmen eine Komplexbildung zwischen Calcium und Enzym an.

Inhibitoren von Bienengift-Phospholipase A sind zwar recht zahlreich; doch ist bisher kein Hemmstoff hoher Selektivität bekannt. Erwartungsgemäß inhibieren Fluorid und Citrat. Phosphat mindert die Aktivität der Bienengiftphospholipase wenig, die von Crotalus-terribilis-Gift dagegen sehr stark. Auch Schwermetallsalze hemmen, z.B. Zn^{++} und Hg^{++} ; doch sind bei den angewandten Konzentrationen (um 10^{-3} M) bereits unspezifische Reaktionen mit dem Enzymprotein zu erwarten (HABERMANN, 1957 b).

γ) Aktivitätsmessung. Die älteste Methode besteht in der Bestimmung des Lyssolecithins an Hand seines hämolytischen Vermögens. Der entscheidende Vorteil liegt in der großen Empfindlichkeit des Verfahrens; doch ist es schwierig, das gebildete Lyssolecithin quantitativ zu erfassen. Wegen der variablen Sensibilität der Erythrocytensuspension muß stets ein Enzym-Standard mitgeführt werden (NEUMANN und HABERMANN, 1954 b). Selbst dann können noch Störungen auftreten. So wird Lyssolecithin durch begleitendes, nicht umgesetztes Lecithin oder Cholesterin inaktiviert. Als Ausweg bietet sich die Aufarbeitung der Spaltungsansätze an, etwa durch Chromatographie auf dünnen Kieselgel-Schichten oder auf mit Kieselgel imprägniertem Papier. Anschließend kann man die Lyssolecithinfraktion entweder extrahieren und biologisch bestimmen oder nach Veraschung ihren Phosphatgehalt ermitteln.

Lysolecithin lysiert nicht nur Erythrocyten, sondern ändert infolge seines Tensidcharakters auch die Oberflächen und damit die optischen und thermischen Eigenschaften der verschiedensten Suspensionen bzw. Emulsionen von Lipoproteinen. Als sehr handlich hat sich der Dottercoagulationstest erwiesen, der auf einer Beobachtung von BOQUET *et al.* (1950) beruht: Eigelb gerinnt in der Hitze nicht mehr bzw. stark verzögert, wenn man es zuvor mit Cobragift behandelt hat. FLECKENSTEIN u. Mitarb. (1952) standardisierten das Verfahren und stellten Beziehungen zum dehydrasenhemmenden Prinzip der tierischen Gifte fest. HABERMANN und NEUMANN (1954a) verglichen hämolytisches Vermögen und Verzögerung der Dottercoagulation durch Bienengift im nämlichen Ansatz und fanden strengen Parallelismus bei Variation der Inkubationsbedingungen und bei Einsatz von Inhibitoren. Einige Abweichungen wurden bei Zusatz basischer Substanzen, wie Chinin, Antistin⁶ und Atosil⁸ beobachtet, die selbst die Gerinnung von Eigelb beschleunigten. Lysolecithin und Ölsäure, beides Reaktionsprodukte der Phospholipase A, verzögerten die Hitzecoagulation. — Auf dem gleichen Prinzip bauen turbidimetrische Methoden zur Bestimmung des Enzyms auf, welche an Empfindlichkeit bisher noch nicht übertroffen werden. HABERMANN und NEUMANN (1954a) fanden eine deutliche Eigelb-Klärungsreaktion bereits bei Einsatz von 10^{-3} µg Gesamtgift, also etwa 10^{-4} µg Enzym/ml Ansatz. DOIKAKI und ZIEVE (1964) setzten in neuerer Zeit turbidimetrische Verfahren zur Bestimmung körpereigener Phospholipasen ein.

Man kann auch in konventioneller Weise das Verschwinden von Esterbindungen bzw. die Zunahme der freien Carboxylgruppen verfolgen. Hierzu bieten sich Hydroxamatreaktion (RAPPORT und FRANZL, 1957), titrimetrische Verfahren (FAIRBAIRN, 1945; HANAHAN, 1952; KOCHOLATY, 1966) oder manometrische Techniken (ZAMCNIK *et al.*, 1947; HABERMANN, 1957b) an.

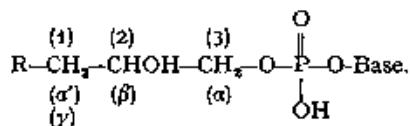
c) Zur Biochemie der Lysophospholipoide

Die pharmakologischen Effekte von Phospholipase A wie auch ihre Wirkungen auf Enzymsysteme kommen durch Phospholipoidspaltung zustande; doch darf man nicht damit rechnen, daß sie durch Lysophospholipoide völlig reproduziert werden könnten. Zunächst muß man das zweite Spaltprodukt, die Fettsäuren, in Rechnung stellen; außerdem weiß man nicht, welches Lysophospholipoid im einzelnen entsteht; schließlich ist es oft unklar, ob das Verschwinden funktionell wichtiger Phospholipoide oder das Auftreten ihrer Spaltprodukte die pharmakologische Reaktion auslöst. Dennoch muß hier zum besseren Verständnis der Phospholipase A-Effekte kurz auf die Struktur der Lysophospholipoide und deren Beziehung zu den pharmakologischen Effekten eingegangen werden. Ihre Wirkungen auf einzelne Systeme sollen später im Zusammenhang mit denjenigen von Phospholipase A abgehandelt werden.

⁶ 2-Phenyl-benzyl-aminomethyl-imidazolin.

⁸ N-(2'-Dimethylamino-propyl)-phenothiazin.

Lysophosphatide besitzen die allgemeine Struktur



Daraus geht unmittelbar ihr Tensidcharakter hervor; denn bei dem Rest R handelt es sich entweder um einen Acyl-(Esterphosphatide) oder einen Vinylrest (Plasmalogene); er stellt also die hydrophobe Seite dar. Auch der basische Rest auf der hydrophilen Seite ist variabel. Lysolecithin, Lysophosphatidylserin (RATHBONE *et al.*, 1962), Lysophosphatidylcholin, Dihydro-Lysophosphatidylcholin (HARTREE und MANN, 1960) sind in ihrem hämolytischen Vermögen vergleichbar. Die übrigen möglichen Lysoverbindungen scheinen bisher nicht näher untersucht worden zu sein. Vom Lysophosphatidyläthanolamin existieren ältere Angaben (LEVENE *et al.*, 1924); es scheint ähnlich stark wie Lysolecithin zu hämolysieren; Lysophosphatidyläthanolamin, das aus Eigelb mit Hilfe des Giftes von *Trimeresurus flavoviridis* dargestellt worden war, hämolysierte Erythrocyten vom Schaf sogar stärker als Lysolecithin (MATSUMOTO, 1961). Es liegen jedoch noch keine systematisch vergleichenden Untersuchungen über die relative pharmakologische bzw. biochemische Aktivität der einzelnen Lysoverbindungen vor. Nicht nur die Bedeutung der Substitution im hydrophilen Anteil müßte abgeklärt werden. Kettenlänge und Sättigungsgrad des Acylrestes wären in die Betrachtung einzubziehen, schließlich auch dessen Position; denn in Organen kommen nebeneinander 1-Acyl- und 2-Acyl-Lysolecithine vor. Da jeweils noch optische Antipoden möglich sind, stellen die Lysoverbindungen eine reiche, bisher noch nicht systematisch untersuchte Gruppe von Pharmaka dar.

Einige Linien dieser Struktur-Wirkungs-Beziehungen wurden von GOTTFRIED u. RAPPORT (1963) verfolgt. Die Art der Bindung zwischen Paraffinkette und Glycerin war ohne wesentlichen Einfluß auf das lytische Vermögen; 1-Acyl, 1-Alkenyl- und 1-Alkyl-Lysoverbindungen unterschieden sich nicht wesentlich. Differenzen zwischen im fibrigen gleichartig gebauten Cholin- und Colaminverbindungen waren eher auf unterschiedliche Löslichkeit als auf unterschiedliche Wirksamkeit zu beziehen. Dagegen wurde das hämolytische Vermögen erheblich gesteigert, wenn man die ungesättigten Acylreste hydrierte. Nachdem sich bei den natürlichen Phospholipiden die ungesättigten Fettsäurereste vorwiegend in 2-Stellung befinden, ist die zunächst geringere hämolytische Aktivität natürlicher 2-Acyl-Lysolecithine einleuchtend. Hydrierung nähert ihr hämolytisches Vermögen demjenigen der 1-Acyl-Lysolecithine. Auch in den Versuchen von REMAN u. VAN DEENEN (1967) erwies sich Oleyl-Lysolecithin gegenüber Stearoyl-Lysolecithin als schwächer hämolytisch; Myristoyl-Lysolecithin lag in der Aktivitätsreihe zwischen beiden, Decanoyl-Lysolecithin war kaum wirksam. Im untersuchten Bereich besteht also eine deutliche

Abhängigkeit des hämolytischen Vermögens von der Kettenlänge. Neue Aspekte bringt jedoch die Entdeckung, daß auch Didecanoyl-Lecithin (etwa so stark wie Myristoyl-Lysolecithin) hämolsiert, und zwar viel stärker als sein Lyso-Derivat. Diheptanoyl-Lecithin lysiert kaum, Dimyristoyl-Lecithin im untersuchten Bereich nicht, (1-Oetyl-2-Butyryl)-Lecithin nur schwach. Die Resultate zeigen, daß die Lysolecithinstruktur keine Vorbedingung für das hämolytische Vermögen von Phospholipoiden ist; sie lassen vermuten, daß der hydrophobe Anteil des Moleküls eine optimale Größe im Vergleich zum hydrophilen Anteil aufzuweisen hat. Ob sich die Kohlenstoffatome auf eine aliphatische Kette oder auf deren zwei verteilen, mag nur für quantitative Betrachtungen bedeutsam sein.

An Schichten von Erythrocyten-Lipoiden ergibt sich die Aktivitätsreihe: Stearoyl-Lysolecithin = Oleyl-Lysolecithin > Didecanoyl-Lecithin > Myristoyl-Lysolecithin (REMAN u. VAN DEENEN, 1967). Das Modell gibt also die Verhältnisse an der Erythrocyten-Membran nicht genau wieder.

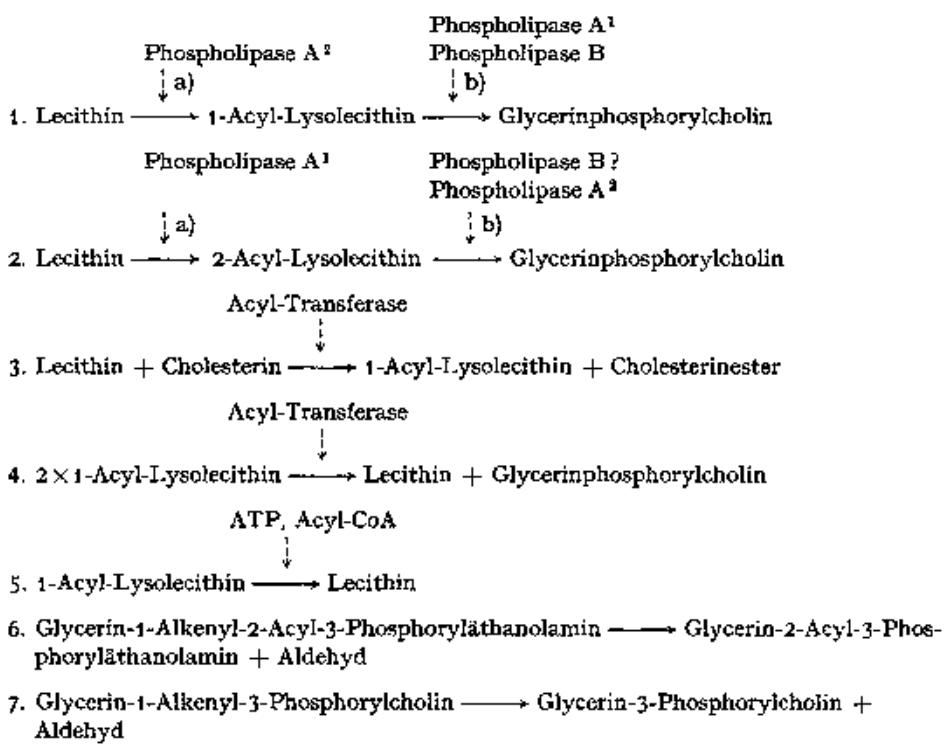
PERRIN und SAUNDERS (1964) ermittelten den Aggregatzustand von Eigelb-Lysolecithin in wässriger Lösung durch Messung von Diffusion und Sedimentation. Das „Molekulargewicht“ einer wasserfreien Micelle wurde zu 95 000 errechnet; auf Grund von Viscositätsmessungen wurde auf ein Achsenverhältnis der Ellipsoide von 3,5 (prolates Ellipsoid) bzw. 4,0 (oblates Ellipsoid) geschlossen. Die Werte stehen in guter Übereinstimmung mit dem von ROBINSON und SAUNDERS (1959) mit der Lichtstreuungsmethode gefundenen Micellargewicht von 92 400. Es würden sich demnach rund 180 Lysolecithinmoleküle zu einer Micelle zusammenlegen. Die Verteilung des Micellargewichts um den Mittelwert wurde offenbar noch nicht studiert.

SAUNDERS (1966) hat in letzter Zeit die bisher vorliegenden physikalisch-chemischen Daten wässriger Dispersionen von Phospholipiden mit der Annahme dreier Aggregat-Typen gedeutet; er unterscheidet sphärische, helikale und gefaltete lamelläre Aggregate. Entscheidende Faktoren sind dabei die Raumfüllung der einzelnen Moleküle, vor allem die Größe der Steradiane/Molekül, weiter die elektrostatische Wechselwirkung zwischen benachbart liegenden phosphor- und stickstoffhaltigen Gruppen, schließlich hydrophobe und van der Waalssche Kräfte. Nach SAUNDERS dürfte Lysolecithin sphärische Micellen bilden, ein Gemisch aus Lysolecithin und Lecithin (2:1) helikale. Eigelb-Lecithin soll sich zu gefalteten Lamellen anordnen; dies gilt jedoch nur für solche Lecithine, deren Acylreste sich bei der Dispersion in „flüssiger“ Form befinden, nicht z.B. für Dipalmitoyl- und Distearoyl-Lecithin. Die Micellen-Form für 1-Butyryl-2-Oleyl-Lecithin dürfte intermediär zwischen Helix und gefalteter Lamelle liegen, die für 1-Oetyl-2-Butyryl-Lecithin dagegen helikal sein. Diese Zustandsformen würden nicht nur das Verhalten der Phospholipoide und ihrer Gemische, auch mit Cholesterin, in Dispersion erklären; auch auf die Angreifbarkeit für Phospholipase A lassen sich Rückschlüsse ziehen.

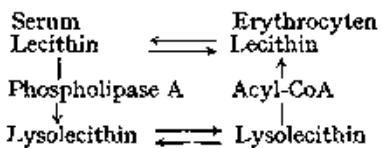
Es würde den Rahmen dieses den Hymenopterengiften gewidmeten Referates sprengen, wollte man alle Reaktionen des Organismus aufführen, welche zur Bildung und zum Abbau von Lysophospholipiden führen. Das Schema ist bis heute noch nicht vollständig, weil die einzelnen Reaktionsgleichungen meist nur an Lecithinen abgeleitet wurden. Es muß jedoch auf die Möglichkeit

des Abbaus von Lysolecithin bzw. seiner Resynthese zu Lecithin hier besonders eingegangen werden, weil davon auch Dauer und Stärke der pharmakologischen Wirkung exogen zugeführter Lysoverbindungen, z.B. bei der Hämolysse (MUNDER *et al.*, 1965) abhängen kann. Folgende Reaktionen sind möglich (Übersicht bei VAN DEENEN und DE HAAS, 1966; MUNDER *et al.*, 1965; s. auch S. 271) (Tabelle 13).

Tabelle 13. Einige Reaktionsmöglichkeiten von Phospholipoiden



MULDER und VAN DEENEN (1965) kombinieren die in Serum und Plasma ablaufenden Prozesse zu folgendem System:



Lysolecithin kann nach den heutigen Kenntnissen nur aus Lecithin entstehen, nicht aus seinen Bausteinen synthetisiert werden. Der Entstehungsort des im Blutplasma vorkommenden Lysolecithins (Übersicht bei MISRA, 1965) und Lysophosphatidylthanolamins (MISRA, 1965) ist unbekannt.

Welche der aufgeführten Reaktionen vorzugsweise abläuft, hängt vom Gehalt an Enzymen und Cofaktoren ab. So ließ sich bisher in reifen Erythro-

cyten im Gegensatz zu zahlreichen anderen Geweben keine Phospholipase A-Aktivität (Reaktion 1 bzw. 2) nachweisen; auch Reaktion 4 scheint in diesem System zurückzutreten. Dagegen konkurrieren Reaktion 1 b (bzw. 2b) mit Reaktion 5: der ATP-Gehalt des Systems bestimmt offenbar, ob eine Resynthese zu Lecithin oder ein weiterer Abbau des Lysolecithins stattfindet (ein analoger Befund wurde auch am Gehirn erhoben; s. S. 272). Die stärkere lytische Wirkung von Lysolecithin bei Erniedrigung der Temperatur wird auf die dabei verminderte Umsetzung durch die zelleigenen Enzyme bezogen. Der stabilisierende Effekt von sublytischen Lysolecithinkonzentrationen auf gealterte menschliche Erythrocyten könnte mit seinem — in Gegenwart von höheren ATP-Mengen verstärkten — Einbau in das zelleigene Lecithin zusammenhängen (MUNDER *et al.*, 1965).

STEIN und STEIN (1965) haben die Verteilung von biosynthetischem, radioaktiv (^{32}P) markiertem Rattenleber-Lysolecithin zwischen Perfusionsflüssigkeit und Rattenherz untersucht. Lysolecithin wird schnell vom Herzen aufgenommen und verteilt sich darin gleichmäßig; zum Teil wird es zu Lecithin acyliert. Das markierte Lysolecithin wird durch proteinfreies Perfusionsmedium nicht eluiert, wohl aber durch albuminhaltige Lösungen; markiertes Lecithin verläßt das Herzgewebe nicht. Bei Zusatz von Lysolecithin zu Ratten- oder Humanserum und anschließender Papierelektrophorese wandert die Hauptmenge des Lipids mit Albumin, eine kleinere Menge im β -Lipoproteinkontinentbereich. Es wird postuliert, daß Lysolecithin an einen Gewebs-Akzeptor gebunden werde und beim Aufbau von Zellmembranen wichtig sei. Die bei diesen Versuchen unmittelbar sichtbare Albuminbindung macht die Minderung der Lysolecithin-Aktivität gegenüber Erythrocyten bzw. Thrombocyten bei Albuminzusatz verständlich (s. S. 275).

Zugeführtes Lysolecithin wird offenbar in den Orten seiner Bindung weitgehend zum Teil zum Aufbau von Lecithin verwendet, zum Teil hydrolysiert. Das gilt für Schnitte und isolierte Präparate des Dünndarms von Ratte und Hamster (NILSSON und BORGSTRÖM, 1967), für Ascitestumorzellen (Maus) *in vitro* und *in vivo* bei intraperitonealer Injektion (STEIN u. STEIN, 1967), für die Leber (Maus) *in vivo* bei intravenöser Injektion (STEIN u. STEIN, 1967), für die Umbilicalarterie des Menschen und die A. carotis des Hundes (EISENBERG *et al.*, 1967). Für die Lysolecithinase der Tumorzellen und der Nabelschnurarterie wird eine Position an der Zellmembran postuliert.

Zusammenfassende Bemerkungen über den Lysolecithin-Stoffwechsel im Zentralnervensystem finden sich bei WEBSTER (1966). Folgende Reaktionen wurden nachgewiesen: Lecithin \rightarrow Lysolecithin + Fettsäure. Kolaminkephalin \rightarrow Lyso-Kolaminkephalin + Fettsäure (GALLAI-HATCHARD *et al.*, 1962). Kolamin-Plasmalogen \rightarrow Lysokephalin + Aldhyd (ANSELL und SPANNER, 1965). Lysolecithin \rightarrow Glycerinphosphorylcholin + Fettsäure (DAWSON, 1956; MARPLES und THOMPSON, 1960); Lysolecithin + (CoA-)Fettsäure \rightarrow Lecithin.

Nachdem zur Acylierung von Coenzym A Energie bereitgestellt werden muß, reichert sich Lysolecithin unter anaeroben Bedingungen wesentlich stärker an als unter aeroben (WEBSTER und THOMPSON, 1965).

Beträchtlichen Lysolecithinabbau fand DAWSON (1956) in Präparaten von Niere, Milz und Blut der Ratte, während Herz oder Skelettmuskel nur wenig Glycerinphosphorylcholin freilegte. Auch in Leber, Schilddrüse und Niere des Schafes wurde das Enzym gefunden.

Als nicht-enzymatische Veränderung des Lysolecithinmoleküls ist die sog. Acyl-Wanderung anzusprechen, die sich z. B. in einer Änderung der Substrat-eigenschaft für Pankreas-Phospholipase manifestiert. Wie erwähnt, greift das Enzym nur 2-Acyl-Lysolecithin an. Bei Aufbewahrung in der Kälte als Substanz oder bei Zimmertemperatur in Lösung, auch bei Chromatographie an Kieselgel ändert sich die Phospholipaseempfindlichkeit beträchtlich (SLOTHBOOM *et al.*, 1967).

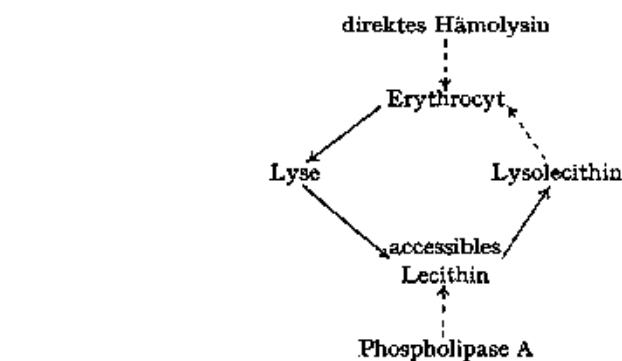
d) Wirkungen von Phospholipase A und ihren Reaktionsprodukten auf pharmakologische Systeme

Nicht alle Phospholipasen vom Typ A können als in pharmakologischer bzw. biochemischer Hinsicht gleichwertig betrachtet werden. Phospholipase A von Crotalus-terrificus-Gift wird im Gegensatz zum Bienengiftenzym durch Phosphat stark gehemmt; darauf wurde bereits (s. S. 266) hingewiesen. Cobragift-Phospholipase A spaltet ganz allgemein die Lipoide von relativ gering vor-geschädigten Zellstrukturen; damit Vipera-palestinae-Phospholipase angreifen kann, müssen massivere Schädigungen eingetreten sein. So kann das Cobragift-Enzym die Phospholipoide osmotisch hämolysierter Erythrocyten spalten, nicht dagegen das Enzym aus Vipera-palestinae-Gift (CONDREA, MAMMON *et al.*, 1964; CONDREA, DE VRIES u. MAGER, 1964). Analoges gilt für Blutplättchen (KIRSCHMANN *et al.*, 1964) und Leukocyten (KLIBANSKY, SHILOAH *et al.*, 1967). In den Giften von Agkistrodon halys und Crotalus adamanteus wurden zwei, im V.-russelli-Gift sogar drei verschiedene Phospholipasen nachgewiesen, die als Isozyme zu bezeichnen wären. Zwei von den drei Russell viper-Enzymen hydrolysierten Phospholipoide in osmotisch gewonnenen Erythrocytenmem-branen, das dritte Enzym war hierzu nicht imstande (Übersicht bei CONDREA und DE VRIES, 1965).

a) *Hämolyse*. Früher brachte man die Hämolyse durch Bienen- und Schlangengift nahezu ausschließlich mit Phospholipase in Zusammenhang. Das Enzym sollte das Lecithin der intakten Erythrocytenwand spalten und so auf doppeltem Wege — durch Zerstörung eines Bausteins der Zellwand und durch Freilegung des hämolysierenden Lysolecithins — den Austritt des Zellinhalts ermöglichen. Diese auf den ersten Blick plausible Deutung wurde aber der alten Beobachtung nicht gerecht, daß manche phospholipasereiche Gifte nicht bzw. nur schwach hämolysierten. Es wurden überdies für Schlan-

gengift (ROY, 1945) und Bienengift (GROSSE und TAUBÖCK, 1942) Hinweise beigebracht, daß sie neben Phospholipase A noch einen unmittelbar zellschädigenden Faktor enthalten könnten. Der Nachweis eines „direkt“, d.h. in Abwesenheit von extracellulärem Lecithin hämolysierenden Faktors neben der nur indirekt, d.h. bei Substratzusatz lytischen Phospholipase A wurde erstmalig beim Bienengift erbracht (s. S. 233). Inzwischen wurde die Existenz direkt wirkender basischer und darin dem Melittin vergleichbarer Hämolsine für die Gifte von *Naja nigricollis* und *Naja haje* evident (HABERMANN, 1954d; HABERMANN und NEUMANN, 1954b), später für eine Reihe weiterer Gifte (Übersicht bei CONDREA und DE VRIES, 1965). Phospholipasen von

Tabelle 14. Autokatalytisches Schema der Kombination „direkter“ und „indirekter“ Hämolsine



—→ = ergibt; - - - - → = wirkt auf.

Schlangengiften, z.B. *Crotalus adamanteus* (HEEMSKERK und VAN DEENEN, 1964), auch Pankreasphospholipase A (IBRAHIM und THOMPSON, 1965) können zwar die Phospholipide von Erythrocytenschatten, nicht aber diejenigen von intakten Erythrozyten spalten; analoge Verhältnisse wurden früher (HABERMANN, 1955a, 1958a) für Bienengift-Phospholipase A nachgewiesen. Rohe Schlangengifte, wie sie von IBRAHIM und THOMPSON verwendet wurden, enthalten die bereits genannten „direkt lytischen“ Faktoren. Man kann nicht daraus, daß derartige Gemische „direkt“ hämolysieren, auf eine — vielleicht spezifisch — direkt hämolysierende Phospholipase A schließen. Der von IBRAHIM und THOMPSON betonte Parallelismus zwischen Phospholipidspaltung und „direkter“ Hämolysen durch erhitztes *Naja-naja*-Gift läßt sich viel einfacher dadurch erklären, daß die darin enthaltene Phospholipase A die Lipide der Erythrocytenschatten, welche bei der durch das „direkte“ Hämolsin ausgelösten Zellschädigung anfallen, sofort zu Lysolecithin abbauen kann. Die Befunde von CONDREA, DE VRIES und MAGER (1964) mit *Naja-naja*-Gift zeigen, daß sich das „autokatalytische“ Schema (Tabelle 14) der Bienengift-hämolysen auch auf Schlangengift anwenden läßt. Allerdings unterscheiden

sich manche Phospholipasen vom Typ A voneinander in ihrer Substrataffinität, so daß z. B. Vipera-palestinae-Phospholipase A die Phospholipoide osmotischer Hämolsate nur nach zusätzlicher Ultraschallbehandlung zu lysieren vermag, während Cobragift-Phospholipase A auch „simple“ osmotisch gewonne Schatten angreift. Vipera-palestinae-Phospholipase A ruft auch eine geringere Mitochondrienschwellung hervor als das Cobragiftenzym. Das Zusammenspiel zwischen „direkt lysierendem“ Faktor des Cobragifts und Phospholipase A läßt sich bei vergleichenden Untersuchungen an Erythrocyten verschiedener Tierarten zeigen. So bestimmt die Empfindlichkeit gegen den „direkt“ lytischen Faktor, ob eine Erythrocytenart überhaupt lysiert wird. Die Phospholipoide von gegen Cobragift empfindlichen Erythrocyten sind erst dann mit der Phospholipase vom Vipera-palestinae-Gift spaltbar, wenn man (nach osmotischer Hämolyse) den direkt lytischen Faktor zugesetzt hat (CONDREA, MAMMON *et al.*, 1964). Die Phospholipase A des südindischen Skorpions *Heterometrus scaber* greift — wie die entsprechenden Enzyme höherer Tiere — zwar die Phospholipoide von Erythrocytenschatten an, nicht aber die von intakten Erythrocyten; übrigens läßt sie sich durch Ammonsulfat-Fällung vom toxischen Prinzip trennen (KURUP, 1966). Die Analogie zwischen Bienengiftphospholipase A und dem entsprechenden Enzym aus *Crotalus-adamanteus*-Gift reicht bis in die experimentellen Details. Nach HABERMANN (1957a) werden gewaschene Meerschweinchenerthrocyten (im Gegensatz zu denjenigen der bisher untersuchten übrigen Tierspecies und des Menschen) durch Phospholipase A des Bienengiftes lysiert; das gleiche gilt für *Crotalus-adamanteus*-Gift (HEEMSKERK und VAN DEENEN, 1964). Der bereits diskutierte „Lawinenmechanismus“ läßt sich auch für *Crotalus*-Phospholipase demonstrieren. Die Lipide zunächst phospholipase-resistenter Zellen werden zum Teil gespalten, wenn man die Zellen osmotisch hämolsiert; noch besser schließt eine Lysolecithin-Hämolyse auf. Wahrscheinlich genügt eine Auflockerung der Zellwand durch nicht-hämolsierende Konzentrationen an Desoxycholat, um die Lipide für Phospholipase A zugänglich zu machen. — Die Mechanismen der Hämolyse durch Cobragift bzw. Bienengift entsprechen sich also im Prinzip, allerdings mit dem Unterschied, daß bei der cobragiftbedingten Lyse der direkt hämolsierende Faktor relativ schwach ins Gewicht fällt. WILLE und VOET (1965) rechnen damit, daß der basische „direkt lytische“ Faktor des Cobragifts selbst nicht lysiert, sondern die Zelle durch Blockade saurer Gruppen, s. B. Neuraminsäure, für Phospholipase A zugänglich macht. Sie schließen dies aus der Temperaturabhängigkeit und der Aktivierbarkeit durch Ca^{++} .

Die Hämolyse ist der Endzustand einer Permeabilitätserhöhung, die sich unmittelbar auf strukturelle Veränderungen der Zellwand zurückführen läßt. So erklärt sich der prolytische Kaliumverlust, der „osmotische“ Typ der Lysolecithinhämolyse, auch ihre relativ geringe Abhängigkeit vom Salzgehalt

der Suspensionslösung (HABERMANN, 1958a). Der unspezifische Charakter der Primärwirkung des Lysolecithins — einfacher ausgedrückt seine universelle Grenzflächenaktivität — bedingt, daß die Lysolecithinhämolyse durch Zusatz zahlreicher vor allem lipoider Stoffe abgeschwächt werden kann, z. B. durch Cholesterin (DELEZENNE und FOURNEAU, 1914) und Lecithin (HABERMANN, 1958a), aber auch durch Albumin (KLIBANSKY und DE VRIES, 1961).

Um wirksam zu werden, muß Lysolecithin an Erythrocyten gebunden werden. So könnte erklärt werden, daß die Lysolecithinhämolyse wie die Digitoninhämolyse in der Kälte durch niedrigere Lysin-Konzentrationen ausgelöst werden kann als in der Wärme; eine andere Deutung basiert auf der bei höherer Temperatur rascheren Umsetzung von Lysolecithin zu nicht bzw. schwach hämolysierenden Produkten (s. S. 270). Der hämolytische Prozeß selbst wird durch Wärme begünstigt (COLLIER, 1952; HABERMANN, 1958a; JUNG, 1959). Zur Auslösung der Hämolyse ist die Adsorption einer Mindestmenge Lysolecithin pro Zelle erforderlich. Dementsprechend sinkt die Menge an freigesetztem Hämoglobin, wenn man bei konstanter Lysolecithin-Konzentration die Zellzahl erhöht (HABERMANN, 1958a). KLIBANSKY u. Mitarb. (1962) haben papierchromatographisch die Menge an Lysolecithin pro Zelle bestimmt, die für die Erzeugung bestimmter morphologischer Veränderungen erforderlich ist. Wenn weniger als $0,6 \times 10^{-11} \mu\text{Mol}/\text{Zelle}$ fixiert sind, bleibt die Zellform normal; Mengen bis $1,3 \times 10^{-11} \mu\text{Mol}/\text{Zelle}$ erzeugen Formveränderungen, etwa Crenation; $2 \times 10^{-11} \mu\text{Mol}/\text{Zelle}$ führen zu der bereits von BERGENHEM und FAHRAEUS (1936) beschriebenen Sphärocytose; Steigerung der Hämolysinmenge auf $2 \times 10^{-10} \mu\text{Mol}/\text{Zelle}$ führt schließlich zur Hämolyse. Auch JUNG (1959) errechnet ca. $10^{-10} \mu\text{Mol}/\text{Zelle}$ als eben hämolysierende Dosis für menschliche Erythrocyten. Nach KLIBANSKY u. Mitarb. (1962) verlieren Kaninchen-Erythrocyten bei Behandlung mit Lysolecithin einen Teil ihres Cholesterin. Die Solubilisierung des Cholesterins könnte demnach der entscheidende, zur Hämolyse führende Prozeß sein. Allerdings müßte zur Stützung des Primats des Cholesterins ermittelt werden, wie sich andere lipide und auch nichtlipide Bestandteile der Zellmembran bei der Hämolyse durch abgestufte Lysolecithin-Konzentrationen verhalten. Wir haben bereits betont, daß sich bei Einsatz hoher Lysolecithin-Mengen elektronenoptisch ein fast völliger Zerfall der Zellmembran darstellt, also prinzipiell alle Bestandteile des Erythrocyten freigesetzt werden können. Damit wird auch die Frage (JUNG, 1959) gegenstandslos, ob die Lysolecithinmoleküle in Form einer monomolekularen Schicht die Erythrocytenmembran überziehen. Wir müssen uns vorstellen, daß sich das Lysolecithin seifenähnlich zwischen die Membranlipoide schiebt, mit ihnen also gemischte Aggregate (Übersicht bei ROBINSON, 1961) bildet. Die Bindungsfähigkeit der Zelle reicht für mehr als die hämolysierende Lysolecithinmenge aus; die Bindung von viel überschüssigem Lysolecithin dürfte jedoch partiell reversibel sein. Ob die Geschwindigkeit der Bindung etwas

mit der charakteristischen Latenz der Lysolecithinhämolyse zu tun hat, ist unbekannt; eine Schwellung scheint in dieser prähämolytischen Phase nicht einzutreten (JUNG, 1959).

Möglichkeit und Ausmaß einer *in vivo*-Hämolyse durch Phospholipase A war lange Zeit umstritten. Die Tatsache, daß das Enzym gewaschene Erythrocyten *in vitro* praktisch nicht angreift, bedeutet nicht, daß es auch *in vivo* unwirksam sein müßte; denn dort findet es in den zirkulierenden Lipiden reichlich Substrat, allerdings auch Inhibitoren seines Reaktionsproduktes, des Lysolecithins. Injiziert man gereinigte Bienengiftphospholipase beim Kaninchen, so sinkt der Spiegel des Plasma-Lecithins abrupt ab (HABERMANN und KRUSCHE, 1962), ebenso wie nach Injektion von *Vipera-palestinae*-Gift (KLIBANSKY *et al.*, 1962). Der Anstieg des Lysolecithinspiegels entspricht nicht quantitativ dem Lecithinverlust. Der Fehlbetrag an Lipoidphosphor erklärt sich wohl dadurch, daß das oberflächenaktive Lysolecithin unmittelbar nach seiner Entstehung an Zellgrenzflächen gebunden wird; so wird ein Teil des durch Schlangengift freigesetzten Lysolecithins an den Erythrocyten wiedergefunden. Kleine Mengen von Bienengiftphospholipase führen zur Sphärocytose und Erhöhung des Hämatokritwertes (HABERMANN und KRUSCHE, 1962), höhere zu mäßiger intravitaler Hämolyse und Hyperkaliämie, die aber zur Zeit des Todes im Abklingen ist. Die Sektion ergibt massive, fleckförmige, zur Erklärung des Todeseintritts hinreichende Hämorragien und Ödem in der Lunge. Mikroskopisch findet man in den entnommenen Blutproben Erythrozytenaggregate, die Anlaß zu multiplen Mikroembolien sein könnten (STOCKEBRAND, 1965). Aus Plasma hergestellte Lysophosphatide rufen, intravenös appliziert, bei Kaninchen Sphärocytose und Anämie hervor (DE VRIES, 1951). Die Zellschädigung durch zur Hämolyse nicht hinreichende Phospholipase-Dosen äußert sich in einer ca. 2 Std anhaltenden Sphärocytose sowie in einer Verkürzung der Überlebensdauer der Erythrocyten (DE VRIES *et al.*, 1962).

Die LD 50 der Bienengift-Phospholipase A liegt bei Mäusen (7 mg/kg; HABERMANN, 1957a) wesentlich höher als bei Kaninchen (um 0,5 mg/kg). Die Mäuse sezernieren hämoglobinhaltigen, erythrocytenfreien Harn; roter Schaum vor der Nase und Sektionsbefund weisen auf intravitale Hämolyse und die erwähnten Störungen der Lungenzirkulation hin. Kaninchen können aus noch ungeklärten Gründen gelegentlich an wesentlich kleineren Phospholipase-Mengen akut sterben. Die LD 50 für Lysolecithin liegt bei rund 150 mg/kg (Maus, intravenös); die Symptome sind im Prinzip die gleichen wie bei Injektion von Phospholipase A. Schon ein Bruchteil der LD 50 (Maus) von Phospholipase A würde ausreichen, um unter optimalen Bedingungen binnen 30 min eine derartige Lysolecithinmenge zu bilden. Zirkulierendes Lysolecithin wird aber schnell inaktiviert; also muß das Hämolsin durch Injektion einer hohen Enzymmenge in extrem kurzer Zeit erzeugt werden, soll eine nennenswerte Toxicität zustande kommen.

Erstaunlich ist die Diskrepanz zwischen der extrem stark *in vivo und in vitro* hämolsierenden Phospholipase C und der *in vivo* verhältnismäßig schwach, gewaschene Zellen nicht hämolsierenden Phospholipase A. Die LD 50 des erstgenannten Enzyms (Maus, intravenös) liegt um 0,001 mg/kg, also ca. 7000mal niedriger als bei Phospholipase A. Phospholipase C greift *in vivo* in erster Linie die Erythrocyten-Phospholipoide an, kaum die Plasmalipoide; bei Phospholipase A ist es umgekehrt (HABERMANN und KRUSCHE, 1962).

Phospholipase A spaltet einen hydrophoben Rest (Fettsäure), Phospholipase C dagegen einen räumlich entgegengesetzt angeordneten hydrophilen Teil (Phosphorylcholin) aus dem Lecithinmolekül. So drängt sich folgende Hypothese auf: Die Lecithinmoleküle sind in die Erythrocyten-Wand derart eingelagert, daß der hydrophile Teil des Moleküls nach außen gerichtet und somit enzymatisch, d.h. für Phospholipase C, zugänglich ist, während der hydrophobe Anteil im lipoiden Innern der Membran begraben ist. Er muß durch osmotische Hämolyse oder auch durch „direkte“ Hämolysine, wie Melittin oder Lysolecithin, für Phospholipase A zugänglich gemacht werden. Bei Meerschweinchenzellen scheinen allerdings besondere Texturverhältnisse der Membran vorzuliegen.

Die Oberflächenaktivität von Lysolecithin (HABERMANN, 1955 b, 1958b) regt zu einem Vergleich mit Melittin an, dem oberflächenaktiven Bienengiftpeptid (s. S. 233). Wie aber schon bei der Besprechung der Melittinhämolyse herausgestellt wurde, sind die Diskrepanzen zahlreich; die starke Basizität gibt dem Melittin einen zusätzlichen Akzent. Die pharmakologischen Eigenschaften des Lysolecithins lassen sich leichter aus seiner Oberflächenaktivität ableiten als dies beim Melittin möglich ist. Zwanglos ergibt sich seine klärende Wirkung auf Suspensionen bzw. Emulsionen von Cholesterin, Ölsäure, Lecithin (HABERMANN, 1955 b, 1958b), Hirnhomogenate (GROSSE und TAUBÖCK, 1942; WEBSTER, 1957; MARPLES *et al.*, 1959), Eigelb (HABERMANN und NEUMANN, 1954a), Leberpartikel-Präparate (HABERMANN, 1958b), auch die Auflösung von Erythrocytenstromata (HABERMANN und MÖLBERT, 1954; BLOMFIELD *et al.*, 1966) und die Freilegung von Enzymen, z.B. echter und Pseudocholinesterase, aus Hirnschnitten (MARPLES *et al.*, 1959). Inkorporiert man Lysolecithin in ultradünne Lipidmembranen, so fällt ihr elektrischer Widerstand um zwei Zehnerpotenzen; derartige Prozesse könnten bei der Regelung von Membranfunktionen *in vivo* mitwirken, zumal Lysolecithin und metabolisierende Enzyme in zahlreichen Organen nachgewiesen sind (s. S. 270) (VAN ZUTPHEN u. VAN DEENEN, 1967).

Es liegt nahe, die Schädigung der Erythrocytenwand als Modell für Lysolecithin-Effekte an anderen Membranen zu betrachten (NEUMANN und HABERMANN, 1957; s. auch S. 237); doch läßt sich die Organwirkung aus dem Tensidcharakter nicht ohne weiteres erschließen. Die pharmakologischen Effekte von Phospholipase A und Lysolecithin sind gelegentlich, z.B. am isolierten Meerschweinchen-Ileum, konträr. Hier wirkt Phospholipase A kontrahierend, Lysolecithin dagegen erschlaffend (s. S. 284).

β) Schädigung von Thrombocyten und Leukocyten. Kaninchen-Thrombocyten verhalten sich gegenüber Phospholipase A und Lysolecithin wie Erythrocyten: Lysolecithin zerstört sie und legt das darin enthaltene Serotonin frei. Gegen Phospholipase A sind sie erwartungsgemäß resistent (HABERMANN und SPRINGER, 1958). MARKWARDT u. Mitarb. (1966) studierten das gleichzeitige Verhalten von Serotonin, Histamin und ATP bei Inkubation von Kaninchen-Thrombocyten. Während bereits 1 µg Melittin/ml den Serotonin gehalt um 77 %, den Histamingehalt um 73 %, den ATP-Gehalt um 54 % senkte, führten 10 µg/ml Bienengift-Phospholipase A nur zu einer Minderung des Serotonin- und ATP-Gehaltes um 17 bzw. 15 %, während Histamin nicht signifikant vermindert wurde. Die starke Aminfreisetzung durch das Gift von *Crotalus terrificus* beruht nicht auf dessen Phospholipasegehalt. Während unter dem Einfluß von Bienengift die Thrombocyten zerfallen, sind sie nach Crotalusgift phasenkontrastmikroskopisch intakt.

Das morphologische und funktionelle Verhalten menschlicher Blutplättchen unter dem Einfluß von Lysolecithin studierten KIRSCHMANN *et al.* (1963). In hämolysierenden Konzentrationen löst Lysolecithin auch isolierte, in Salzlösung suspendierte Thrombocyten; sublytische bzw. partiell lytische Konzentrationen machen die Plättchen wirksamer im Thromboplastin-Generationstest: Generationszeit und Gerinnungszeit werden beide verkürzt. Die „clot-retracting“-Aktivität wird vermindert. Radioaktives Lysolecithin wird in annähernd linearer Abhängigkeit von der zugesetzten Menge bis zum lytischen Grenzwert (5×10^{-6} µg/Plättchen) adsorbiert. Im Verband des Gesamtblutes sind die Thrombocyten resistent gegen Lysolecithin, selbst wenn man Konzentrationen anwendet, die reine Plättchensuspensionen längst lysieren. Bei Inkubation von menschlichem Gesamtblut mit *Vipera-palestinae*-Phospholipase wird zwar ein Teil des Lysolecithins an Erythrocyten gebunden; doch bleibt ca. 1 mg/ml ungebunden im Plasma. Wenn man fast das gesamte Plasmalecithin von Kaninchen durch Injektion von *Vip.-palestinae*-Phospholipase A in Lysolecithin überführt, liegt der maximal erreichbare Spiegel dieses Lipids niedriger (180 µg/ml), weil ein Teil des Lysolecithins die Blutbahn verläßt, aber immer noch höher als zur Lyse isolierter Plättchen erforderlich; dennoch bleiben --- wie beim in vitro-Versuch mit Humanblut — Plättchenzahl, Retraktionszeit des Gerinnsels und Thromboplastin-Generationszeit unverändert. Die Erklärung für diese Diskrepanzen fanden KIRSCHMANN *et al.* (1963) darin, daß in Gegenwart von Humanblut weniger radioaktiv markiertes Lysolecithin an die Plättchen adsorbiert wird; lytische Mengen ($> 5 \cdot 10^{-6}$ µg/Plättchen) werden selbst bei Zusatz von 0,4 mg Rattenleber-Lysolecithin/ml Blut nicht gebunden. Die Hemmung der Lysolecithin-Bindung durch Plasma beruht zum Teil, aber nicht ganz auf dessen Albumingehalt. Eine „direkte“ Lyse gewaschener Plättchen durch Phospholipase A (aus *Vipera-palestinae*-Gift) ist nicht möglich, ebensowenig wie eine Serotoninfreisetzung (HABERMANN und

SPRINGER, 1958) durch Bienengift-Phospholipase A. Dementsprechend wird auch keine Störung der Gerinnungsfähigkeit des Blutes bei intravenöser Verabreichung von Bienengift-Phospholipase A an Kaninchen gesehen (STOCKER RAND, 1965).

Phospholipasen (Typ A) unterschiedlicher Herkunft scheinen die Lipide von Granula und Membranen der Plättchen verschieden gut zu spalten. An einem Ende der Skala steht Vipera-palestinae-Gift, das intakte Plättchen nicht angreift, isolierte Granula und Membranen nur wenig. Russell-Viper-Gift hydrolysiert die Lipide von gewaschenen Plättchen sowie von Granula und Membranen, nicht aber diejenigen intakter Erythrocyten. Diese Versuche, welche auf eine verschieden gute „Zugänglichkeit“ der Lipide intakter Plättchen bzw. Erythrocyten schließen lassen, sollten aber mit gereinigten Enzymen wiederholt werden; denn es könnten begünstigende oder störende Faktoren in den verwendeten Giften vorhanden sein (BRADLOW u. MARCUS, 1966). Der Unterschied zwischen den (elektrophoretisch gereinigten) Phospholipasen von Cobragift und Vipera-palestinae-Gift bestätigt sich auch bei Verwendung gewaschener menschlicher Blutplättchen als Substrat; wie osmotisch gewonnene Erythrocytenschatten werden sie durch das Cobraenzym, nicht durch das Vipernenzym lysiert und ihre Phospholipoide gespalten. Setzt man jedoch den direkt lytischen Faktor von Cobragift oder oberflächenaktive Agentien (Digitonin, Saponin) zu, so gewinnen die Plättchen Substrafunktionen auch für das VipernGift (KIRSCHMANN *et al.*, 1964). Hypotonie-Behandlung verbessert die Substrafunktion für dieses Gift nicht, ebensowenig wie beim Erythrocyten.

Auch Leukocyten gesunder oder an Leukämie erkrankter Menschen adsorbieren Lysolecithin (enzymatisch aus Rattenleber-Homogenat hergestellt) aus physiologischer Kochsalzlösung. Die pro Zelle gebundene Substanzmenge ist der Lysolecithinkonzentration direkt proportional und steht in inverser Beziehung zur Zellkonzentration. Durch Albuminzusatz gelingt eine komplette Desorption. Hohe Lysolecithinkonzentrationen (ca. 1,5 µg/10⁶ Zellen) führen zur Lyse, kleinere stören bereits die Membranfunktionen, was sich an der Färbbarkeit mit Trypanblau zeigt (KLIBANSKY, SMORODINSKY *et al.*, 1967). Isolierte Leukoytentgranula (Lysosomen) werden durch Lysolecithin wie auch durch andere Hämolytica zerstört (WEISSMANN *et al.*, 1964). Da Lysosomen aus Rinderleukocyten nicht nur endogenes Lysolecithin (ca. 5 % des Lipid-Phosphors) enthalten, sondern auch Phospholipase A, Phospholipase B und eine Acyltransferase, erscheint eine Beteiligung von Lysoverbindungen beim Lysomenzerfall diskutabel (HEGNER u. FRIMMER, 1967).

An menschlichen Leukocyten lassen sich deutliche Unterschiede zwischen Phospholipase A von Cobragift und Vipera-palestinae-Gift aufzeigen. Zwar sind beide Enzyme inaktiv gegenüber intakten Zellen; auch werden die Zellen durch den (phospholipasefreien) direkt lytischen Cobragiftfaktor oder Homogenisieren für beide Enzyme aufgeschlossen. Doch macht milde Schädigung,

z. B. durch wiederholtes Zentrifugieren oder durch Hypertonie-Behandlung, die Zellen selektiv für Phospholipase A aus Cobragift empfindlich (KLIBANSKY *et al.*, 1967).

y) *Lokaltoxicität und Mastzellzerfall*. Lysolecithin schädigt das Kaninchenauge bei Injektion in die Vorderkammer. Binnen einiger Stunden trübt sich die Cornea; es tritt Conjunctivitis und Exophthalmus auf (FELDBERG *et al.*, 1938). Die Lokaltoxicität von Bienengift-Phospholipase A ist wesentlich geringer als die von Melittin; doch erhöht das Enzym die Gefäßpermeabilität der Rattenhaut bei intracutaner Injektion, z. B. für zuvor intravenös injiziertes Evans-Blau (HABERMANN, 1957a). Es zerstört wahrscheinlich am Ort der Injektion die Mastzellen; denn der Effekt ist durch kombinierte systemische Applikation von Antagonisten gegen Histamin (Diphenhydramin) und Serotonin (BOL 148)⁶ hemmbar. Die Histaminfreisetzung läßt sich an isolierter Rattenhaut *in vitro* direkt nachweisen; das gelingt auch nach Blockade des Stoffwechsels mit 2,4-Dinitrophenol sowie in Abwesenheit von Glucose. Dagegen kann man aus den Zellen der Peritonealflüssigkeit von Ratten mit Phospholipase nur wenig Histamin freilegen, ganz im Gegensatz zu der massiven Wirkung von Melittin oder Lysolecithin. Auch die Zellen des Rattenmesenteriums werden durch das Enzym nur in verhältnismäßig geringem Ausmaß degranuliert. Phospholipase A kann also von außen her den Mastzellzerfall nur dann induzieren, wenn extracelluläre, lysolecithinliefernde Phospholipoide im Gewebe bzw. im Inkubationsmedium vorhanden sind (ROTHSCHILD, 1965). Die Mastzelle verhält sich gegenüber Phospholipase A wie ein Erythrocyt oder Thrombocyt. Die früheren Befunde von HÖGBERG und UVNÄS (1957, 1960) sind unverständlich, wenn man nicht annimmt, daß ein melittinhaltiges Phospholipasepräparat benutzt wurde. FREDHOLM (1966, 1967), ein Mitarbeiter von UVNÄS, hat inzwischen gleichfalls eine mastzelldegranulierende Bienengiftfraktion von Phospholipase A abtrennen können. Damit entfällt die erste und wichtigste Stütze der Phospholipasetheorie des Mastzellzerfalls. Auch von KELLER (unveröffentlicht) wurden die Rothschildischen Befunde, soweit sie die Peritonealzellen betreffen, bestätigt. Zur Rettung der Phospholipasetheorie, die in ihrer ursprünglichen Form sicher nicht haltbar ist, könnte man die spekulative Erwägung anstellen, daß vielleicht eine Aktivierung *intracellulärer* Phospholipase A am Beginn des Mastzellzerfalls stünde; ein solches Enzym hätte genügend Substrat. Aus dem isolierten Rattenzwerchfell setzt Bienengift-Phospholipase A Histamin frei; wahrscheinlich liegt in diesem Präparat — im Gegensatz zum Mesenterium, aber ähnlich wie in der Rattenhaut — genügend extracelluläres bzw. accessibles Phospholipoid vor (STRIEBECK, 1958) (Abb. 13; Tabelle 15).

Ein als Phospholipase A angesprochenes Enzym wurde an Rattenmastzellen gefunden (GIACOBINI *et al.*, 1965). Gruppen von 2—6 Zellen wurden

⁶ 2-Bromlysinsäure-Diäthylamid.

mit Lecithin inkubiert und die Säureproduktion mittels des Cartesianischen Schwimmers gemessen. Kephalin, Phosphatidsäure und Lysolecithin wurden nicht hydrolysiert. Spinalganglionzellen waren in diesem System ebenfalls

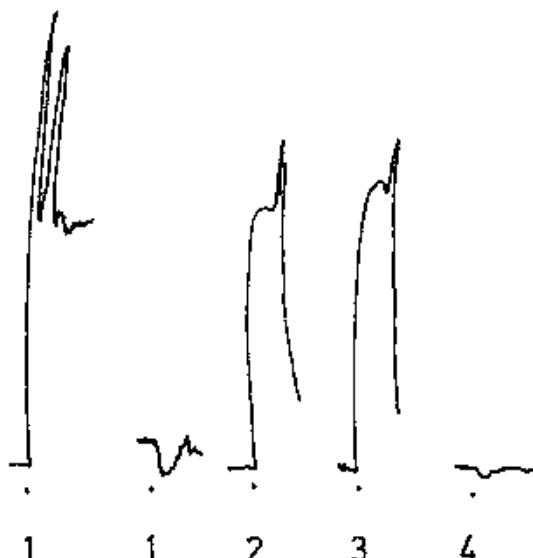


Abb. 13. Histaminfreisetzung durch Bienengift-Phospholipase A (10 µg/ml) aus dem isolierten Rattenzwerchfell (nach STRIEBECK, 1958). 1 0,5 ml Phospholipase A (man beachte die extreme Tachyphylaxie!); 2 0,5 ml enzymhaltige Badeflüssigkeit; 3 0,5 µg Histamin; 4 0,5 ml enzymfreie Badeflüssigkeit

Tabelle 15. Histaminfreisetzung durch einige tierische Gifte und deren Komponenten aus dem isolierten Rattenzwerchfell. (Nach STRIEBECK, 1958)

Substanz		Gesamtgehalt der Testlösung [abzüglich Kontroll-Lösung (µg)]
Gereinigtes Bienengift	1: 100000	3,5
	1: 100000	4,0
	1:1000000	0,5
Naja-tripudians-Gift	1: 10000	5,0
	1: 100000	2,5
Melittin	1: 100000	2,5
	1:1000000	0,5
	1: 10000	1,0
Crotamin aus Crotalus-terrificus-Gift	1: 20000	0,75
	1: 10000	1,5
Crotactin aus Crotalus-terrificus-Gift	1: 10000	1,25
	1: 10000	1,5
	1: 10000	1,25
Fraktion II aus Bienengift	1: 10000	5,0
	1: 100000	4,5
	1: 100000	5,0
Phospholipase A aus Bienengift	1: 100000	5,0
	1: 100000	4,0
	1:1000000	1,5
	1:1000000	1,0
Phospholipase A aus Crotalus-terrificus-Gift	1: 100000	4,0
	1: 100000	2,5
	1:1000000	1,0
	1:1000000	1,0

aktiv, nicht dagegen Erythrocyten oder Leukocyten von Ratten. In den Zellen des Peritonealexssudats des Kaninchens wurden folgende Reaktionen nachgewiesen (ELSBACH *et al.*, 1965):

- a) Lecithin → Lysolecithin + Fettsäure; diese Reaktion wird überlagert von dem Prozeß,
- b) Lysolecithin → Glycerinphosphorylcholin + Fettsäure. Zusatz von Deoxycholat hemmt Reaktion b), so daß dann mehr Lysolecithin im Reaktionsansatz verbleibt.
- c) Lysolecithin → Lecithin + Glycerinphosphorylcholin.

Aus Kolamin-Kephalin entsteht die entsprechende Lyso-Verbindung.

Den Gewebsmastzellen stehen die basophilen Zellen des Blutes morphologisch und in ihrem Histamin- und Heparingehalt nahe. Nach Bienengift-Phospholipase, in Mengen von 100 µg/ml Blut zugesetzt, wird ein „Schmelzen“ der Granula beschrieben, zusätzlich, besonders nach längerer Inkubationszeit, auch eine Degranulation. Kleinere Dosen wirken nicht signifikant, höhere hämolysieren generell. Auf Grund der Versuchsbedingungen kann nicht entschieden werden, ob es sich um einen „direkten“ Effekt an den Zellen handelt oder ob die Lyse des Plasma-Lecithins vorausgehen muß. Versuche mit Lysolecithin wurden offenbar nicht angestellt (SHELLEY und JUHLIN, 1962).

Neben den Mastzellen werden auch Makrophagen und Mikrophagen durch Lysophosphatide beeinflußt. So wird die Phagocytoseaktivität von Peritonealmakrophagen durch kleine Lysolecithindosen gesteigert; durch hohe Konzentrationen werden die Zellen so weit geschädigt, daß keine Phagocytose mehr stattfindet (KABOTH und AX, 1966). Inkubiert man Makrophagen mit silikogenem Staub, so werden die Zellen irreversibel geschädigt. Dabei werden gewebstoxische und hämolysierende Substanzen freigesetzt. Möglicherweise sind Lysolecithin und Lysokephalin die verantwortlichen Agentien, da sie — wie die dünnenschichtchromatographische Analyse des Lipidmusters zeigt — stark zunehmen. Übrigens begünstigt bereits Inkubation von dichtgepackten Makrophagen das Auftreten von Lysoverbindungen (MUNDER *et al.*, 1966). Substanzen, welche den Lysolecithinstoffwechsel beeinflussen, sollten dementsprechend entzündliche Prozesse abschwächen. Auch dafür gibt es Hinweise. So können Salicylate und Indomethazin die Lysophospholipase von Erythrocytenmembranen aktivieren. Noch stärker aktiviert allerdings Dipyridamol (Persantin). Dem „Sicherheitsezym“ Phospholipase B wird eine doppelte Rolle zugeschrieben: es soll den hypothetischen Entzündungsstoff Lysolecithin entfernen und durch Minderung des Gehalts der Membranen an „physiologischem“ Lysolecithin den Widerstand, z.B. für den Phosphattransport, erhöhen (FERBER, 1966).

Das Zusammenspiel zwischen Phospholipase A und oberflächenaktiven Substanzen manifestiert sich an einem weiteren pathogenetischen Modell: der experimentellen Pankreasschädigung der Ratte. Injiziert man Lysolecithin

(als 0,2—2%ige Lösung) in den Pankreasgang, so treten dosisabhängig leichte bis schwerste Schädigungen der Drüse auf. Phospholipase A (250 µg/0,6 ml), für sich allein appliziert, schädigt nicht eindeutig. Kombiniert man sie aber mit 0,4% Taurocholat, das für sich allein nur geringfügig schädigt, so findet man schwerste, über den ganzen Bauchraum verteilte Nekrosen. In nekrotischem menschlichem Pankreasgewebe wird sehr viel mehr Lyssolecithin gefunden als in normalem; die menschliche Bauchspeicheldrüse enthält verhältnismäßig viel Phospholipase A. Daher wird dem Enzym eine möglicherweise entscheidende Rolle bei der Pathogenese der akuten Pankreatitis beigemessen (SCHEMIDT *et al.*, 1967).

8) *Kontraktile Organe*. Die Hämolyse durch Lyssolecithin, der Abbau von Phospholipoiden vorgeschädigter Erythrocyten durch Phospholipase A sind besonders leicht zugängliche Beispiele einer Zellschädigung, die auch an anderen Populationen abläuft. Man wird dann mit dem Zusammenbruch des Membranpotentials, mit Austritt von Kalium und Eintritt von Wasser rechnen müssen. Für die isolierte *quergestreifte Muskulatur* des Frosches wurden diese Vorgänge unter Lyssolecithin bewiesen (HABERMANN, unveröffentlicht; LAM-PARTER, 1954; HEYDENREICH, 1957). Nähere Untersuchungen über die Wirkungen von Lyssolecithin auf die mechanischen und elektrischen Phänomene des Skelettmuskels stehen noch aus; das isolierte Phrenicus-Zwerchfellpräparat der Ratte ist weitgehend resistent gegen Phospholipase A und Lyssolecithin. Bienengiftphospholipase A erregt den Froschrectus nicht, ändert auch nicht seine Empfindlichkeit gegen Acetylcholin. Das isolierte Froschherz gerät unter Lyssolecithin 1:1000 allmählich in Kontraktur, während Phospholipase A lediglich die Hubhöhe verstärkt (HABERMANN, 1957a). Man sollte erwarten, daß Phospholipase A die quergestreifte Muskulatur erheblich stärker beeinflussen würde, nachdem sie bereits in Verdünnungen 1:50000 die Na⁺-K⁺-aktivierbare ATPase isolierter Zellmembranen des Herzmuskels hemmt (PORTIUS und REPKE, 1963 b). Ein gleichartiger Effekt wird von Lyssolecithin (1:33 000) hervorgerufen, das somit am Zustandekommen der Enzymwirkung beteiligt sein könnte. Wenn beide Substanzen am intakten Organ nicht digitalisartig wirken, wie das nach der Hypothese von REPKE der Fall sein müßte, so mag das daran liegen, daß die sensible ATPase durch eine intakte Zellmembran vor dem Enzym geschützt ist, ähnlich wie eine intakte Erythrocytenmembran resistent gegen Phospholipase A ist. Das direkt hämolysierende Melittin hemmt nach PORTIUS und REPKE die Transport-ATPase noch in Verdünnungen 1:100000 sehr stark (s. S. 249).

Die *Herzwirksamkeit* von Lyssolecithin gewann besonderes Interesse, als man Palmitoyl-Lyssolecithin als einen positiv inotrop wirksamen Faktor in Organextrakten identifizierte und am Froschherzen Analogien zu Digitalis fand (HAJDU *et al.*, 1957). Nach GOVIER u. BOADLE (1967) ist jedoch die Vergrößerung von Hubhöhe (rechter Ventrikel) bzw. Schlagfrequenz (rechter

Vorhof des Meerschweinchens) indirekt, durch Freisetzung adrenerger Transmitter, zu erklären; denn vorherige Gabe von Reserpin bzw. Pronethalol hebt den Effekt auf. Lysolecithin verhält sich auch insofern anders als Digitalis, als die Empfindlichkeit in der Reihe Meerschweinchen > Ratte > Kaninchen abnimmt. Lysolecithin ist nicht durch Cetavlon, ein oberflächenaktives Agens, zu ersetzen, so daß nicht die Oberflächenaktivität allein für die cardialen Effekte von Lysolecithin verantwortlich sein kann. Es fällt weiter auf, daß die positiv inotrope Wirkung durch Spülung aufgehoben wird, was gegen eine simple Lyse von Membranen spricht. Die benötigten Lysolecithinmengen ($5 \mu\text{g}/\text{ml}$) liegen so niedrig, daß an eine Regelfunktion dieser zelleigenen Substanz gedacht werden kann.

Phospholipase C hat — wie an Erythrocyten — auch an der Muskulatur (isolierte Muskelfasern der Ratte) deutliche Membranwirkungen. Das Enzym erhöht die Ionenpermeabilität in Konzentrationen über $1.5 \mu\text{g}/\text{ml}$, erniedrigt das Ruhepotential und setzt ($1.5 \gamma/\text{ml}$) den das Aktionspotential erzeugenden Mechanismus außer Funktion. Es wird vermutet, daß Phospholipase C die polaren Enden der Membranphospholipoide modifiziert und dadurch den Ionentransport beeinflußt. Die Empfindlichkeit chronisch deprivierter Muskulatur gegen Acetylcholin bleibt unter Phospholipase C-Konzentrationen intakt, welche die elektrische Erregbarkeit bereits aufheben (ALBUQUERQUE u. THESLEFF, 1967). Phospholipase A wurde noch nicht in vergleichbarer Versuchsanordnung geprüft.

Erhitztes Habu-Gift (*Trimeresurus flavoviridis*) und daraus gewonnene Phospholipase A rufen bei i.m. Injektion in den Mäuseschenkel eine Myolyse hervor, die sich makroskopisch in Schwellung, histologisch in Zerfall der Muskelfasern äußert. Die Mitochondrien der Muskulatur schwollen zunächst und zerfallen dann. Hämorragien sind nach Injektion von Gesamtgift regelmäßig vorhanden, nicht aber nach Applikation der Phospholipase-Fraktion. Der Lecithingehalt des mit Phospholipase vergifteten Areals wurde indirekt bestimmt, indem *in vitro* weiteres Enzym zugesetzt und das gebildete Lysolecithin ermittelt wurde. Der Lecithingehalt fiel mit zunehmender Myolyse. Das phospholipasefreie Gift von *Trimeresurus okinavensis* rief eine viel schwächere Myolyse hervor als Habu-Gift (MAENO *et al.*, 1962).

An der glatten Muskulatur sind die Effekte von Lysolecithin und Phospholipase A konträr. Lysolecithin bringt den Meerschweinchendarm zur Er schlaffung, der nur gelegentlich eine kurze Kontraktion vorausgeht (ALBL, 1956); dabei wird der Darm gleichzeitig weniger empfindlich gegen Acetylcholin und Histamin (FELDBERG *et al.*, 1938). Phospholipase A bringt ihn zur Kontraktion; wiederholte Applikation des Enzyms ruft Tachyphylaxie hervor, die wesentlich stärker ist als z.B. nach Melittin (HABERMANN, 1957a). Zur Deutung der Diskrepanz zwischen dem Verhalten des Darms gegen Lysolecithin und Phospholipase A wäre anzuführen, daß das Enzym nicht nur Lysolecithin entstehen läßt, sondern auch freie Fettsäuren, die zum Teil als slow reacting substances (SRS) wirken können. So lassen sich aus giftbehandeltem Eigelb organische Säuren extrahieren, welche die Wirkungen von Phospholipase A imitieren können; ihrer Anwendung folgt selbstverständlich

keine Tachyphylaxie (FELDBERG *et al.*, 1938; VOGT, 1957). SCHÜTZ und VOGT (1961) perfundierten isolierte Meerschweinchenlungen mit Tyrodelösung, welche Cobragift bzw. Phospholipase A (aus Bienengift) enthielt, und fanden im Eluat SRS-Aktivität. Auf Grund der Löslichkeit, des Verhaltens bei Verteilung und Chromatographic sowie pharmakologischer Eigenschaften schlossen sie, daß es sich um ungesättigte Fettsäuren handelte, die aus Gewebsphosphatiden abgespalten werden. Nach DAKHIL und VOGT (1962) sind vor allem Oxidationsprodukte der hochungesättigten Fettsäuren, aber auch Hydroxyfettsäuren, SRS-aktiv. Es ist eine Definitionsfrage, ob man Oxidationsprodukte der Fettsäuren, die unter den Redoxbedingungen des Gewebes entstehen, als Artefakte auffassen will. Man sollte annehmen, daß die Fettsäuren durch Bindung in β -Position des Lecithins nicht vor Oxidation geschützt werden und demnach derartige SRS-Substanzen zelleigen in gebundener Form vorliegen können. Ob die Kontraktion des virginellen Meerschweinchenterus unter hohen Konzentrationen eines Lysolecithin-Präparates (FELDBERG *et al.*, 1938) tatsächlich auf Lysolecithin und nicht auf begleitender SRS beruht, wäre — am besten mit synthetischem Material — nachzuprüfen. DELEZENNE und LEDEBT (1911) sowie BOQUET *et al.* (1950) arbeiteten mit Eigelb, das mit Gesamtgift von Schlangen inkubiert war. Infolgedessen kann nicht mit Sicherheit gesagt werden, ob die Effekte der „DL-Substanz“ (von BOQUET *et al.* zu Ehren von DELEZENNE und LEDEBT so genannt) auf Lysolecithin, Fettsäuren, einer Kombination von beidem oder auf einem Reaktionsprodukt beruhen, das nichts mit Phospholipase A zu tun hat. BOQUET *et al.* weisen auf Unterschiede zwischen den Effekten von DL-Substanz und Lysolecithin hin. Retrospektiv betrachtet, erscheint eine Kombination von Lysolecithin- und Fettsäure-Effekten als wahrscheinlich; so trat eine Erschlaffung des Meerschweincheneileums ein, dabei eine Abschwächung der Kontraktionen nach Histamin, Acetylcholin und Cobragift. Am Kreislauf von Kaninchen und Katzen fiel eine ausgeprägte Einengung der Lungenstrombahn auf, die sich auch am isolierten Herz-Lungen-Präparat des Kaninchens reproduzieren ließ.

Injiziert man Lysolecithin in die Perfusionsflüssigkeit des *isolierten Ganglion cervicale superius* der Katze, so fällt die Empfindlichkeit für exogenes Acetylcholin stärker ab als für die elektrische Reizung; der Effekt ist reversibel (SEIFERT, 1958; HABERMANN, 1954a). Am intakten Tier wird weder ein derartiger Effekt noch eine curareähnliche Wirkung auf Zwerchfell oder Extremitätenmuskulatur nach Applikation von Gesamtgift gesehen (v. BRUCHHAUSEN, 1955). Der intakte Nervus phrenicus der Ratte wird selbst durch hohe Bienengiftkonzentrationen nicht blockiert, also auch nicht durch Phospholipase A (HOFMANN, 1952b). Dagegen scheinen freie Axone des Tintenfisches durch Schlangengifte für anschließend applizierte Pharmaka, z.B. Curare, sensibilisiert zu werden. Es wird vermutet, daß Phospholipase A der verantwortliche Faktor ist; eine an Phospholipase A reiche Fraktion aus Cobragift war an

diesem Testobjekt aktiv (ROSENBERG und NG, 1963; ROSENBERG und PODLESKI, 1963). Lysolecithin war jedoch unwirksam, desgleichen Phospholipase C, während die aus Kohl isolierbare Phospholipase D im Sinne von Phospholipase A wirkte.

Lysolecithin und Phospholipase A sind beide *kreislaufwirksam*, wobei die Aktivität des Enzyms sowohl auf Lysolecithin als auch auf SRS-artige Fett-säuren bezogen werden könnte, also indirekter Art wäre. Für einen indirekten Mechanismus spricht die massive Tachyphylaxie, die am Blutdruck der Katze

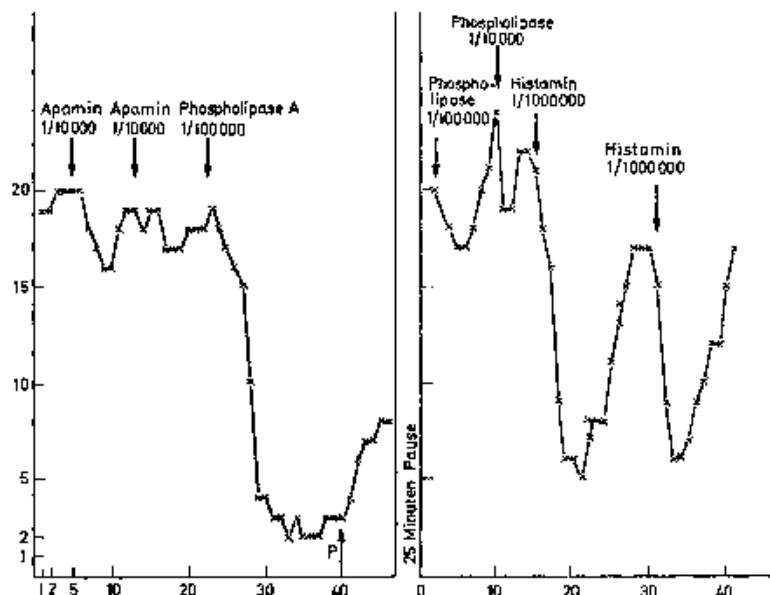


Abb. 14. Tachyphylaxie gegen Phospholipase A, Unwirksamkeit von Apamin am isoliert durchströmten Kaninchenohr (nach STOCKEBRAND, 1965). P Druckerhöhung. Ordinate: Tropfenzahl/min. Abszisse: Zeit (min)

beobachtet wurde (HABERMANN, 1957a). Lysolecithin senkt den Blutdruck verschiedener Versuchstiere ohne konsekutive Tachyphylaxie; es erhöht den Druck in der A. pulmonalis der Katze und den Portalvenendruck des Hundes. Wiederholte Applikation hoher Dosen führt bei der Katze (wie auch bei anderen Versuchstieren, s. S. 276) zu hämorrhagischem Lungenödem (FELDBERG *et al.*, 1938). Die Gefäße des isolierten Kaninchenohres kontrahieren sich bereits, wenn man um 10 µg Bienengift-Phospholipase A der Durchströmungsflüssigkeit zusetzt (Abb. 14); der ersten Applikation folgt massive Tachyphylaxie (STOCKEBRAND, 1965). Die Gefäße der Hinterextremität von Hunden werden bei intraarterieller Injektion von Lysolecithin (ca. 1 mg) kurzfristig erweitert (FELDBERG *et al.*, 1938). Eine phospholipasehaltige Fraktion von Naja-naja-Gift rief bei Katzen biphasische Blutdrucksenkung hervor, die von vorübergehender Depression des Elektrokardiogramms und Störungen des Atemrhythmus begleitet war (BICHER *et al.*, 1965).

e) *Intracelluläre Membranen* werden ebenfalls durch Phospholipase A und Lysolecithin angegriffen. Isolierte Lysosomen aus Rinder-Lukukocyten werden nicht nur durch Melittin, sondern auch durch Phospholipase A und Lysolecithin zerstört (HEGNER, unveröffentlicht; WEISSMANN *et al.*, 1964). Ob derartige Prozesse bei der lokalen Reaktion auf Bienengift als Primärwirkung oder beim Untergang vorgeschädigter Zellen eine Rolle spielen, sei dahingestellt. Die lysosomalen Effekte von Phospholipase A und Lysolecithin leiten über zu den im folgenden besprochenen Wirkungen auf enzymtragende Zellpartikel.

Eine besondere Stellung nimmt die Freilegung der an Zellstrukturen gebundenen, pharmakologisch aktiven Substanzen durch Lysolecithin ein. Auf den Austritt von K⁺ und Histamin wurde bereits eingegangen, ebenso auf den Austritt von Serotonin und Adenosintriphosphat aus Blutplättchen. Nach GAUTRELET und CORTEGGIANI (1939) setzt Lysolecithin (wie Saponin und die Gifte der Cobra und der Vipera aspis) Acetylcholin aus Meerschweinchengchirn frei. KELLAWAY und TRETHEWIE (1940) berichteten über das Auftreten von Adenylderivaten bei Perfusion isolierter Herzen, FELDBERG (1940) schließlich über die Freilegung von Adrenalin aus der Nebenniere der Katze, wenn man das Organ *in situ* oder bei Perfusion mit Lysolecithin behandelte. Auch aus Zellpartikeln setzte das Phospholipoid Adrenalin frei. Der Effekt des Lysolecithins hielt wesentlich länger an als der von Acetylcholin; auf wiederholte, höhere Dosen sprach die Nebenniere nicht mehr an. Auch Bienengift setzte bei intraarterieller Injektion Adrenalin frei; es wäre noch zu beweisen, daß der Prozeß durch dessen Phospholipase via Lysolecithinbildung ausgelöst wird und nicht durch das „direkt“ cytolytische Melittin. Die massive Zellschädigung äußert sich bei Perfusion der Hundeleber im Austritt nicht nur von Histamin, sondern auch von koagulierbarem Protein und Pigmenten sowie in starker Schwellung (FELDBERG *et al.*, 1938).

e) **Wirkungen von Phospholipase A und Lysolecithin auf Zellpartikel und damit verbundene Enzymsysteme**

Gifte von Bienen und Schlangen mindern die Aktivität zahlreicher Enzyme und Enzymsysteme. Prinzipiell sind dabei mehrere Wirkungsmechanismen möglich. Melittin kann z.B. auf Grund seines Invertseifencharakters mit molekularen und supramolekularen Strukturen des Organismus reagieren (s. S. 248). Schlangengifte können mit Hilfe von Phosphatasen metabolisch wichtige Coenzyme spalten. Sie können z.B. Acylierungsreaktionen durch Minderung der Coenzym A-Konzentration beeinflussen, Redoxsysteme durch Spaltung von NAD bzw. NADP. Sie können ferner ATP, ADP und AMP spalten. Auch Ribonucleinsäure, Desoxyribonucleinsäure, Inosin-5'-Phosphat und Ribonucleosid-2', 3'-Phosphat werden hydrolysiert (Übersicht bei NEUMANN und HABERMANN, 1960).

In Hymenopterengiften wurden derartige Phosphatasen bisher nicht nachgewiesen. Sie wirken auf enzymatische Reaktionen vor allem mit Hilfe von

Phospholipase A ein; umgekehrt wurde eine Enzymhemmung durch Phospholipasen erstmals für Bienengift gezeigt (NEUMANN, HABERMANN und AMEND, 1952; NEUMANN und HABERMANN, 1954a; HABERMANN, 1954b), und zwar an mehreren Beispielen: an Gewebsthromboplastin, Bernsteinsäuredehydrierung und oxidativer Phosphorylierung. Für eine Enzymhemmung durch Phospholipasen müssen drei Bedingungen erfüllt sein: Das Enzym muß an lipidhaltige Zellpartikel gebunden sein, die Lipide dieser Zellpartikel müssen für Phospholipase A zugänglich und für die Funktion der gebundenen Enzyme wesentlich sein. Damit tritt das gleiche Problem auf, das bereits am Beispiel der Hämolyse demonstriert wurde.

Von der Definition her können wir eine unmittelbare Wirkung der Phospholipase A auf die Enzymsysteme von einer mittelbaren, durch Lysolecithin ausgelösten unterscheiden. In praxi gibt es aber kein Enzymsystem, dessen Aktivitätsminderung ausschließlich auf eine Herabsetzung des Gehalts an ursprünglich partikelgebundenen Lipiden oder — als Gegenstück hierzu — auf die Bildung von Lysolecithin bezogen werden könnte.

α) *Thromboplastin-Inaktivierung*. Gewebs-Thromboplastin ist ein Gerinnungs-Aktivator cellulärer Herkunft. Wahrscheinlich ist die Thromboplastinaktivität menschlichen Gehirns mit der Mikrosomenfraktion verknüpft. Sicher ist, daß es sich dabei um sehr große, lipidhaltige Komplexe handelt, für deren Gerinnungsaktivität nicht nur die chemische Zusammensetzung (s. HECHT und SLOTTA, 1962), sondern auch der physiko-chemische Zustand von Bedeutung ist. Durch Desoxycholat lassen sie sich solubilisieren; bei Entfernung des Lösungsvermittlers tritt Re-Aggregation ein (HVATUN und PRYDZ, 1966). Die Förderung und Hemmung des Gerinnungssystems durch Schlangengifte ist schwer durchschaubar; denn besonders die Viperidengifte enthalten eine Vielzahl von koagulierenden, koagulationshemmenden und fibrinolytischen Faktoren. Bienengift wirkt jedoch mit nur zwei Komponenten auf das Gerinnungssystem ein. Während Melittin erst in relativ hohen Konzentrationen die Koagulation beeinflußt (s. S. 248), tritt bei niedrigen Giftkonzentrationen die Phospholipase-Wirkung ganz in den Vordergrund. Nun spielen Lipide als Komponenten des sog. Gewebsthromboplastins eine entscheidende Rolle, wenn auch bis heute keine einheitliche Auffassung darüber besteht, ob die Gerinnungsförderung ganz auf dem Lipidanteil der bisher erhaltenen gereinigten Thromboplastine beruht, ob ihrem Proteinanteil eine apoenzymähnliche Wirkung zukommt oder ob dieser nur als Hilfsmittel zur optimalen Dispersion der Lipide dient. Plättchen enthalten in ihren Granula ebenfalls gerinnungsfördernde Lipide; doch ist möglich, daß sie für die Gerinnung weniger wichtig sind als Membranlipide (Übersicht bei DAVIE und RATNOFF, 1965). Versuche mit Schlangengift lassen den Verdacht auftreten, daß die Intaktheit der phospholipaseempfindlichen Plättchenlipide für die Gerinnung von geringerer Bedeutung ist als gemeinhin

angenommen wird (s. S. 278). Welcher Gruppe von Phospholipoiden die Gewebsaktivatoren zugehören, ist ebenfalls noch strittig. Nach HECHT und SLOTTA (1962) sind reines Lecithin und Kolaminkephalin unwirksam, reines Serinkephalin wahrscheinlich ebenfalls. Die Kombination von Serinkephalin und Lecithin ist inaktiv, am günstigsten ist die Kombination von Serinkephalin + Kolaminkephalin. Die Versuche wurden unter Einsatz von synthetischem Lecithin und Kolaminkephalin bestätigt.

Bienengift zerstört Organthromboplastine aus Hirn und Lunge. Für den Phospholipasecharakter des aktiven Agens spricht sein Vorkommen in der elektrophoretischen Fraktion II, welche Phospholipase A und Hyaluronidase als einzige bekannte Wirkstoffe enthält (NEUMANN, HABERMANN und AMEND, 1952; NEUMANN und HABERMANN, 1954a), ferner die Abhängigkeit der Thromboplastin-Inaktivierung von Zeit und Temperatur bei der Inkubation sowie vom Ca^{++} -Gehalt des Ansatzes. Das inaktivierende Prinzip wird wie Phospholipase A durch Zink-Ionen blockiert. Auch in seiner Temperaturempfindlichkeit entspricht es dem genannten Enzym. Im Sauren (pH 2,2–6) ist es thermostabil, im Alkalischen ($\geq \text{pH } 7,9$) ist es thermolabil. Durch Lysolecithin läßt sich Lungenthromboplastin ebenfalls inaktivieren (HABERMANN, 1954c), übrigens auch durch andere oberflächenaktive Substanzen wie Digitonin (HERMANNSDORFER, 1916; DYCKERHOFF und MARX, 1944) oder Melittin (s. S. 248). Umgekehrt entsteht aus handelsüblichem Lungenthromboplastin hämolysierendes Material, wahrscheinlich Lysophospholipid, wenn man es mit Schlangengift umsetzt (HABERMANN, 1954c). Schließlich zerstören auch Präparate von Phospholipase C (*B. cereus* bzw. *C. perfringens*) die Aktivität von Thromboplastin-Suspensionen (OTTOLENGHI, 1967). Die Beweiskette scheint also geschlossen; doch bleibt hier — wie bei der Wirkung von Phospholipase A auf zahlreiche andere Enzymsysteme — die Frage offen, ob die Bildung von oberflächenaktiven Lysoverbindungen oder die Hydrolyse funktionell unentbehrlicher Phospholipide der entscheidende Prozeß ist.

HOUESSAY (1930) hat vor bald 40 Jahren die Thromboplastin-Inaktivierung durch Schlangengifte als phospholipasebedingt gedeutet. In der Zwischenzeit wurde diese Ansicht mehrfach in Frage gestellt, so von KRUSE und DAM (1950), nach denen die gerinnungshemmende Wirkung von *Naja-naja*-Gift beim Erhitzen in Wasser oder Ringer-Lösung fast ganz zerstört wird, während die Phospholipase-Aktivität weitgehend erhalten bleibt. Wir haben daher die alten Untersuchungen von HIRSCHFELD und KLINGER (1915) nachgeprüft, nach denen das gerinnungshemmende Prinzip von Cobragift im Sauren relativ thermostabil, im Alkalischen thermolabil ist, und sie für Bienengift, *Naja-nigricollis*- und *Naja-naja*-Gift bestätigen können. Wir zweifeln demnach nicht daran, daß die Gerinnungsverzögerung sowohl durch Bienengift als auch durch Schlangengift im wesentlichen auf der phospholipasebedingten Thromboplastin-Inaktivierung beruht. Ob basische Schlangengiftkomponenten, ähnlich wie

höhere Melittinkonzentrationen (HABERMANN, 1954c) eine *direkte* Thromboplastin-Inaktivierung hervorrufen, ist noch nicht untersucht worden.

In *vivo* sind die Gerinnungseffekte von Phospholipase A bzw. Bienengesamtgift unbedeutend. Zwar berichteten DYCKERHOFF und MARX (1944) über die Aufhebung der Gerinnbarkeit von Kaninchenblut nach intravenöser Injektion von Bienengift; doch konnte STOCKEBRAND (1965) bei Kaninchen durch subletale bzw. letale Dosen von Bienengift-Phospholipase (oder Melittin) keine eindeutige Veränderung der Blutgerinnung erzielen; ebensowenig konnte REDELBURGER (1958) die Gerinnungs- bzw. Blutungszeit von Mäusen durch intravenöse Injektion von Bienengift-Phospholipase ändern. Da lipoide Co-Faktoren der Bildung von Blutthromboplastin nur in den Thrombocyten vorkommen, wäre es interessant zu erfahren, ob Zahl und Funktion der Thrombocyten unter dem Einfluß der Bienengift-Phospholipase sich ändern. Die Wirkung von Schlangengift auf Thrombocyten wurde S. 278 diskutiert.

β) Beeinflussung von Mitochondrienfunktionen. Phospholipoide sind essenzielle Bestandteile der Mitochondrienmembranen. Man nimmt an, daß sie dort Doppel-Lagen bilden, in denen die Fettsäurereste zweier Moleküle einander zugekehrt sind, die hydrophilen Partien der Moleküle dagegen an den Oberflächen der Membranen liegen. Diesem Skelet, das eher einer Schalenstruktur entspricht, sollen die Strukturproteine und vor allem die Enzyme angelagert sein. Dabei üben die Bindungsbereiche der Proteine wahrscheinlich allosterische Funktionen aus; die Lipidmembranen erhalten möglicherweise durch räumliche Ordnung der spezifischen hydrophilen Anteile (Cholin, Colamin, Serin, Inosit etc.) eine mosaikartige Struktur, die für die Bindung bestimmter Proteine besonders vorteilhaft sein könnte (LEHNINGER, 1966). Man darf demnach erwarten, daß durch alle Eingriffe, welche die Struktur der Mitochondrien schädigen, auch die Funktion der an ihre Membranen gebundenen Enzyme modifiziert wird, und zwar desto massiver, je stärker die allosterische Funktion der Bindung an die Membran ist. Besonders stark werden Enzymketten in Mitleidenschaft gezogen werden, weil ihr Funktionieren von der räumlichen Zuordnung der einzelnen Glieder abhängig ist.

Die Beeinflussung der Mitochondrienfunktionen durch oberflächenaktive Substanzen, wie Lysolecithin oder Melittin (s. S. 248), ist also verständlich; sie ist in Analogie zur Erythrocytenschädigung durch derartige Pharmaka zu setzen. Dagegen ist nicht ohne weiteres einzusehen, weshalb die Mitochondrienmembranen im Gegensatz zu denjenigen der Erythrocyten phospholipaseempfindlich sein sollen. Man könnte annehmen, daß die Lipidhülle stellenweise „Texturfehler“ aufweist, welche dem Enzym den Zutritt zu β -ständigen Fettsäuren (s. S. 262) erlauben, die ja ins Innere der Membran gerichtet sein sollen. Näher liegt allerdings die Annahme, daß die Mitochondrien bei der Präparation, wenn auch nur leicht, geschädigt und dadurch für Phospholipase A zugänglich werden, und daß aus derart vorgeschädigten Partikeln

Lysolecithin entstehen kann, welches weitere, vielleicht noch intakte Mitochondrien angreift. Auch hier ist also, wie schon mehrfach, zu fragen, ob das entstehende Lysolecithin oder der Zerfall der essentiellen Lipoide für die Störung von Funktion und Struktur der Mitochondrien entscheidend ist.

Die Störung der Mitochondrienstruktur durch Lysolecithin bzw. Schlangengift läßt sich unmittelbar elektronenoptisch sichtbar machen (NYGAARD *et al.*, 1954). Ein Gemisch von Lysolecithin und Lysokephalin (aus Eigelb), in höheren Konzentrationen angewandt (bis 10 mg/ml), führt zu einem dosisabhängigen Zerfall der Mitochondrien, wohl entsprechend seinen allgemein solubilisierenden Eigenschaften. Es erstaunt nicht, daß Phospholipase A („Crotoxin“) keine derart massiven Veränderungen hervorruft, da eine so große Lysophosphatidmenge auch bei komplettem Umsatz der Mitochondrien-Lipide nicht zu erwarten ist. Die Außenschicht der Mitochondrienmembran löst sich unter Phospholipase A in auffallender Weise ab; schon ca. 20 µg Enzym/Ansatz reichen dazu aus. Das sind Konzentrationen, die eine nur geringe Lyse mitochondrialer Phospholipoide bewirken, aber den Elektronenübergang zwischen Cytochrom b und c (Succinoxidase-System) bereits komplett hemmen. Für Bienengift bzw. dessen Komponenten liegen keine entsprechenden Abbildungen vor. Jedoch lassen sich die Strukturveränderungen auf einfache Weise demonstrieren. Präparate von Zellpartikeln aus Rattenleber erhöhen ihre Extinktion, wenn man sie mit Melittin oder hohen Bienengiftkonzentrationen inkubiert; sie vermindern sie unter dem Einfluß von kleinen Giftkonzentrationen oder von Lysolecithin. Zellpartikel verhalten sich demnach analog wie Eigelbsuspensionen. Im Endeffekt resultiert eine weitgehende Solubilisierung (HABERMANN, 1955a, 1958b). Kleine Lysolecithinkonzentrationen rufen eine Schwellung von Mitochondrien aus Rattenlebern hervor, die durch Zusatz von ATP teilweise gehemmt werden kann. Interessant ist, daß bei diesen Versuchen keine Mitochondrienschwellung durch Crotalus-adamanteus-Gift erzielt wurde; vielleicht gelang den Autoren die Darstellung einer extrem reinen Mitochondrienfraktion, die aus oben erwähnten Gründen resistent gegen das Gift sein sollte (WITTER und COTONE, 1956). Die Mg-abhängige ATPase wird bei Lysolecithinbehandlung von Mitochondrien aktiviert, die DNP-aktivierte gehemmt (WITTER *et al.*, 1957). Inkubiert man Rattenlebermitochondrien mit Lysolecithin oder erhitztem Gift von Vipera ammodytes und zentrifugiert sie dann, so erscheint ATPase und Pyrophosphatase vermehrt im Überstand. Pyrophosphatase wird dabei partiell inhibiert (HABERMANN, 1955a).

Die aus den optischen Daten, den elektronenoptischen Bildern und der Solubilisierung von Desmoenzymen zu erschließende Störung der Mitochondrienfunktion manifestiert sich vor allem in der Unterbrechung der Atemkette und der Entkopplung der oxidativen Phosphorylierung. Man darf keinen selektiven Angriff der Gifte bzw. von Phospholipase A an einem bestimmten Glied erwarten, da jedes Einzelenzym strukturgebunden, also ein „Desmo-

enzym“ ist; doch ist es möglich, daß bestimmte Abschnitte der Atemkette empfindlicher sind als andere. Die Störung des Elektronentransports durch Phospholipase A und Lysolecithin wurde nahezu gleichzeitig in mehreren Laboratorien um 1950 nachgewiesen. Voraus gingen Untersuchungen von CHAIN (1939), der die Hemmung verschiedener Dehydrogenasen durch black tiger-snake-Gift beschrieb. Er bezog sie aber auf die Spaltung von NAD durch eine gifteigene Nucleotidase. FLECKENSTEIN *et al.* (1950) inaktivierten durch Bienengift die anaerob ablaufende Dehydrierung verschiedener Substrate (Milchsäure, Bernsteinsäure, Brenztraubensäure, Citronensäure, Oxalessigsäure, Glycerinphosphorsäure) in Froschmuskelbrei. Über den Wirkungsmechanismus konnte FLECKENSTEIN noch keine näheren Angaben machen; er vermutete eine Analogie zu den von ihm früher geprüften schmerz- und entzündungserregenden Zellgiften bekannter chemischer Konstitution. GHOSH und BHATTACHARYA (1952) und CHATTERJEE (1949) beobachteten ebenfalls eine Hemmung oxidierender Prozesse durch Schlangengift. Das Verständnis für diese Reaktionen nahm zu, als man fand, daß die Inaktivierung der Bernsteinsäuredehydrierung durch Bienengift nicht bzw. nur zum kleinen Teil auf dem schmerz- und entzündungserregenden Melittin (damals noch FI genannt) beruhte, sondern auf der pharmakologisch weniger auffälligen Fraktion II, welche Phospholipase A (neben Hyaluronidase) als wirksames Agens enthält. Bienengift hat sich also, wie bei der Thromboplastininaktivierung, so auch bei der Störung der Mitochondrienfunktionen, als besonders einfaches und transparentes Modell der Schlangengiftwirkungen erwiesen (NEUMANN, HABERMANN und AMEND, 1952; HABERMANN, 1953). Es handelt sich bei der Beeinflussung der Bernsteinsäuredehydrierung aus Rinderhirn durch Bienengift-Phospholipase A nicht um eine Hemmung, sondern um eine Inaktivierung des Enzyms durch Strukturveränderung infolge Spaltung des lipoiden Trägers; Zusatz von Lysolecithin imitiert den Effekt in den untersuchten Dosisbereichen nicht (SCHOETENSACK, 1953). BRAGANCA und QUASTEL (1953) fanden gleichzeitig, daß erhitztes Cobragift nur gebundene, d.h. Desmoenzyme, nicht dagegen freie, d.h. Lyoenzyme inaktiviert und bezogen dies auf die im erhitzten Gift noch vorhandene strukturschädigende Phospholipase A. Calciumzusatz beschleunigte die Inaktivierung der mitochondrialen Enzyme. Untersucht wurde die Oxidation von Glucose, Brenztraubensäure, L-Glutamat, Succinat, α -Ketoglutarat, Fructose sowie die Dehydrierung von Brenztraubensäure im Hirnhomogenat, die Bernsteinsäuredehydrierung in Hirn und Herz, die Cytochromoxidase des Gehirns, die Cholinoxidase der Leber (BRAGANCA u. QUASTEL, 1953). Kleine Mengen an Phospholipase A bzw. Lysolecithin lockern offenbar die Struktur der Mitochondrien auf, ohne sie zu zerstören; so wird verständlich, daß dann die Atmung gesteigert (NYGAARD und SUMNER, 1953) bzw. die Oxidation von Cytochrom c begünstigt wird (WITTER *et al.*, 1957). Im einzelnen wurden folgende Reaktionsschritte als lysolecithinempfindlich

befunden: Oxidation von Succinat und β -Hydroxybutyrat mit Ferricyanid, nicht dagegen von Succinat mit Phenazin-Methosulfat (WITTER *et al.*, 1957); Succinoxidase wurde stärker gehemmt als Succinodehydrogenase, Cytochromoxidase, Cholinoxidase, Malicoxidase; die Hemmung (Rattenlebermitochondrien, Crotoxin als Enzymquelle) ließ sich nicht durch Zusatz von Cytochrom c überwinden. Es wurde vermutet, daß die Bindung zwischen Succinodehydrogenase und Cytochrom c durch spaltbare Phospholipide mitbedingt ist. Schon nach Hydrolyse von nicht mehr als 10% des Gesamtlecithins soll die Inaktivierung komplett sein. Allerdings wurde diese Zahl aus der hämolytischen Wirksamkeit ermittelt; dadurch wird aber nur ein Teil des entstandenen Lysins erfaßt. Weiteres Lysolecithin könnte an Cholesterin oder noch intaktes Lecithin der Mitochondrien gebunden sein (NYGAARD und SUMNER, 1953). Eine weitere Einengung des Angriffspunktes wurde von NYGAARD (1953) versucht, der den Elektronenübergang von Succinat auf Cytochrom c und von NADH auf Cytochrom c in Rattenleberhomogenat prüfte. Beide Reaktionen wurden durch Crotoxin gehemmt. Der Block wurde auf Grund spektralphotometrischer Untersuchungen zwischen Cytochrom c und b lokalisiert: Die Reoxygenation von reduziertem Cytochrom b wurde gehemmt, nicht dagegen seine Reduktion. In einer Präparation aus Schweineherz ließ sich die NADH-Cytochrom c-Reduktion komplett unterbrechen, ohne daß dabei die Succinat-Cytochrom c-Reduktion nennenswert beeinflußt wurde. Lösliche „NADH-Cytochrom c-Reduktase“ wurde erwartungsgemäß (s. S. 292) nicht angegriffen (NYGAARD, 1953). EDWARDS und BALL (1954) bestätigten die stärkere Hemmbarkeit der Succinoxidase durch Cobragift im Vergleich zur Succinodehydrogenase und Cytochromoxidase. Auch erhitztes Gift war wirksam, Ca^{++} war erforderlich; es darf also angenommen werden, daß Phospholipase A das aktive Agens war. Sie diskutierten eine mögliche Bedeutung der ungesättigten Fettsäuren, die aus Lecithin abgespalten werden: 1 μMol Oleat/mg der verwendeten Enzympräparation hemmt die drei genannten Prozesse weitgehend. Stearat war sehr viel schwächer wirksam.

Dieser Deutung ist aber entgegenzuhalten, daß Phospholipase C, welche die in dieser Hinsicht sicher unwirksamen (EDWARDS und BALL, 1954) Spaltprodukte Glycerinphosphorylcholin und Diglycerid erzeugt, ebenfalls Succinoxidase, Succinodehydrogenase und Cytochromoxidase von Kaninchemitochondrien inaktiviert (MACFARLANE, 1950), sowie Mg-aktivierte ATPase aus Muskulatur (KIELLEY und MEVERHOF, 1950).

Die Bedeutung struktureller Faktoren geht aus Befunden von ARAVINDAKSHAN und BRAGANCA (1961 b) hervor, die erhitztes Cobragift bzw. daraus gewonnene kristallisierte Phospholipase A auf submitochondriale Fragmente aus Rattenleber einwirken ließen. Hier trat keine Entkopplung der oxidativen Phosphorylierung ein, sondern eine gleichmäßige Minderung von Oxidation (β -Hydroxybutyrat als Substrat) und Phosphorylierung. In die gleiche Rich-

tung deutet die Beobachtung, daß Phospholipase A bzw. erhitztes Cobragift eine Schwellung von Mäuseleber-Mitochondrien bewirkt; übrigens tendierten auch Mitochondrien von *in vivo* vergifteten Mäusen zu verstärkter Schwellung. Obwohl Lysolecithin selbst die oxidative Phosphorylierung entkoppelt (HABERMANN, 1954b, 1955a; WITTER *et al.*, 1957; ARAVINDAKSHAN und BRAGANCA, 1961b), ist seine Entstehung wohl nicht für die Entkopplung durch Phospholipase A entscheidend. Zwar wirkt es — wie Phospholipase A — nur auf komplett Mitochondrien, nicht auf Fragmente; die zur Entkopplung und auch zur Schwellung erforderliche exogene Lysolecithinmenge ist jedoch wesentlich höher als der aus entsprechend geschädigten Mitochondrien extrahierbare Betrag an endogenem Lysolecithin (ARAVINDAKSHAN und BRAGANCA, 1961b).

GREEN und FLEISCHER (1963) haben die Beziehungen zwischen Lipiden und Mitochondrienfunktionen ausführlich dargestellt, die zum Teil unter Zuhilfenahme von Phospholipase A ermittelt wurden. Über 90 % der Mitochondrienlipide sind Phospholipoide. Man kann ihnen kaum die Rolle von Cofaktoren zuschreiben; dazu ist die Relation Lipid/Enzymprotein zu hoch, der relative Lipidgehalt in einzelnen, funktionell unterschiedlichen mitochondrialen Partikeln zu gleichförmig (22—29 % der Trockensubstanz) und auch die Zusammensetzung der Lipidfraktion aus Phosphatidylcholin (37 %), Phosphatidyläthanolamin (31 %), Cardiolipin (16 %), Phosphatidylinositol ($\leq 10\%$) in Gesamtmitochondrien und daraus hergestellten Untereinheiten zu wenig differenziert. Man kann aber Glieder der Atemkette, die man durch Extraktion von Lipiden inaktiviert hatte, durch Zusatz von Phospholipoiden partiell ($> 50\%$) bis vollständig reaktivieren. Das gilt für Cytochromoxidase, für die Verbindungen zwischen Succinat und Cytochrom c, Succinat und Coenzym Q, reduziertem Coenzym Q und Cytochrom c. Es kommt dabei erst in zweiter Linie auf die molekulare Struktur der zur Reaktivierung verwendeten Lipoide an, vor allem jedoch darauf, daß überhaupt eine Bindung erreicht wird. So kann man acetoneextrahierte Mitochondrien mit Lecithin, Kolaminkephalin, Cardiolipin oder einem Gemisch reaktivieren. Die vier Komplexe der Atemkette ($\text{NADH} \rightarrow \text{Q}$; $\text{Succinat} \rightarrow \text{Q}$; $\text{QH}_2 \rightarrow \text{Cytochrom c}$; red. $\text{Cytochrom c} \rightarrow \text{O}_2$) sind wahrscheinlich sämtlich lipidbedürftig; für die Reaktion $\text{NADH} \rightarrow \text{Q}$ wurde dies allerdings noch nicht nachgewiesen. Die Funktion der mitochondrialen Phospholipoide mag darin bestehen, daß sie entsprechend ihrem physiko-chemischen Charakter Micellen bilden, auf welche die mit hydrophoben Arealen versehenen Proteine der Atemkette aufgezogen sind. So wird einerseits eine räumliche Zuordnung erzielt, andererseits die Atemkette in die Nähe eines Mediums mit niedriger Dielektrizitätskonstante gebracht. Es gibt Gründe für die Annahme, daß dadurch die Reaktionsfähigkeit einzelner Kettenglieder außerordentlich erhöht wird. Neben dieser „ordnenden“ Funktion der Lipide muß noch an eine Mittler-Funktion zwischen hydrophilen und hydrophoben

Bereichen gedacht werden. Paradoixerweise lassen sich Mitochondrien und bestimmte Proteine hieraus nur dann gut in Wasser suspendieren bzw. lösen, wenn sie lipidhaltig sind; der hydrophile Anteil ist also nach außen gerichtet. Umgekehrt wird der hydrophobe Anteil nach dem Prinzip eines „flüssigen Kristalls“ funktionieren und darin gelösten Substanzen, z.B. Coenzym Q, eine erhebliche Beweglichkeit lassen. Bei Würdigung der Anschauungen von GREEN und FLEISCHER ist es verständlich, daß weder der Phospholipase A noch dem Lysolecithin ein spezifischer Angriffspunkt in Mitochondrien zugeschrieben werden kann, gleichgültig ob man die Glieder der Atemkette oder die Kopplungsstellen der oxidativen Phosphorylierung diskutiert.

Allosterische, von den Lipiden ausgehende Effekte könnten für einen von PESCH und PETERSON (1964) mitgeteilten Befund verantwortlich sein. Rattenherzmitochondrien wurden unter Zuhilfenahme von Lysolecithin zerkleinert; die Suspension setzte man mit gereinigter Phospholipase A aus Crotalus-adamanteus-Gift um. Während unbehandelte Mitochondrienteilchen NADH und NADPH oxidieren können (als Elektronenakzeptor diente 3-Acetyl-pyridin-Adenin-Dinucleotid), setzten phospholipasebehandelte Präparate nur noch NADH um. Unterstellt man, daß dasselbe Enzym die Reaktionen von NADH und NADPH katalysiert, so wäre durch die Einwirkung von Phospholipase A ein Wandel der Substratspezifität eingetreten.

Exogene Phospholipase A hat also an isolierten Systemen eine Vielzahl von Effekten. Daher wurde schon mehrfach eine Beteiligung von endogener Phospholipase A an der Regelung der Mitochondrienfunktionen diskutiert. Zwar wurde Lysolecithin bisher nur in sehr geringer Menge in Mitochondrien gefunden. Nach Auffindung der Lysolecithin metabolisierenden Enzymsysteme hatte man dafür eine einfache Deutung: Der Organismus setzt dieses Lipid sofort weiter um. Als Quellen des endogenen Lysolecithins kämen zwei Prozesse in Betracht. Serum enthält deutliche Mengen an diesem Lipid, es könnte also von den Zellen aufgenommen worden sein (s. S. 270). Wesentlich interessanter wäre der Nachweis einer Bildung in der Zelle, etwa durch eine zelleigene Phospholipase A. Ein solches Enzym wurde bisher in den Erythrocyten nicht gefunden (MULDER und VAN DEENEN, 1965; MUNDER *et al.*, 1965), wohl aber in den Mitochondrien (ROSSI *et al.*, 1965; SCHERPENHOF und VAN DEENEN, 1965; BJÖRNSTAD, 1966a) und Mikrosomen (BJÖRNSTAD, 1966b) aus Rattenleber. Auch in lysosomenreichen Fraktionen polymorphkerniger Leukocyten ließ sich inzwischen Phospholipase A nachweisen (ELSBACH und RIZAK, 1963; HEGNER u. FRIMMER, 1967), ferner in den Lysosomen und der Fraktion der großen Granula des Nebennierenmarks vom Rind (BLASCHKO *et al.*, 1967; s. S. 297). Das mitochondriale Enzym spaltet nur in Gegenwart von Ca^{++} , und zwar Kolaminkephalin besser als Lecithin; es ist thermolabil. Exogenes Kolaminkephalin soll schlechter gespalten werden als endogenes (BJÖRNSTAD, 1966a); doch wird auch das Gegenteil angegeben (SCHERPENHOF und VAN DEENEN, 1965). Rossi *et al.* (1965) verfolgten den Abbau von endogenem Lecithin.

Die endogene mikrosomale Phospholipase A verhält sich im Prinzip der mitochondrialen ähnlich, was die bevorzugte Spaltung von Kolaminkephalin und die Aktivierbarkeit durch Ca^{++} (und Mg^{++}) betrifft. Da die entstehenden Lyso-Verbindungen weiter gespalten werden können, ist als zweites Enzym eine Phospholipase B anzunehmen (BJÖRNSTAD, 1966b). Eine ribosomale (oder mikrosomale) Fraktion aus der Mucosa des Rattendünndarms spaltet Lecithin. Eine nähere Charakterisierung des Angriffspunktes steht noch aus (OTTOLENGHI, 1964).

Welche Bedeutung besitzt die Zerstörung mitochondrialer Enzymsysteme für die Intoxikation durch Phospholipase A bzw. Gesamtgifte von Insekten und Schlangen? Es leuchtet ein, daß in vorgeschädigten Geweben, deren Mitochondrien nicht mehr durch Zellgrenzen vor dem Angriff zellfremder Enzyme geschützt sind, die Phospholipasen der Gifte zur Wirkung kommen können. Die relativ geringe Lokalwirkung isolierter Phospholipase A spricht umgekehrt gegen eine wesentliche Mitochondrienschädigung; es ist nicht anzunehmen, daß ein Molekül mit einem Gewicht um 19000 (STOCKEERAND, 1965) in die intakte Zelle permeieren kann. Um so überraschender sind die Angaben von ARAVINDAKSHAN und BRAGANCA (1959, 1961a, 1961b), daß Mitochondrien aus Hirn und Leber von mit Cobragift behandelten Mäusen einen erniedrigen P/O-Quotienten aufweisen. Nach Injektion von unerhitztem Cobragift lag er bei 0 (!); wieso die Tiere mit diesem Defekt einige Stunden weiterleben konnten, ist unklar. Während für diesen Befund Nucleotidasen des Cobragiftes verantwortlich gemacht werden, wird die Erniedrigung des P/O-Quotienten (von normalerweise 2,6—3,0 auf 1,3 bis 1,6) durch erhitztes Gift auf dessen Phospholipase A-Gehalt bezogen; Extrakte aus Hirn und Leber hämolysierten, nachdem die Mäuse 75 µg erhitztes Cobragift erhalten hatten. Von den drei Phosphorylierungsprozessen, die mit der Kette des Elektronentransports verbunden sind, war der mit der Cytochromoxidase gekoppelte am empfindlichsten (Versuche mit *in vitro* und *in vivo* vergifteten Mitochondrien); die Kopplung im Bereich der NADH-Cytochrom c-Region war *in vivo* relativ resistent gegen Phospholipase A. Das wurde als Grund dafür angeführt, daß keine komplette Entkopplung *in vivo* erzielbar wurde. *In vitro* war jedoch auch dieser Schritt, wenn auch geringer, gegen erhitztes Cobragift empfindlich.

γ) *Endoplasmatisches Reticulum und Ribosomen*. Auch die Strukturen des endoplasmatischen Retikulums sind empfindlich gegen Phospholipasen. Bisher wurde vor allem mit Phospholipase C gearbeitet; das Enzym inaktiviert die Mg⁺⁺-aktivierbare ATPase aus Muskulatur weitgehend (KIELLEY und MEYERHOF, 1950). Die im elektronenoptischen Bild sichtbaren mikrosomalen Strukturen (aus Kaninchen- oder Rattenmuskulatur dargestellt) werden erstaunlich wenig beeinflußt; es treten lediglich die zu erwartenden Diglycerid-Tröpfchen auf. ATPase-Aktivität und Ca⁺⁺-Aufnahme sinken um mehr als 90 % des Ausgangswertes ab. Auch nach Solubilisierung der ATPase durch Deoxycholat kommt die phospholipasebedingte Inaktivierung zustande. Na-Oleat aktiviert in niedrigen Konzentrationen (10^{-4} M) die ATPase und inhibiert in diesen Bereichen die Ca⁺⁺-Aufnahme; höhere Konzentrationen hemmen dann die ATPase. Lysolecithin mindert in Verdünnungen 1:10000 die ATPase-Aktivität phospholipasefreier (aber ölsäurebehandelter) Mikrosomen (MARTONOSI, 1964). Die durch Phospholipase C bedingte Schädigung der Mikrosomen läßt sich durch Zusatz von Lecithin verschiedener Zusammensetzung (MARTONOSI,

1963), besonders gut aber durch Lysolecithin (MARTONOSI, 1964) in kleinen Konzentrationen (1:20000—1:30000) restituierten: ATP-Spaltung und Ca^{++} -Aufnahme finden wieder statt. Die geschädigte mikrosomale ATPase gewinnt unter dem Einfluß von Lecithin auch ihre Hemmbarkeit durch Calcium und ihre Aktivierbarkeit durch DNP wieder.

Beim Angriff von Phospholipase A an Ribosomen sind zwei Mechanismen diskutabel: Abbau von Lipoproteinen und Aktivierung ribosomaler Ribonuclease. Leber-Polyribosomen von Ratten werden durch erhitztes Naja-naja-Gift desaggregiert; Präparate aus normalen Rattenlebern sind gegen das Enzym empfindlicher als solche aus regenerierenden Organen. Beide Präparationen werden durch Lysolecithin etwa gleich stark angegriffen. 10 $\mu\text{g}/\text{ml}$ sind kaum wirksam; 25 $\mu\text{g}/\text{ml}$ und mehr desaggregieren zunehmend (TSUKADA u. LIEBERMANN, 1965; TSUKADA *et al.*, 1966).

d) Chromaffine Granula. Die chromaffinen Granula des Nebennierenmarks vom Rind sind besonders reich an Lysolecithin; sie enthalten 16 % des gesamten Lipid-Phosphors als Lysolecithin, während z. B. in den Mikrosomen und Mitochondrien jeweils nur ca. 2 % enthalten sind (BLASCHKO *et al.*, 1966). Lysolecithin setzt Adrenalin aus der perfundierten Nebenniere frei (FELDBERG, 1940); dem Prozeß dürfte jedoch eine Schädigung der Zellmembran mit gleichzeitiger Fixierung des Lysins vorausgehen. Exogenes Lysolecithin greift primär wohl nicht dort an, wo sich das endogene Lysolecithin angereichert findet.

Der hohe Lysolecithingehalt des Nebennierenmarks steht vielleicht in Zusammenhang mit dem Vorkommen von Phospholipase A in diesem Organ. In der Lysosomenfraktion läßt sich ein entsprechendes Enzym mit Aktivitäts-Optimum im Säuren (um pH 4) nachweisen, das Lecithin und Phosphatidyl-äthanolamin zu spalten vermag. Darüber hinaus enthält die Fraktion der großen Granula (und zwar wahrscheinlich ihr chromaffiner Anteil) eine zweite Phospholipase A mit einem Aktivitätsoptimum bei pH 6,5 (BLASCHKO *et al.*, 1967).

e) Zellmembranen. Die Membranschädigung durch Lysolecithin wurde am Beispiel der Hämolyse bereits abgehandelt. Jede schwerwiegende Permeabilitätserhöhung wird zum Austritt bzw. zur Aktivitätsänderung von normalerweise intracellulär vorliegenden Enzymen führen, wie am Beispiel des Erythrocyten gezeigt wurde (HABERMANN, 1955a).

In diesem Zusammenhang interessiert aber vor allem die Beeinflussung von membranständigen Enzymen, deren Aktivität für die Funktion der Zelloberfläche von entscheidender Bedeutung sein dürfte; ionenaktivierte ATPasen nehmen dabei den ersten Platz ein. Membranpräparate der Herzmuskulatur werden durch Phospholipase A aus Bienengift und auch durch Lysolecithin stark geschädigt; die $\text{Na}^+ \cdot \text{K}^+$ -abhängige „Transport“-ATPase-Aktivität fällt ab. Phospholipase C hemmt nicht nur die Transportaktivität, sondern auch die ionenunabhängige Basisaktivität (PORTIUS und REPKA, 1963b). Die Verhältnisse an der Erythrocytenwand liegen ähnlich; erhitztes Schlangengift

Tabelle 16. Gegenüberstellung der wichtigsten biochemischen und pharmakologischen Eigenschaften der Biogenengiftkomponenten sowie von Lyssolecithin

	Histamin	Melittin	Apeamin	MCD-Peptid	Hyaluronidase	Phospholipase A	Lyssolecithin
Gehalt (%) ^a	0,1—1	50	2	2	1—3	12	0
Aminosäurereste/Molekül	—	26	18	22	?	?	—
Molekulargewicht	111	2840	2038	2593	>20000	19000	524
Schwefel	0	0	++	++	+	+	0
Oberflächenaktivität	0	++	?	?	0	0	+
Allgemeintoxizität ^b	192—445 ^c	4	4	>40	0	7,5	150
Lokaltoxizität							
Schmerzerzeugung	++	++	?	?	0	?	?
Erhöhung der Capillarpermeabilität	++	++	+	++	+	+	++
Zellschädigung	0	++	?	+	0	+	0
Neurotoxicität	0	++ ^d	++	0	0	0	0
„Direkte“ Hämolysse	0	++	0	0	0	0	++
Kreislaufeffekte	++	++	0	+(Ratte)	0	+	++
(Neuro-) Muskuläre Effekte	0	++	0	?	0	0	++
Glattmuskuläre Effekte	++	++	0	0	0	+	++
Ganglienblockade	0	++	0	?	0	0	++
Histaminfreisetzung	0	++	0	++	0	+	++
„Indirekte“ Hämolysse	0	0	0	0	0	+	0
Thromboplastin (Inaktivierung)	0	+	?	?	0	++	+
Elektronentransport (Unterbrechung)	0	+	?	?	0	++	+
Oxidative Phosphorylierung (Entkopplung)	0	+	?	?	0	++	+
Spreading	0	0	0	0	++	0	0
Antigenität	0	?	?	?	++	++	?

^a Deutlich; ^b nicht nachweisbar; ^c nicht geprüft.^d Annähernd, im Trockengift. ^e mg/kg, Maus i.v. ^c Nach NARANJO (1966).^d Nur für Mastzellen erwiesen. ^e Nur bei lokaler Anwendung erwiesen. ^f Wahrscheinlich indirekt.

hemmt, wohl durch seine Phospholipase A, die ouabainsensitive, kationenaktivierte ATPase menschlicher Erythrocytenschatten fast vollständig, die ouabainresistente, kationenunabhängige ATPase nur unbedeutend (TATIBANA, 1963). Phospholipase C von *Clostridium perfringens*-Gift inaktiviert nicht nur die ouabainsensitive, sondern auch die insensitive ATPase von Erythrocytenschatten (SCHATZMANN, 1962).

Tabelle 16 faßt die wichtigsten Daten der Bienengiftkomponenten zusammen.

III. Wespengifte

Über Wespengift ist erheblich weniger bekannt als über Bienengift. Während Bienengift in technischem Maßstab in Gramm-Mengen gewonnen wird, macht die Bereitstellung entsprechender Wespengiftmengen Schwierigkeiten. Man hat Mühe, eine hinreichende Zahl von Tieren zu sammeln. Außerdem trägt der Wespenstachel keine Widerhaken: eine Reihe von besonders „reinlichen“ Entgiftungsverfahren (s. S. 224), die sich bei der Biene im halbtechnischen Maßstab anwenden lassen, entfällt demnach. Eine dritte Schwierigkeit begegnet vor allem dem Nicht-Zoologen. Während die zur Imkerei verwendeten Bienenrassen sich nicht grundsätzlich unterscheiden, vor allem nicht in ihrer Giftzusammensetzung (HABERMANN, unveröffentlicht), ist die Zahl der Wespenarten sehr groß; „Wespe“ ist ein volkstümlicher, kein zoologischer Ausdruck. Zu den Vespiden gehört z.B. auch die Hornisse (*Vespa crabro*). Die antigenen Eigenschaften verschiedener Wespen werden später (S. 310) mit denen von Bienen verglichen. ABRAHAMS (1955) gibt folgende Werte für Wespen-Rohgift (*V. polistes*) an: pH 6,8—6,9; Trockengiftmenge/Tier 0,14 mg bzw. 0,17 mg; Rückstand des Extrakts aus dem Stechapparat 0,68 mg.

A. Niedermolekulare Substanzen

1. Biogene Amine

Die formale Zusammensetzung des Wespengiftes entspricht derjenigen des Bienengiftes; doch bestehen im einzelnen erhebliche Unterschiede. So enthält auch Wespengift Histamin; bei Aufbereitung nach Code findet man das Amin zu 1,6 % in der Trockensubstanz, nach papierchromatographischer Abtrennung wurden 2 % ermittelt (JAQUES und SCHACHTER, 1954). Der Histamingehalt liegt also deutlich höher als im Bienengift; doch fand diese Arbeitsgruppe (SCHACHTER und THAIN, 1954) auch im Bienengift etwas höhere Werte (1 % der Trockensubstanz) als andere (vgl. S. 228). Als zweites biogenes Amin kommt Serotonin in den Giften verschiedener Wespenarten vor. Zunächst wurde es von JAQUES und SCHACHTER (1954) im Gift von *V. vulgaris* zu etwa 0,32 mg/g durch biologische Testung nach vorangegangener Papierchromatographie bestimmt. Vielleicht enthält auch Bienengift Spuren von Serotonin; WELSH und BATTY (1963) führen einen Gehalt von 21 µg/g Giftapparat (Feuchtgewicht) auf, wobei allerdings nicht sicher ist, ob das Amin ins Gift

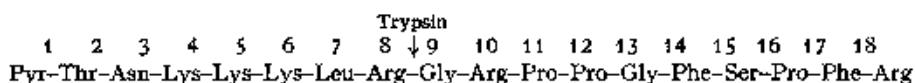
übertritt. Im Stechapparat solitärer Wespen war kein Serotonin nachweisbar; doch wurde es bei allen untersuchten Sozialwespen gefunden: *Polistes fuscatus* 0,81 µg/Stechapparat; *Polistes versicolor* 1,04 bzw. 1,2 µg/Stechapparat; *Polistes versicolor vulgatus* 1,94 µg/Stechapparat; *Vespa maculata* 1,29 µg/Stechapparat; *Synoeca surinama* 2,74 µg/Stechapparat. Auch das entsprechende Organ der Hummel enthält kleine Mengen (0,02 µg/Stechapparat). Das Amin wurde an Hand seines charakteristischen Fluoreszenzspektrums identifiziert und bestimmt (WELSH und BATTY). Das Vorkommen von Acetylcholin wurde für das Gift von *Polistes omissa* beschrieben (SAID, 1960).

2. Kinine

SCHACHTER u. Mitarb. (JAQUES und SCHACHTER, 1954; SCHACHTER und THAIN, 1954; SCHACHTER, 1960, 1963) entdeckten ein Wirkprinzip im Gift von *Vespa vulgaris*, das den Kininen, die aus Blutplasma durch eine Reihe von Enzymen freizulegen sind (ROCHA E SILVA et al., 1949), chemisch und in seinem Wirkungsspektrum sehr nahesteht. Kinine erhöhen die Gefäßpermeabilität, erzeugen Schmerz, senken den Blutdruck und rufen eine langsame Kontraktion glatter Muskulatur hervor, welche — auch nach Applikation hoher Dosen — keine bzw. eine nur wenige Minuten währende Tachyphylaxie nach sich zieht. Melittin teilt zwar die beiden erstgenannten Fähigkeiten mit den Kininen, ist aber von ihnen am Kreislauf und an glatter Muskulatur sowie vor allem an Hand seiner zusätzlichen pharmakologischen Wirkungen und seiner physiko-chemischen Eigenschaften zu unterscheiden. Melittin ist also kein Kinin; wollte man es einer bestimmten Substanzgruppe zuordnen, so kämen dafür am ehesten basische Proteine in Frage, z. B. Protamine und Histone, von denen es aber in seiner Aminosäurezusammensetzung und seinen physikochemischen Eigenschaften ebenfalls recht verschieden ist.

Die Wespengiftkinine stehen demgegenüber auch chemisch den Plasmaininien nahe. Wahrscheinlich gibt es mehrere Wespinkinine, die sich in den an das „Rückgrat“ Bradykinin geketteten Aminosäuren unterscheiden. So erhielten MATHIAS und SCHACHTER (1958) bei Ionenaustauschchromatographic an Amberlite XE 64 Hinweise auf die Existenz von drei verschiedenen Kininen, von denen das am stärksten basische 85—90 % der wiedergefundenen Aktivität umfaßte. In ihrem Verhalten gegen Trypsin (Abschwächung) und Chymotrypsin (Inaktivierung) waren sie nicht zu unterscheiden, auch nicht in ihrer relativen Aktivität an Rattenuterus und Meerschweinchenileum. Alle drei Komponenten waren jedoch von Bradykinin und Kallidin verschieden. PRADO et al. (1966) griffen die Arbeiten von SCHACHTER u. Mitarb. auf und analysierten die Kinine der Wespenspecies *Polistes exclamans*, *Polistes annularis* Linnæus und *Polistes fuscatus*. Sie erhielten fünf säulenchromatographische Fraktionen mit Kininaktivität, die sich sämtlich durch Chymotrypsin, nicht dagegen durch Pepsin, Trypsin oder Kollagenase inaktivieren

ließen und deren Wirkung nicht durch LSD oder Atropin abgeschwächt wurde. Alle kontrahierten sie das Meerschweinchenileum und den Rattenuterus, brachten das Rattenduodenum zum Erschlaffen und senkten den Blutdruck der Ratte. Zwei der Kinine waren (im Gegensatz zu den Befunden von SCHACHTER) von Bradykinin und Kallidin nicht zu unterscheiden. Zwei weitere Fraktionen wurden nicht identifiziert. Das am stärksten basische Prinzip konnte rein erhalten werden und enthielt wahrscheinlich die Sequenz Gly-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, also Glycyl-Bradykinin. Trypsinbehandlung änderte die chromatographischen Eigenschaften an Carboxymethylsephadex und auf Dünnschichtplatten; die biologische Aktivität nahm etwas zu. Am intakten Peptid wurde kein N-Terminus gefunden; es wird vermutet, daß sich dort ein Pyroglutaminsäurerest befindet. UDENFRIEND *et al.* (1967) geben folgende Struktur für „Kinin 3“ an:



Nach SCHACHTER u. Mitarb. ist Wespenkinin relativ thermostabil im neutralen oder mäßig sauren Bereich; es ist langsam dialysabel, also relativ kleinmolekular, und durch Trypsin oder Chymotrypsin zerstörbar. Die Zerstörbarkeit durch Trypsin paßt allerdings nicht zu der von PRADO *et al.* (1966) vorgeschlagenen Bradykinin-Struktur, deren biologisch entscheidendes Kernstück bekanntlich trypsinresistent ist. Käufliches Trypsin enthält chymotryptische Aktivität, die vielleicht für die Inaktivierung in SCHACHTERS Versuchen verantwortlich war.

Der Gehalt des Wespengiftes an Kinin wird von SCHACHTER u. Mitarb. nicht angegeben; sicher ist er sehr viel niedriger als etwa der Gehalt des Bienengiftes an Melittin. PRADO u. Mitarb. erhielten aus dem Gift von 3000 Wespen 1,3 mg des im chromatographischen Gipfel Nr. 3 gefundenen Prinzips (Tabelle 17).

Tabelle 17. Eigenschaften von Bradykinin, Kallidin und Wespen-Kininen (Gipfel 1, 2 und 3).
(Nach PRADO *et al.*, 1966)

	Bradykinin	Gipfel 1	Kallidin	Gipfel 2	Gipfel 3
Fraktionsnummer bei Elution aus Carboxymethylcellulose	21—25	19—23	31—35	26—35	43—51
Papierelektrophoretische Wanderung (cm)	47—51	40—47	55—58	45—51	44—54
Papierchromatographische Wanderung (cm) in Butanol-Eisessig-Wasser	8—10	5—10	3—6	0—5	0—2,5
Empfindlichkeit gegen					
Chymotrypsin	+	+	+	+	+
Trypsin, Pepsin, Kollagenase,	0	0	0	0	0
LSD, Atropin					

B. Höhermolekulare Substanzen

Wie Bienengift entwickelt das Gift von *Vespa vulgaris* Hyaluronidase-Aktivität (JAQUES, 1955), desgleichen das Gift von *Polistes omissa* (SAID, 1960); neben Phospholipase A enthält das Gift von *Vespa vulgaris* auch Phospholipase B (CONTARDI und LATZER, 1928), was von uns (HABERMANN, unveröffentlicht) bestätigt wurde. CONTARDI und LATZER geben an, daß auch Glycerinphosphorsäure gespalten wird, was auf eine Phosphatase, vielleicht sogar auf eine Phospholipase C im Wespengift hinweisen würde; diesem Fingerzeig wurde bisher nicht nachgegangen, auch nicht der Frage, ob die Abspaltung der beiden Acylreste des Lecithins tatsächlich auf der sukzessiven Einwirkung zweier verschiedener Enzyme (A und B) oder auf der Einwirkung eines einzigen Enzyms (A + B) beruht. Eine Trennung der Wirkqualitäten von Phospholipasen A und B wurde offenbar noch nicht versucht. Desgleichen ist nichts darüber bekannt, ob das Wespengiftenzym α -Acyl-Glycerophosphorylcholin oder β -Acyl-Glycerophosphorylcholin vorzugsweise spaltet oder ob vielleicht der Spaltung eine (katalytische?) Acylwanderung $\alpha \rightarrow \beta$ vorausgeht. Im Gift von *Polistes omissa* fand SAID (1960) Aliesterase-Aktivität.

Im Laufe der letzten Jahre sind Lysophospholipasen in zahlreichen Geweben nachgewiesen worden (s. z. B. MARPLES und THOMPSON, 1960). In einer Enzympräparation aus *P. notatum* waren Phospholipase A- und B-Aktivität assoziiert; dagegen spaltet das Enzym aus Leber (DAWSON, 1956) und Pankreas (SHAPIRO, 1953) nur Lysolecithin, kein Lecithin. Die Bedeutung der Lysophospholipasen des Gewebes für den Stoffwechsel endogener Substrats sowie für die Wirkungsdauer exogenen Lysolecithins wurde bereits S. 270 gewürdigt, weitere Abbau- und Synthesewege des Lysolecithins sind dort aufgeführt. Unklar bleibt der Sinn des Phospholipase B-Gehaltes des Wespengiftes; sollte man doch damit rechnen, daß Lysolecithin ein für die pharmakologischen und biochemischen Effekte des Wespengiftes wichtiges Intermediärprodukt sei, jedenfalls ein wichtigeres als die beim weiteren Abbau entstehenden Fettsäuren.

JAQUES und SCHACHTER (1954) berichteten über (nicht regelmäßig reproduzierbare) Histaminfreisetzung aus der perfundierten Katzenhaut durch Wespengift. Eine Zuordnung des Effekts zu den besprochenen Komponenten ist einstweilen nicht möglich. Wespengift und eine daraus durch Pikrinsäurefällung gewonnene Fraktion hämolysieren gewaschene menschliche Blutkörperchen. Durch Erhitzen in 0,85 % NaCl (siedendes Wasserbad) wird die Wirksamkeit auf etwa $1/8$ herabgesetzt; bei Verwendung von n/20 HCl wird sie aufgehoben. Eine nähere Identifizierung der beteiligten Faktoren, auch der bei lokaler Instillation in den Conjunctivalsack reizenden Komponenten, steht noch aus. Das sog. Pikratgift wirkt etwa 10—16mal stärker hämolytisch als getrocknetes Tropfen-Nativgift; dem entspricht, daß aus getrocknetem Giftsekret nur ca. 5 % Pikratgift dargestellt werden konnten, während ca. $3/4$ der Trockensubstanz des Bienengiftes als Pikrat fallen. In Verdünnungen um 1:10000 bewirkt Wespengift eine positiv inotrope Reaktion des isolierten Froschherzens; es ist mindestens 10mal schwächer herzwirksam als Bienengift.

oder dessen Fraktion I. Am isolierten Froschrectus ist es wirkungslos (ABRAHAMS, 1955).

IV. Hornissengift

Hornissengift läßt sich einfacher gewinnen als Wespengift, weil die großen, verhältnismäßig ruhigen Tiere gut zu handhaben sind, besonders im unterkühlten Zustand. Ein Darstellungsverfahren ist bei ABRAHAMS (1955) beschrieben. Man erhält dabei im Mittel 3,68 mg Giftsekret mit 1,04 mg Trocken gift/Tier.

Die Zusammensetzung des Hornissengiftes (*Vespa crabro*) ist derjenigen des Wespengiftes (*Vespa vulgaris*) sehr ähnlich. An niedermolekularen Stoffen ist Histamin vorhanden, das von NEUMANN und HABERMANN (1956) zusammen mit ALBL (1956) papierchromatographisch sowie durch biologische Testung unter Zuhilfenahme von Inhibitoren nachgewiesen wurde. BHOOA *et al.* (1961) fanden 3—30 mg Base/g getrocknetem Stechapparat. Daneben erscheint Serotonin (NEUMANN und HABERMANN, 1956; ALBL, 1956) in Mengen um 7—19 mg/g (BHOOA *et al.*, 1961). Als drittes niedermolekulares Pharmakon tritt Acetylcholin hinzu, das durch Paralleluntersuchungen an verschiedenen isolierten Organen, Verhalten gegen Cholinesterase und Inhibitoren sowie durch Papierchromatographie mit synthetischem Material identifiziert wurde (ABRAHAMS, 1955; NEUMANN und HABERMANN, 1956; ALBL, 1956; BHOOA *et al.*, 1961). Trockengift bestand zu 5 % aus Acetylcholin. Hornissen gift dürfte demnach das acetylcholinreichste Material der Tierwelt sein.

Man fragt sich, welche Bedeutung dieser Massierung niedermolekularer Substanzen nicht nur im Hornissengift, sondern auch im Wespen- und (in geringerem Maß) im Bienengift zukommt. Für die Allgemeintoxicität spielen sie in Anbetracht ihrer raschen Metabolisierung wohl keine Rolle; dagegen könnten sie, da sie sämtlich, und besonders in Kombination, schmerzerzeugend sind, für die Sofortreaktionen von Kreislauf und Nervenendigungen verantwortlich sein. Die prolongierten Effekte dürften demgegenüber durch die hochmolekularen Toxine bzw. Enzyme bedingt sein. Vielleicht liegt gerade darin ihr biologischer „Sinn“: Beim Gegner — ehe er von den hochmolekularen Stoffen geschädigt wird — einen *sofortigen* Warnreiz zu setzen.

Auch Hornissengift enthält ein kininähnliches Prinzip, das aber nicht mit dem Wespengift-Kinin identisch sein dürfte. Bei Papierchromatographie in Butanol-Essigsäure-Wasser soll das Wespengift-Kinin am Start liegen bleiben, während das Hornissengift-Kinin wandert; nach PRADO *et al.* sollen jedoch sämtliche Wespengiftkinine in dem genannten Laufmittel beweglich sein. Hornissengift-Kinin ist am Meerschweincheneileum relativ schwächer wirksam; im übrigen sind die pharmakologischen Effekte qualitativ nicht unterscheidbar (BHOOA *et al.*, 1961; SCHACHTER, 1963).

Über die höhermolekularen Bestandteile des Hornissengiftes ist kaum etwas bekannt. Es enthält Phospholipase A und B (HABERMANN, unveröffentlicht).

Auf Hyaluronidase und andere in tierischen Giften vorkommende Enzyme wurde bisher noch nicht geprüft. In seinem direkt hämolysierenden Vermögen entsprach es etwa dem Wespengift; durch Erhitzen fiel die Wirksamkeit ab. Eine Zuordnung dieses Effekts steht noch aus; ebenso fehlt die Identifizierung des lokal am Kaninchenauge entzündungserregenden Faktors (ABRAHAMS, 1955).

V. Hummelgift

Über dieses Gift ist noch nicht ausführlicher gearbeitet worden. Sicher ist, daß Stiche von Hummeln beim Menschen lokale und allgemeine Reaktionen auslösen können. Ob die Lokaleffekte auf hypererger oder toxischer Basis entstehen, ist unbekannt. Die Allgemeinreaktionen beim Menschen gehören nach ihrer Symptomatologie in den Bereich des allergischen Formenkreises. WELSH und BATTY (1963) fanden bei verschiedenen *Bombus*-Species fluorimetrisch im Mittel 0.02 µg Serotonin/Stechapparat. JAQUES (1955) wies im Giftapparat von *Bombus pratorum* Hyaluronidase und Phospholipase A (Dottercoagulationstest) nach. Cholinesterase fehlte, auf Phospholipase B wurde nicht geprüft.

VI. Lähmende Vespidengifte

Die Gifte von Bienen, Hummeln, *Vespa vulgaris* und *Vespa crabro* dienen ausschließlich der Verteidigung. Einige andere Wespenarten benutzen jedoch ihr Gift, um die Beute zu lähmen, die dann zur Eiablage benutzt wird. Die Erforschung der Inhaltsstoffe dieser Gifte hat noch nicht begonnen; im wesentlichen liegen Beschreibungen ihrer Wirkungen am Ganztier vor. Eine Übersicht findet sich bei BEARD (1963). So hat das Gift von *Vespa arenaria arenaria* (FABRICIUS) bei Injektion in die Larve von *Galleria mellonella* eine Erregung mit konsekutiver Lähmung von Herz, Nervensystem und Muskulatur zur Folge. Das Gift von *Habrobracon juglandis* (*Bracon hebetor*) zirkuliert nach Injektion in der Leibeshöhlenflüssigkeit der genannten Larve; der Stich muß also nicht eine definierte Stelle des Nervensystems treffen. Die spezifische Aktivität des Giftes ist außerordentlich hoch: BEARD schätzt, daß 1 Teil Gift in 2×10^8 Teilen Larvenblut zur permanenten Paralyse hinreicht. Die Aktivität der visceralen Muskulatur bleibt unbeeinflußt, die Körpermuskulatur unterliegt wahrscheinlich einem neuromuskulären Block. Larven von *Galleria*, *Anagasta* und *Plodia* waren sehr empfindlich, während z.B. Larven von *Popillia japonica* und *Pyrausta nubilalis* resistent waren (BEARD, 1952). PIEK (1966a) führte die Untersuchungen BEARDS an ligierten Larven von *Philosamia cynthia* weiter. Die Resultate gleichzeitiger Registrierung von extracellulären Aktionspotentialen und Verkürzung wurden dahingehend gedeutet, daß weder Nerven noch Muskeln, sondern die neuromuskulären Verbindungen der Larven paralysiert wurden. Die durch Reizung des ventralen Nervenstamms auslösbarren Muskel-Aktionspotentiale verschwanden unter dem

Einfluß des Giftes; der paralysierende Faktor war auswaschbar. Einen prinzipiell gleichen Angriffspunkt wies das Gift der Grabwespe *Phylanthus triangulum* am Nervensystem der Heuschrecke auf (PIEK, 1966 b); die neuro-muskuläre Synapse der „schnellen“ Fasern wurde gelähmt, während die Synapse der „langsam“ und „hemmenden“ Fasern offenbar nicht beeinflußt wurde (Versuche mit intracellulären Elektroden). Das Muskel-Aktionspotential blieb auslösbar. Es ist zu betonen, daß die Lähmungen bereits durch Giftmengen verursacht werden können, die einem einzigen Stich entsprechen. Vermutlich handelt es sich bei dem curareähnlichen Agens um ein Protein.

Es gibt noch zahlreiche weitere paralysierende Wespengifte, darunter solche, die nur eine Wirkungsdauer von einigen Minuten aufweisen, was für die Eiablage hinreicht. Über Chemie, Wirkungsweise und Abbaumodus ist nichts bekannt (BEARD, 1963).

VII. Zur Immunologie der Hymenopterengifte

A. Neutralisierende Antikörper gegen Bienengift

Dem Bienengift werden schon seit alten Zeiten immunisierende Eigenschaften nachgesagt. Nach den heutigen Kenntnissen ist zu erwarten, daß sich seine Antigene in zwei Gruppen einteilen lassen. „Gute“ Antigene werden voraussichtlich die hochmolekularen Inhaltsstoffe sein, wie Phospholipase A und Hyaluronidase, zumal derartige Enzyme anderer Herkunft ebenfalls antigene Eigenschaften aufweisen (CINADER, 1957); dagegen werden Melittin, Apamin und die sonstigen Peptide nur unter besondren Bedingungen die Antikörperproduktion anregen. Melittin, MCD-Peptid und Apamin haben Molekulargewichte unter 3000; Apamin und MCD-Peptid enthalten keine aromatischen Aminosäuren, Melittin enthält nur einen Tryptophanrest pro Molekül.

Die bisher vorliegenden Befunde entsprechen diesen Erwägungen. Immunisiert man z.B. Kaninchen oder Meerschweinchen mit Bienengesamtgift, so lassen sich im Serum inaktivierende Antikörper gegen Phospholipase A und Hyaluronidase nachweisen; der Antihyaluronidase- und Antiphospholipase-Titer steigt auf ein Vielfaches des Ausgangswertes an. Präcipitatbildung wurde bei diesen Versuchen nicht beobachtet; dazu reichte die Antikörperproduktion noch nicht aus. Im Gegensatz dazu wurden Melittin-Effekte durch das Serum immunisierter Tiere nicht stärker abgeschwächt als durch das Serum normaler Tiere; das betrifft „direkte“ Hämolys, Allgemeintoxizität für Mäuse, Kontraktion von Meerschweinchenileum und -uterus, Effekte an Froschherzen, Froschsartorius und Vorhof des Meerschweinchenherzens (HABERMANN und EL KAREMI, 1956). Auch Tierversuche anderer Autoren erbrachten keinen Hinweis auf zirkulierende Antikörper gegenüber Melittin. So setzte DEREVICI (1965) Meerschweinchen einer steigenden Zahl von Bienenstichen (bis 40/dosi) aus und gewann dann deren Serum; es schützte Mäuse weder im Gemisch mit dem Gift noch bei separater, vorheriger Applikation gegen eine eben noch

tödliche Giftdosis. Wie S. 233 dargestellt, ist Melittin als das Hämolyisin des Bienengifts anzusprechen. Bei der direkten Hämolyse durch Gesamtgift sind Melittin *und* Phospholipase A beteiligt; daher ist verständlich, daß Anti-Bienengift-Seren eine antihämolytische Wirkung unter diesen Bedingungen entfalten können.

Der Antigencharakter von Schlangengift-Phospholipase ist bekannt; die Dehydrasenhemmung durch Schlangengifte, die im wesentlichen auf dieses Enzym zurückzuführen ist, läßt sich durch spezifisches Antiserum hemmen (FLECKENSTEIN und SCHNEITER, 1951).

Das Naturexperiment der sog. Imker-Immunität sollte erlauben, die Verhältnisse beim Menschen näher zu studieren. Die Antigenität aller Komponenten müßte unter diesen Bedingungen besonders gut zum Tragen kommen; denn die Dauer der Antigenzufuhr beträgt im Tierversuch nur Wochen, beim Imker dagegen Jahre oder Jahrzehnte. Die während dieser Zeit aufgenommene Antigenmenge ist beträchtlich, der Applikationsweg intracutan; Melittin könnte als entzündungserregendes Adjuvans betrachtet werden. Dennoch scheint bisher nur das Auftreten von Anti-Hyaluronidasen als gesichert (BARKER *et al.*, 1966), und zwar ebenfalls nicht als präcipitierende, sondern nur als neutralisierende Antikörper im Bereich der γ -Globuline. Eine Anti-Phospholipase wurde nicht gefunden. Präcipitatbanden, die sich bei der Reaktion zwischen Phospholipase A und Serum bildeten, beruhten auf einer Umsetzung der Lipoproteine; sie traten auch im normalen Serum auf und lassen sich zum Nachweis des Enzyms heranziehen. Demgegenüber behaupteten MOHAMMED und EL KAREMI (1961), inhibitorische Antikörper gegen Phospholipase A im Serum von Imkern gefunden zu haben; die in ihren Tabellen wiedergegebenen Zahlen zeigen aber, daß der Effekt bestenfalls sehr schwach ausgeprägt war. GASTPAR *et al.* (1956) verglichen das antihämolytische Vermögen von Normalserum und Imkerserum, indem sie eine konstante Giftmenge mit fallenden Verdünnungen der Seren versetzten, ehe sie eine Suspension gewaschener Erythrocyten zufügten. Imkerserum hemmte etwa doppelt so stark wie Normalserum. Außerdem waren Imker-Erythrocyten etwas resistenter gegen Bienengift als Normal-Erythrocyten. Der Versuch ist jedoch mehrdeutig; denn, wie S. 233 auseinandergesetzt, steht die „direkte“ Hämolyse neben der „indirekten“, die durch Umsatz der Lipide von melittinhämolierten Erythrocyten (bzw. des Serums) mit der Phospholipase des Giftes zustande kommt. Dazu tritt die Beeinflussung der beiden Hämolysearten durch Serumbestandteile, wie Lecithin und Cholesterin. Derartige Experimente müßten mit der γ -Globulin-Fraktion des Antiseraums und mit gereinigten Giftkomponenten wiederholt werden.

Neben der allgemeinen, durch zirkulierende Antikörper bedingten Immunität wäre eine auf den Bereich wiederholter Bienengiftapplikationen beschränkte Empfindlichkeitsminderung denkbar. DOLD (1917) hatte sich ohne Erfolg

bemüht, durch wiederholte Instillation von Bienengift in den Conjunctivalsack des Kaninchens eine Minderung der entzündlichen Reaktion zu erzwingen; übrigens traten auch keine zirkulierenden, gegen die phlogistische Bienengiftwirkung gerichteten Antikörper auf.

Gegen die Doldischen Versuche könnte man einwenden, daß verhältnismäßig niedrige Giftdosen angewandt wurden, und diese überdies nur auf Schleimhautoberflächen. ANTON (1946) ist jedoch in sorgfältigen Untersuchungen an Kaninchen, Mäusen und Menschen zu gleichen Ergebnissen gekommen. Zunächst versuchte er bei Kaninchen, eine Hautimmunität durch vorausgehende Bienengiftbehandlung zu erzielen. Vor der Immunisierungsperiode bestimmte er durch intracutane Applikation die Dosis necroticans minima (DNM), dann erhielten die Tiere binnen 14 Tagen siebenmal je 1 mg/kg Trockengift zum Teil intracutan, zum Teil subcutan. Die DNM war anschließend nicht verändert. Auch eine humorale Immunität kam nicht zustande. ANTON setzte in einem Teil der Versuche über einen Zeitraum von 5 Wochen intracutane Quaddeln von Trockengift bei Kaninchen; insgesamt entsprach die verabreichte Menge etwa 60 Bienenstichen. Das anschließend entnommene Serum neutralisierte die dermatonekrotische Wirkung nicht stärker als das zuvor entnommene Kontrollserum. In einer weiteren Versuchsreihe applizierte er während der Immunisierungsperiode je 670—790 Bienenstiche/Tier, ohne zirkulierende, gegen die dermatonekrotische Wirkung gerichtete Antikörper zu erzielen. Auch die Allgemeintoxicität an der weißen Maus wurde durch derartig gewonnene Antiseren nicht beeinflußt. Bemerkenswert ist ANTONS Beobachtung, daß Kaninchen ohne Zeichen von Allgemeinvergiftung in einer Sitzung 100 Bienenstiche in die epilierte Bauchhaut erhalten können. Auch bei einem Imker, der als praktisch immun gegen Bienengift galt, war der antitoxische Titer (Letalität bei Mäusen und Dermatonekrose bei Kaninchen als Tests) nicht erhöht, desgleichen nicht bei Patienten, die sich einer Bienengift-Therapie unterzogen hatten. Schließlich prüfte ANTON, ob mit Bienengift (entweder intracutan oder subcutan appliziert) behandelte Kaninchen resisterter gegen intravenöse Vergiftung seien als unbehandelte; auch das war nicht der Fall. Man konnte demnach im Tierversuch weder eine örtliche Empfindlichkeitsminderung noch eine allgemeine aktive oder passive Immunisierung erzeugen. Das steht mit unseren Untersuchungen zur Produktion der Antikörper im Einklang: Nur die enzymatischen Komponenten erwiesen sich als Antigene, nicht dagegen das Haupttoxin Melittin.

Wie eine Umfrage unter 40 Imkern ergeben hat, ist es verfehlt, von einer regelmäßigen „Giftfestigkeit“ gegen Bienengift zu sprechen. Der Umgang mit Bienen führt dazu, daß Stiche mit der Zeit als Selbstverständlichkeit betrachtet und allein schon deshalb weniger intensiv empfunden werden. Alle Befragten geben an, daß der Stich nach wie vor schmerhaft sei; die Heftigkeit des Schmerzes wird meist als schwächer, gelegentlich auch

als stärker im Vergleich zu früher empfunden. Für die Dauer des Schmerzes werden 10—20 min veranschlagt. Das spricht gegen Histamin als vorwiegende Schmerzursache; bei der sehr geringen, im Gift vorhandenen Histaminmenge (ca. 0,1—1 µg/Stich) dürfte die Schmerzschwelle kaum erreicht bzw. schnell wieder unterschritten werden. Man muß eher an den lang anhaltenden Melittin-Schmerz denken. Die weitaus überwiegende Mehrzahl der Imker berichtet, daß Rötung und Schwellung im Laufe der Jahre erheblich geringer geworden sei. Nachdem im Imkerserum Antihyaluronidase und vielleicht auch Antiphospholipase gefunden wurde (s. S. 306), ist die Verkleinerung des geschädigten Areals gut verständlich. Manches weist aber auch auf eine lokale Empfindlichkeitsminderung hin, so die Angabe, daß Stiche in häufig getroffene unbekleidete Körperteile wesentlich mildere Folgen hätten als Stiche z.B. in den Rücken. Bemerkenswert ist im Hinblick auf das folgende Kapitel, daß mehrere Imker im Lauf der Jahre gegen Bienenstiche allergisch wurden (HABERMANN, unveröffentlicht).

B. Allergische Reaktionen gegenüber Hymenopterengiften

Die Trockensubstanz des Bienengiftes besteht zum größten Teil aus Proteinen und Polypeptiden; es sind also nicht nur immunologische, sondern auch allergische Reaktionen zu erwarten. Letztere verdienen aus zwei Gründen besonderes Interesse. Klinisch wichtig ist, daß fast alle gefährlichen Folgen des Kontaktes zwischen Mensch und Bienengift auf ein allergisches Geschehen hinweisen. Es läßt sich rechnerisch zeigen, daß die eigentliche Toxicität des Giftes nur bei der Lokalreaktion zum Ausdruck kommen kann, wie sie S. 247 beschrieben wurde, während für eine Allgemeinvergiftung von Erwachsenen Tausende von Bienen benötigt würden. Solche Ereignisse werden eine Rarität bleiben. Dagegen ist — wenn wir die anaphylaktischen Reaktionen in die Betrachtung einbeziehen — die Biene als das gefährlichste mittel-europäische Gifttier zu bezeichnen. Selbst in den vergleichsweise schlängenreichen USA kommen durchschnittlich mindestens 17 Todesfälle pro Jahr auf das Konto einer allergischen Reaktion gegenüber den Giften von Bienen, Wespen oder bestimmten Ameisenarten; nur etwa ebensoviele (14 Todesfälle bei 1500 Bissen) sind durch Giftschlängen verursacht. Wahrscheinlich ist die tatsächliche Zahl der Todesfälle nach Insektenstichen wesentlich höher; manche Fälle von „Hitzschlag“ oder plötzlichem Herzversagen mögen der richtigen Diagnose entgangen sein (RUSSELL, 1961).

Die akute Reaktion erfolgt in der Regel auf einen einzigen Stich hin. Es ist dabei gleichgültig, wo der Stich getroffen hat. Meist werden es unbekleidete Körperteile sein, wie Kopf, Hals, Hände, auch Unterschenkel und Füße (z.B. beim Gehen ohne Schuhe bzw. in Sandalen). Stiche in den Hypopharynx durch verschluckte Insekten können zur akuten Verlegung der Luftwege führen. Oft wird angegeben, daß der Stachel bei diesen dramatischen Re-

aktionen den Weg in ein Blutgefäß gefunden habe; doch gibt es bisher keinen histologischen Beweis für diese Behauptung, die wohl nur zur Deutung des schnellen Ablaufs aufgestellt wurde. Sie ist überflüssig; denn es handelt sich bei dem nativen Giftsekret um ein hochkonzentriertes (ca. 30% Trockensubstanz) Antigengemisch, das zudem noch Permeabilitätsfaktoren für das Interstitium (Hyaluronidase) und die Gefäßwand (z.B. Melittin) enthält. Die intracutane Injektion durch den Stechapparat gewährleistet eine schnelle und vollständige Resorption von allen Partien des Organismus.

Unter unseren Umweltbedingungen ist zu erwarten, daß jeder in den gemäßigten Zonen lebende Mensch mehrmals von Bienen gestochen wird. Es werden demnach alle Menschen, die zu allergischen Reaktionen neigen, in das Naturexperiment einbezogen; sie erhalten Gelegenheit, eine Bienengiftallergie zu entwickeln. Oft ist in der Anamnese eine frühere Allgemein-Reaktion auf Bienenstich zu eruieren; gelegentlich ist sie auch leer, was aber eine vorhergegangene Exposition keineswegs ausschließt. Expositionsduauer und Dosierung, wahrscheinlich auch der Applikationsweg, spielen gleichfalls eine erhebliche Rolle. Bei massiver Exposition können auch Menschen, die keine Allergiker sind, spezifisch gegen Bienengift überempfindlich werden. Bei den langjährigen Arbeiten im Laboratorium des Autors ist er selbst extrem gegen Bienengift ohne Parallergie sensibilisiert worden; bei zwei Mitarbeiterinnen trat die Überempfindlichkeit ebenfalls erst nach einigen Jahren kontinuierlichen Kontaktes auf, bei einer dritten, die allerdings eine Anamnese als Allergikerin hatte, bereits nach einigen Wochen.

Die Symptomatologie der akuten Überempfindlichkeitsreaktion weist keine für das Gift charakteristischen Besonderheiten auf. Wird der Mensch nach längerdauernder Sensibilisierung durch Kontakt, z.B. eingeatmeten Giftstaub, von einer Biene gestochen, so reagieren zunächst die Regionen der Sensibilisierung, z.B. die Nasenschleimhaut, auch die Lidwinkel und der äußere Gehörgang, mit starker Schwellung und Jucken; die Stichstelle ist meistens nicht stärker gerötet und geschwollen als beim Nichtsensibilisierten; doch kommen erhebliche Abweichungen von der Norm nach beiden Seiten vor. Die akute Reaktion beginnt wenige Kreislaufzeiten nach dem Stich: Der Betroffene kollabiert, gleichzeitig tritt heftiges Jucken am gesamten Körper auf, besonders in Hautfalten und Gelenkbeugen; ein Lidödem kann sehr ausgeprägt sein. Atembeschwerden resultieren aus der starken Schwellung der Nasenschleimhaut und einer Verengung der Bronchien. Der Höhepunkt ist binnen 15 min überschritten; der weitere Ablauf hängt davon ab, ob bzw. ein wie schwerer Schock sich aus dem anfänglichen Kollaps entwickelt hat. Erholt sich der Patient — was die Regel ist — so kann binnen 1 Std die Restitution ad integrum erreicht sein. Manchmal bleibt ein generalisiertes, juckendes, oft papulöses Exanthem Stunden oder Tage bestehen, wie es auch ohne anaphylaktischen Schock nach Bienen- oder Wespenstichen gesehen wird. Ver-

zögerte Reaktionen [z.B. SHAFFER (1961) Fall 2, bei dem 24 Std Latenz bestanden] sind seltener.

Die Therapie hat in den üblichen Maßnahmen zur Behandlung des anaphylaktischen Schocks zu bestehen; meist kommt sie zu spät, wie aus dem Ablauf der von uns unter „Laborbedingungen“ beobachteten und den von JENSEN (1962) im Detail beschriebenen Fällen hervorgeht. Für besonders gefährdete Personen wird daher ein „emergency kit“ empfohlen, den sie stets mit sich führen sollten (SHAFFER, 1961). Der Stachel ist möglichst schnell zu entfernen, ehe sich der daran hängende Giftapparat leergepumpt hat. Die weitere Behandlung entspricht derjenigen des anaphylaktischen Schocks, so die sublinguale Anwendung von Isoproterenol, Inhalation, eventuell auch Injektion von Suprarenin, Corticosteroiden, Ca^{++} und Antihistaminicis. Nach Abklingen der Reaktion sollte das Allergen aufzufindig gemacht werden und eine spezifische Desensibilisierung durch vorsichtig gesteigerte intracutane Injektion angeschlossen werden.

Informationen über die Kasuistik der Reaktionen auf Insektenstiche sind z.B. bei JENSEN (1962), SHAFFER (1961), BARNARD (1957), LOMER (1958), MOESCHLIN (1964) zu finden. Es sei betont, daß aus dem unspezifischen Bild der anaphylaktischen Reaktion nicht auf das ursächliche Gift geschlossen werden kann; es ist nicht einmal sicher, ob stets das Gift und nicht etwa Leibeshöhlenbestandteile oder dem Stachel anhaftende Exkrete der Insekten das auslösende Agens sind. Bei den von uns beobachteten akuten Reaktionen fällt diese Deutung dahin. Allergische Reaktionen sind nicht nur nach dem Stich der Biene beschrieben worden; auch verschiedene Wespenarten und die Hummel applizieren allergenes Material.

Über die chemische Natur der allergisierenden Agentien ist noch wenig bekannt; zweifellos kommen die antigenen Proteine des Bienengiftes dafür in Frage, also Phospholipase A und Hyaluronidase (s. S. 305). Man wird annehmen dürfen, daß auch die entsprechenden Bestandteile der anderen Hymenopterengifte sowie deren Phospholipase B antigen sind. Damit ist aber die Reihe der möglichen Allergene nicht vollständig. Bei den durch häufigen Kontakt mit Gesamtgift zustande gekommenen Sensibilisierungsfällen in unserem Laboratorium waren mindestens zwei Personen betroffen, die auch gegen Kontakt mit hochgereinigtem Melittin überempfindlich geworden waren. Schließlich ist an giftfremde Bestandteile des Insektenorganismus zu denken.

Seit langem ist bekannt, daß die Überempfindlichkeit nicht auf ein einziges Insekt beschränkt sein muß; FOUBERT und STRIER (1958) haben dafür die experimentelle Basis geliefert. Sie stellten Kochsalzauszüge der Körper von *Apis mellifica*, *Vespa pennsylvanica* (Yellow jacket), *Polistes fuscatus aurifer* und *Dolicho vespula arenaria* bzw. *maculata* (gelbe bzw. schwarze Hornisse) her und immunisierten damit Kaninchen. Bei Geldiffusions-

Studien stellte sich heraus, daß zwar jedes Insekt „spezifische“ Antigene enthält, aber auch gemeinsame Antigene vorkommen. Dem entsprach das Ergebnis des Anaphylaxie-Tests an Meerschweinchen; durch Aluminiumhydroxid präcipitiertes Antigen sensibilisierte die Tiere jeweils auch gegen die Körperextrakte der anderen Insekten, allerdings in unterschiedlichem Maß. Der Bienenextrakt wies die höchste Spezifität auf. Bei 85 % der gegen InsektenGift überempfindlichen Patienten war eine gegen mehrere Insekten gerichtete Hautreaktion auslösbar; also ist eine nachträgliche Identifizierung des für die Allgemeinreaktion verantwortlichen Insekts allein auf Grund des Hauttests selten möglich. Während der 10 Tage nach dem Ablauf der Allgemeinreaktion reagiert der Patient schwächer als normalerweise; Menschen, die auf Grund ihrer Familien- oder Eigenanamnese als Allergiker zu bezeichnen sind, neigen zu besonders schweren Allgemeinerscheinungen auf Insektenstiche und sprechen auch im Intracutantest noch auf starke Antigenverdünnungen an (MUELLER, 1959). In einer anderen Serie reagierten $\frac{2}{3}$ aller sensibilisierten Patienten auf Extrakte verschiedener Insekten. SHULMAN *et al.* (1964) analysierten mittels Papier- und Stärkeblockelektrophorese sowie im Schwerefeld die Extrakte von Biene, Wespen (*Polistes exclamans*) und yellow jacket (*Vespula pennsylvanica*). Sie fanden in Körperextrakten mindestens fünf mit verschiedener Geschwindigkeit sedimentierende Komponenten, im Extrakt des Giftsacks mindestens deren zwei. In Fortführung dieser Versuche zeigten sie (LANGLOIS *et al.*, 1965 a), daß das Serum sensibilisierter Menschen Antikörper enthielt. Sie konnten durch intracutane Injektion derartiger Seren die Haut gesunder Versuchspersonen für die Ganzkörperextrakte der drei genannten Insektenarten sensibilisieren. Patienten, welche nur auf den Extrakt einer einzigen Insektenart reagierten, wiesen — wie sich an Hand dieses Tests zeigen ließ — zirkulierende Antikörper nur gegen diesen Extrakt auf; bei den auf zwei oder drei Insektenarten reagierenden Menschen waren Kreuzreaktionen nachweisbar. Die verantwortlichen Antigene stammten nicht nur aus dem Gift, sondern auch aus dem vom Giftapparat befreiten Insektenleib. Im Blut von 40 % der Patienten fanden sich hämagglutinierende Antikörper (Versuche mit gegerbten und antigenbeladenen Zellen). Titer und Häufigkeit nahm bei desensibilisierender Therapie zu. Eine deutliche Beziehung zwischen Titer der hämagglutinierenden Antikörper, Titer der hautsensibilisierenden Antikörper und Schwere der allergischen Lokal- bzw. Allgemeinreaktion bestand nicht.

Gelldiffusion und Immunelektrophorese bestätigten, daß in den Giftapparaten jeder von den drei Insektenarten mindestens ein, möglicherweise zwei Antigene vorliegen, die sich nicht in das reichhaltigere Antigenmuster (4—5 Präcipitatbanden) des Gesamtkörperextrakts einordnen lassen. Ob dieses spezifische Antigen mit einer der Giftkomponenten identisch ist, läßt sich nicht sagen, weil zur Immunisierung nicht etwa gereinigtes Gift, sondern der Giftapparat benutzt wurde. Desgleichen wurde bei diesen Versuchen nicht beachtet, daß Bienengift bzw. dessen Phospholipase A bereits mit normalem Serum Niederschläge bildet, die ein Antigen-Antikörperpräcipitat vortäuschen können (vgl.

S. 306). Immerhin zeigt auch der Hämagglutinationstest, daß Seren gegen den jeweiligen Giftapparat ebenso wie Serum gegen den davon befreiten Insektenleib relativ spezifisch sind (LANGLOIS *et al.*, 1965b). Durch immunelektrophoretischen Vergleich entdeckte man ein nicht aus dem Giftapparat stammendes Antigen, welches Biene und yellow jacket gemeinsam war; ebenso hatten Bienen und Wespen ein gemeinsames Körperantigen. Wespen und yellow jackets hatten je ein aus dem Giftapparat und ein aus dem übrigen Körper stammendes Antigen gemeinsam (ARBESMAN *et al.*, 1965). Auch nach O'CONNOR u. ERICKSON (1965) sind den Giften von Biene, Polistes, gelben Hornissen und Yellow jackets einige Antigene gemeinsam; andere sind artspezifisch, wie die Immundiffusion zeigt. Manche sind nur im Gift vorhanden, andere nur im Insektenleib.

Die Auf trennung von Gesamtkörperextrakt der Biene und Giftsackextrakt wurde durch Chromatographie an DEAE-Cellulose und anschließende Prüfung der immunologischen Komposition (Gelldiffusion) und Hautreaktivität (sensibilisierter Mensch) weitergetrieben. Aus den Giftsäcken erhielt man 3 Fraktionen, von denen nur eine antigen war. Der Körperextrakt ergab 9 Proteingipfel ($E_{280 \text{ m}\mu}$), von denen Fraktion 1 und Fraktion 6 (beide uneinheitlich) Reaktionen beim Menschen hervorriefen, obwohl auch die übrigen Fraktionen zum Teil antigenes Material enthielten. Fraktion 6 war wahrscheinlich auch für die Kreuzreaktivität mit Wespenpräparaten verantwortlich. Eines der vier in Fraktion 1 (Körperextrakt) enthaltenen Antigene war identisch mit dem aus dem Giftapparat stammenden Antigen (SHULMAN *et al.*, 1966b). Bei den Versuchen mit Extracten von Giftapparaten hatten begleitende Körperantigene gestört. Bei vergleichenden Experimenten mit gereinigtem Gift zeigte sich eine einzige, bei Elektrophorese zur Kathode wandernde antigene Bienengiftfraktion. Sie fehlte in Körperextrakten. Spuren von Körperantigen fanden sich auch im Gift; es wird vermutet, daß das Gift bei der Gewinnung mit kleinen Mengen von Körperbestandteilen verunreinigt wurde (SHULMAN *et al.*, 1966a). In einer neueren Übersicht von SHULMAN (1967) wird ausführlich auf allergische Reaktionen gegenüber Insekten und die Antigenanalyse eingegangen. ARBESMAN *et al.* (1966) fassen die Resultate ihrer vergleichenden Untersuchungen mit antigenem Material von Biene, Wespē und Yellow jacket wie folgt zusammen: a) Species-spezifische Antigene finden sich sowohl in Gesamtkörper als auch Giftsackextrakten. Diese Antigene ergeben, testet man sie an der Haut empfindlicher Patienten, die höchsten Titer. b) Jede Insektenart enthält in ihrem Körper (wahrscheinlich nicht im Gift) mindestens zwei Allergene, welche die kreuzweise Reaktion mit jeweils einer der anderen Insektenarten bedingen.

Zwischen allergischen und immunologischen Prozessen nach Insektenstichen bestehen fließende Übergänge. Es scheint eine Frage der Dosierung des Antigens zu sein, ob ein Schutz oder eine Anaphylaxie aus der Antigen-Antikörper-Reaktion resultieren. LOMER *et al.* (1958) berichteten kurz, daß sie durch steigende Bienengiftdosen bei Kaninchen ein präcipitierendes Antiserum erhalten konnten, das aber — wie auf Grund der Versuche von HABERMANN und EL KAREMI (1956) zu erwarten — nicht entgiftete. Indessen sensibilisierten 0,75 bzw. 1 mg Gift, im Abstand von einer Woche intracutan gegeben, Meerschweinchen gegen eine sicher subletale Bienengiftdosis; sie reagierten darauf mit einem anaphylaktischen Schock.

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