PHYSIOLOGICAL TRIGGERS

Theodore H. Bullock, *Editor*

To the Marine Biological Laboratory
with the compliments of the
Society of General Physiologists
Abraham M. Shaner.
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March 28, 1957.

PHYSIOLOGICAL TRIGGERS

and

Discontinuous Rate Processes

Physiological Triggers

AND DISCONTINUOUS RATE PROCESSES

PAPERS BASED ON A SYMPOSIUM AT THE MARINE BIOLOGICAL LABORATORY, WOODS HOLE, MASSACHUSETTS, SEPTEMBER 1955

THEODORE H. BULLOCK, *Editor*

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Preface

EVER SINCE ITS INCEPTION the Society of General Physiologists has sponsored an annual symposium on some aspect of basic experimental physiology. In keeping with the mission of the Society and the very diverse fields represented in its membership, the symposial topics and participants have been chosen deliberately to cut across disciplinary boundaries in the hope that information from diverse fields will, when brought into juxtaposition, reveal unsuspected correlations and suggest new lines for future investigation. Traditionally, also, an effort has been made to venture into little-exploited fields, where the potentialities of rewarding generalizations, if less predictable than in more popular and homogeneous areas, are proportionately the more stimulating.

The subject of the present Symposium seemed to the Council a particularly happy choice, presenting on the one hand the challenge of pioneering in a field both fresh and important and on the other an exceptional opportunity to include contributions by colleagues versed in mammalian physiology, in connection with our joint meeting of 1955 with the American Physiological Society. We also felt fortunate in being able to persuade as well qualified and imaginative a physiologist as Dr. Bullock to enlist the talent required for the experiment. Our expectations were well rewarded by the heavy attendance and active discussion at the Symposium. Whether our essay has achieved a unified picture of the extraordinarily all-pervading and fascinating field of discontinuous rate processes, or only served to define objectives for the future, the reader must judge. In either case the objectives of the Society will have been served.

JOHN BUCK (*Secretary 1953–55*)
For the Council
Society of General Physiologists

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Introduction

THE TOPIC OF THIS SYMPOSIUM, initiated in and assigned to me by the Council of the Society of General Physiologists, has the advantage that every physiologist can identify himself with it and will be as nearly an expert as those represented in the volume. Hence all will have the gratification of being able to criticize the treatment, for despite the predominance of symposia on steady state aspects of physiology, what field or problem or level of functional biology is without its conspicuous examples of abrupt onsets or sudden changes in rate? Biologists and laymen have long recognized that life is characteristically just one thing after another: fertilization, mitosis, induction, contraction, reception, infection, reproduction. But few are the cases where physiological analysis has given insight into the mechanism of the trigger. Indeed what is a trigger? The subtitle of this volume is an admission that, even after picking our examples and formulating our own definition, analysis reveals apparent triggers are sometimes actually levers—though often coupled in a complex way, like the accelerator of a car.

The present assortment of physiologists, intentionally diverse, as explained in the Preface, have undertaken to pioneer, without guideposts, in the hope of being provocative. Unlike the usual symposium, the authors were not familiar with one another's field. None could weave together the threads they had in common. Each was urged in his own way and in the detail he saw fit to come to intimate terms with the actual mechanism of his 'trigger.' The result is bound to be a specialized treatment of a heterogenous group of fields and obviously unity is not expected. The justification and the dividends are set forth in Dr. Buck's explanation of the philosophy of the Society of General Physiologists' symposia. Any new systematization, comparisons and contrasts, principles or criteria must arise after the symposium and in the minds of those readers willing to cross both the vertical lines dividing fields and the horizontal lines between the levels of analysis.

The symposium was held at Woods Hole, Massachusetts during the Society's 1955 meeting. As reported in *Science* (vol. 122, pp. 1098–1100) it included outstanding contributions by Folke Skoog and Lawrence Blinks which rounded out the botanical side, but which, to our regret, are not printed here. By special invitation the papers of Jean Botts, J. B. Buck and K. S. Cole have been added since the Woods Hole meeting.

Once again, as in the publication of the last annual symposium on *Electrolytes in Biological Systems*, edited by A. M. Shanes, we are indebted to the American Physiological Society and especially to the Board of Publication Trustees for its good offices in seeing the symposium into print.

THEODORE H. BULLOCK
Woods Hole, Summer, 1956.

The Trigger Concept in Biology

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MANY BIOLOGISTS IN MANY CONTEXTS have used the word trigger. Many others have objected because the word was not clearly defined. We may examine the question whether there is at least heuristic value in formulating a concept of triggers in biology and, if so, what sorts of questions it raises.

At the outset we may recognize some justification for the desire to consider what might be called discontinuous rate processes, or events of relatively sudden onset. So many of our symposia quite appropriately have concentrated on the steady state processes of living systems, that inevitably some have raised the question, "What about the sudden changes in living systems?". Events are as characteristic of life as the steady state, especially improbable events. As a tentative beginning, we may ask whether there is a class of events to which the concept of triggers can be properly applied and, if so, what common denominators define them. The conclusion that such a class exists and can be defined is the starting point of this essay. Given this, the assumption is made that there is at least some value for some people in recognizing the distinguishing characters, the variables and the possible subdivisions of triggers. By spelling out the defining features of the class and several types of criteria for recognizing subclasses, it is expected that at least formal comparisons can be made between different cases. The stage of knowledge in specific instances will be more clearly recognized and new measurements may be more obvious.

DEFINITION

The dictionary defines a trigger as a piece connected with a catch or detent as a means of releasing it. We may define a trigger as an arrangement of parts in a system, or an energy state in a system, such that application of an increment of energy (commonly but not necessarily small) will precipitate a change, whose time course (commonly but not necessarily rapid) and magnitude are essentially independent of those of the energy increment. This definition admits a wide class of phenomena: the trigger of a gun (it is not important whether there is a bullet in position or not, but simply that the hammer be triggered); a lever pushing a rock off a cliff (or down a gentle slope); the release of a trap, or of a bomb rack in a plane; a light switch; an electronic circuit with two stable positions, or, indeed, one in which neither position is stable but lasts a long time relative to the transit time between these two positions, (a so-called multivibrator or flip-flop circuit); even a relaxation oscillator.

All these are examples from the inanimate world which would be embraced under this formulation. Of course it is possible, if one prefers not to be so inclusive, to rephrase the definition to exclude a number of these cases. For example, if it is deemed essential that the increment of energy be of relatively small amount compared to the energy released after the critical point, then we can exclude the relaxation oscillator, the light switch and other cases where the energy released is simply that which is put into a spring by the pull upon the lever.

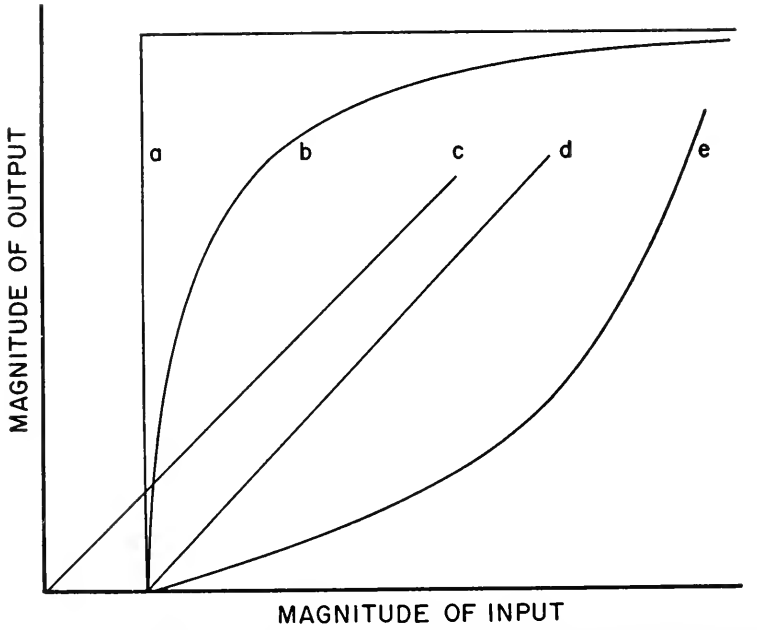

FIG. 1. Diagrammatic input-output relations; *a* is the relation defining a trigger, the others are various classes of linear and nonlinear continuously input-controlled relations, with (*b*, *d*, *e*) or without (*c*) a threshold.

In either case it is apparent that our formulation leaves out of account the nature and quantity of energy which may be released as a consequence of the change. For example, the amount of current which flows after the light switch is flipped or the amount of dynamite with which a cap or a bomb may have been charged, or even the presence of such energy, is unimportant. The essential common denominators in this class of phenomena are: *a*) a pull, that is to say an addition of energy to a system, at a rate which may vary within wide limits, *b*) a critical point, beyond which *c*) there is released a store of energy, *d*) in a manner which is independent of the time course and energy content of the pull and beyond the control of that input, i.e., is all-or-none.

If we recognize the existence of such a class of phenomena in nature, there arises the question how to distinguish triggers from non-triggers. The main contrast, it is clear, is with devices like the accelerator of a car or the key on a piano, in which the application of energy does not lead to a critical point, nor the independence of amplitude and time course of the output from those of the input. The relation between input (pulling the trigger) and output (change in position of the hammer) is a step function in the class of triggers whereas it is graded over some range in non-triggered events (fig. 1).

Put in this way, it will be apparent that there can be intermediate cases. A compound lever system with only a fairly critical zone, an amplifier with some regeneration, indeed any nonlinear system will display somewhat intermediate properties. However, in devices made or selected for a use, in general one or the other alternative will be chosen—either stable dependence of output on input, or a triggering action.

The definition does not require that the output of energy or change of state initiated upon passing the critical point be abrupt in time, only that it be abrupt as a function of increasing input. We have examples of good triggers in which the rate of release of energy is slow—as in launching a battleship—or voting its construction!

There is always an energy hump, but once this is surmounted the built-in features of the system determine the course of events, not the nature of the pull. In useful triggers, there is generally a large amplification.

TYPES OF TRIGGERS

A second question which is automatically posed if we recognize the existence of triggers, is whether all the cases embraced under the class are perfectly equivalent, or whether there are subclasses.

We may propose a subdivision on the basis of the mechanism of the critical point. *a*) Some triggers depend for their critical point upon a spatial arrangement of the parts, which defines the unstable state, as in the trigger in a gun or the rock balanced on the edge of a cliff. *b*) Others depend for their critical point upon the arrangement of parts within molecules or atoms, therefore upon empirically determined properties of these molecules or atoms. Examples would be systems poised close to the ignition point, the boiling point, the melting point or even just above the freezing point because in a useful case it requires an increment of energy to cool further or to seed a super-cooled system. *c*) A third group of cases depends for the critical point upon neither of these conditions fundamentally, but upon the relation between two or more simultaneous rate functions. This will be exemplified by a bistable circuit, an autocatalytic reaction or an explosion, since the critical point in each case depends on the rate of energy release (or forward chemical reaction or plate current) *and* the rate of rise of temperature of neighboring particles (or catalytic effect of products

or amount of feedback). These examples are chain reactions, which are an important family though not the only one in this subclass. We may regard these as three primary subclasses of triggers, and call them respectively mechanical, molecular and kinetic critical points.

On other criteria and in a less fundamental way we can classify triggers according to whether the system is self-restoring or is restorable, or is actually refractory for some time after an event, before another cycle can be initiated.

A further distinction can be made between these instances where the energy released after the critical point is passed, is stored by the pull of the trigger itself or is previously stored and held for long periods in potential form. Obviously the distinction between these cases will not always be easy, and depends upon the process to which we give the name 'pulling the trigger.' But very commonly the difference between the process of storing the energy and the process of pulling the trigger is clear (camera shutter which requires cocking) and in other cases they are obviously the same process (light switch).

Triggers differ among themselves greatly in stability, that is, in the energy required to pull the trigger; put in another form, the probability of random firing. For certain purposes and studies this distinction will be useful or enlightening. Although the difference may only be clear in extreme cases, we can recognize that to control the blasting charge in road-building operations, a plunger requiring large energy input and very unlikely to be set off by accident is used, whereas in other situations the discharge of energy may be inexpensive or harmless and we are more concerned with sensitivity, as in a thermostat which may unavoidably chatter with heavy footsteps.

Triggers differ from one another in their requirements with respect to the time course of pull, especially in their sensitivity to very slow pull. Some triggers, especially electronic ones (e.g., those controlling the raster pattern in a television tube) are AC-coupled and are quite insensitive to slow pull, or, in the terminology of the physiologist, have a significant accommodation. Others are protected from fast pulls, like a chattering thermostat to which we connect a large condenser so that only slow or maintained pulls will trip the trigger.

Various accessories may be attached to triggers; for example, to require a unidirectional pull, or to arm the trigger only under certain conditions, or to protect against accidents, or to channel the form of energy to which the trigger is sensitive.

Triggers made or selected for a use commonly are of a type involving a sequence of different events. Thus a gun, when loaded, represents at least two step functions in series—the release of the hammer and the explosion of a charge. Some cameras and many electronic devices trigger several events seriatim. We shall see that this is particularly characteristic of biological triggered systems.

These considerations mean that if we recognize the class of trigger phenomena and then ask the question whether they are represented in physiological systems, we can immediately recognize a series of questions of intrinsic interest, such as the following. Can we identify the components of a trigger whose existence we know of only from the step-function of input and output (fig. 1)? Can we identify the necessary and sufficient form of the adequate input (stimulus)? Which type of critical point determines the firing of this trigger, mechanical, molecular or kinetic? What are the properties of the trigger system with respect to accommodation, adaptation, refractoriness, sensitivity, and the source of energy released? The most useful question in many cases will be "Is this in fact a trigger?". As will be seen in the papers in this symposium, it is quite possible that a striking or classical case of an abrupt change may turn out, on experiment, to be graded at least in part.

Biological phenomena which appear to satisfy the conditions set forth are not difficult to find. We may think of cases like the activation of the egg by spermatozoa or other means; the initiation of flowering in higher plants; the initiation of infection, muscle contraction, sensory discharge, metamorphosis; the onset and termination of diapause in insects; the initiation of hormone secretion controlling phasic events as well as the initiation of the cellular events in the target organ by the hormone; mitosis; nerve impulses; discontinuous luminescence. These and many other examples come to mind.

Consideration of the present state of knowledge of these cases is likely to lead to some interesting general conclusions. For example, it seems likely that none of these biological triggers involves the type of critical point which we called mechanical (Boettiger has elsewhere described certain insects which use a mechanical snap mechanism in their flight motor) and it seems very doubtful whether they involve the class of molecular critical points exemplified by boiling point, freezing point, and the like. This last conclusion is very likely to need correction in the light of newer knowledge of protein behavior in cell membranes and similar critical loci, but at present it seems likely that at least most physiological triggers will depend for their critical points on the kinetic principle, that is to say, on a critical ratio between two simultaneous rates.

Another generalization is that inanimate triggers are generally degrading, resulting in a more probable distribution of matter and energy, whereas at least many biological triggers significantly increase complexity or initiate a new state which requires more information to describe it than the last state. There is always an energy hump which must be surmounted, but in the inanimate triggers, after passing this hump the new state is typically, like that of the hammer and spring in a gun, at a lower energy level than the cocked state. The same may be true of many biological triggers, but others, like the activation of the

egg by the spermatozoan, may be said to initiate a train of events which, while releasing previously stored energy, create greater complexity of distribution of energy and structure.

This feature may be regarded as the consequence of another generalization about biological triggers: they commonly occur in chains, each the adequate stimulus for the next step, which is likely to be a qualitatively different event and may be either a true trigger or a graded process.

It will be apparent from reviewing the state of knowledge of particular physiological triggers that in many instances we are still seeking the components of the trigger; that is, identifying them anatomically (hypothalamic control of pituitary, Sawyer), or physiologically (cell mechanism altered by hormones, Szego; activating substance in paramecium, Metz; steps in synaptic transmission, Grundfest; linkage between action potential and contraction in muscle, Botts). In other cases the problem is at the stage of searching for the identification of the adequate input (infectious vs. noninfectious virus particles, Rappaport *et al.*; control of luminescence, McElroy *et al.*; intermittent CO_2 release in insects, Buck; chemical, electrical and light triggers in algal cells, Blinks). In virtually every case we still have to solve the problem of the nature of the critical point, except in the analytical picture of the axonal membrane as described by Cole where a kinetic relationship which may well be the prototype of biological triggers in formal terms, is developed. In many cases the inquiries of current interest involve secondary properties such as refractoriness (break of diapause, Schneiderman; block to polyspermy, Metz), sensitivity (kinetins, Skoog), the source of stored energy (cochlear microphonic, Davis), and the time course of its release (high frequency insect muscle, Boettiger).

The trigger concept has one further feature which deserves attention. Trigger mechanisms are compared and analyzed on the basis of functional relations between their components rather than on the structural nature of those components. This means that we can consider cases at different levels of structural complexity from the molecular to the tissue and organ levels. We can hope to establish at least formal connections and contrasts between the types of dependencies, of various examples at different levels, upon the functions of their constituent elements.

SOME COMPARISONS AND CONTRASTS

This is the background and point of departure for this collection of papers. They are a selected sample of cases where some kind of progress has recently been made in elucidation of phenomena which at least superficially appear to be triggered. Those who decry analogical thinking will find here a horrible example, for there is no thread or common denominator except the possibility of functional comparison through the concepts spelled out above. The several instances represented illustrate quite different aspects of the problem of unravelling discontinuous rate process and, put together, their special value

depends on the suggestions which future workers find in chapters outside their own specialties.

Thus, possibly the greatest point of interest growing out of this comparison of events of sudden onset is that so many of them turn out not to be inherently all-or-none triggers, but graded or continuously controlled processes. Even fertilization of the egg and initiation of contraction in insect fibrillar muscle are placed in this category by the present authors. Other cases are the hormonal alterations in permeability of certain target cells, the nervous control of some endocrine glands, and the transducer phase of sensory and postjunctional neuronal response, prior to initiation of impulses.

These cases are not, in their status as non-triggered events, equivalent. For example, the egg and the muscle are denied trigger status for different reasons and—illustrating one of the thought-provoking difficulties of the concept—both debatable. Experimentally, an egg can be activated partially; but a typical trigger may, by experimental manipulation, be altered into a graded device and an inherently graded device may appear to be all-or-none because the minimum normal stimulus is already maximal. One or both of these may be as true of fertilization of the egg as of the initiation of infection by a virus particle or bacterium (not the time course of the infectious process). This triggering by quantal stimuli is different from processes which are continuous functions of their normal input, like the work output of mechanically stimulated insect fibrillar muscle and the microphonic response of the cochlea.

The vibrating insect fibrillar muscle delivers work as a continuous function of the phase difference between its tension and its length. But under normal conditions there is presumably a discontinuous difference between the actively contracting state of a given fibril and the relaxed state. The change from one to the other may be said to be triggered; but the adequate stimulus for this change is at the same time one of the conditions determining the magnitude of the tension and hence does not lose control, experimentally. Not only is the high frequency contraction *in vivo* a modified step function but also the permissive state of the muscle, under control of the few all-or-none nerve impulses per second, is apparently a step function but probably with sloping steps.

Whether other cases, e.g., junctional transmission at synapses, are normally based on quantal input or on graded input is a question which has not been adequately recognized and we possess little information; but it seems probable that some junctions receive graded activity in presynaptic terminals and others all-or-none prespikes. Similarly, in endocrine-controlled events the present knowledge suggests a picture of some which are all-or-none, as in metamorphosis and diapause, while others are normally at least somewhat graded, as in uterine changes. "At least somewhat" means that even though the normal event is as ungraded as the proverbial "slight case of pregnancy," constituent processes may be continuously under the control of their hormonal inputs.

Each example could be similarly examined and at least a number of the

higher level events (metamorphosis, diapause, ovulation, instinctive behavior) would turn out to be essentially triggered over-all phenomena but to consist of a complex of substituent processes, some continuously graded, others triggers, or even chains of triggers within triggers. We have the reciprocal case, too, where a graded event (e.g. the synaptic potential) is believed to consist of a large population of triggered molecular events in the membrane.

The distinction here emphasized is the same as that between digital (triggered) and analog (graded) signals. We shall see in the several papers how complex is the interrelationship between these, each one entering into the makeup of the other and each succeeding the other temporally in different cases. We see this especially clearly in the nervous system and sense organs where, as our authors point out, graded transducers convert stimuli of external origin into triggered impulses, which in turn may contribute at some more central junction to a presynaptic barrage which is again transduced, first to analog and again to digital events.

But for all the wealth of information, it may be well to end by pointing out, as an example of our ignorance of physiological events, the gap between this relative wealth at the neuronal level and the relative wealth at the levels of brain stimulation and recording and of intact animal behavior. The first level is far removed from the second and third. In between we have still to identify, in structural substrata and functional relation, even in formal black boxes and labeled arrows, the parameters of the triggers and levers that integrate, initiate, pattern, associate, memorize and feel.

Process of Infection by Tobacco Mosaic Virus[1]

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IN THE PAST FEW YEARS, information from various fields of virus research has converged to provide us with a rather general and exciting picture of viruses and their mode of replication. This is particularly true for the bacteriophages and some animal viruses. In the realm of plant viruses, though, our facts are somewhat out of balance. We know considerably more of their extracellular properties than we do of their intracellular behavior. The high yields and exceptional degree of purity with which a virus such as tobacco mosaic (TMV) can be prepared has attracted many investigators to this unique rod. As a result, the *in vitro* characteristics of the virus as a macromolecular nucleoprotein are very well-defined. Its size, shape, molecular weight, chemical composition, and numerous other properties associated with its extracellular existence, have been determined with great precision. What is lacking to balance this type of information, however, is a more intimate knowledge of the host-virus relationship. Within its host, TMV exerts a profound influence on the synthetic pathways of the cell. In terms of the vast number of foreign particles each cell is triggered into producing, TMV stands alone. Somewhere between 100,000 and half a million virus rods can be recovered from a single infected plant cell, compared to the hundreds or possible thousands of virus particles produced by animal or bacterial cells.

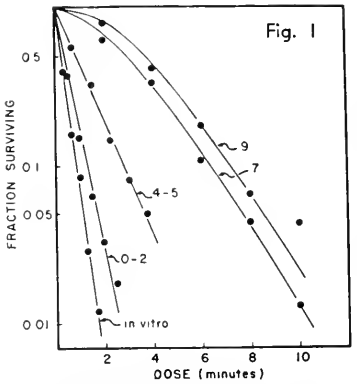
In seeking more information on the intracellular behavior of TMV, our research efforts have been limited to a mild and a severe strain of TMV and to one host—*Nicotiana glutinosa*. *N. glutinosa* is known as a local lesion host. When virus is rubbed on its leaves, small local lesions appear, usually within 2 days. These necrotic areas are composed of cells that died as a result of having supported virus replication. Because the lesions on *N. glutinosa* are discrete and obvious, they serve a three-fold purpose. First of all, the number of lesions obtained is related to the concentration of virus applied, and so forms the basis of the well known plant virus bio-assay. Second, since the area of virus activity is so clearly defined, the rate of spread of the infection from cell to cell can be observed and measured; and third, the entire spectrum of virus activity, from

[1] These studies were supported, in part, by Contract AT(11-1)-34, Project 8 of the Atomic Energy Commission, and USPHS Grant G-4124 from the Division of Research Grants, National Institutes of Health.

beginning infection to cell death, may be observed under the microscope within a perimeter of a few cells at the edge of the necrosis.

The primary act of infection begins with the entry of a single virus particle into a susceptible cell. This initial act of host-virus combination occurs with great rapidity, and corresponds to an all-or-none physiological response. As far as can be ascertained, the infection is either instantaneously successful or it does not occur at all. For example, when the leaf is thoroughly washed immediately after rubbing with virus, no reduction in lesion count is usually observed, compared to an unwashed leaf. However, those infectious virus particles which failed to make the appropriate cellular connections can be recovered from the wash water, and made to infect another leaf.

The events which immediately follow the successful union of virus and host cells have been studied, using a technique developed by Luria and Laterjet for



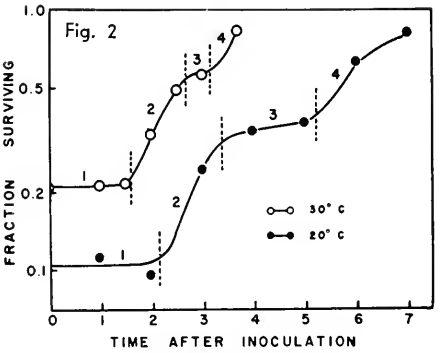


FIG. 1. *In vitro* and *in vivo* ultraviolet survival curves for TMV. Numbers on curves represent hours after inoculation (*Virology* 2: 69, 1956).

FIG. 2. Ultraviolet survival of infectious centers as a function of time in hours after inoculation. Numbers on curves indicate phases of sensitivity (*Virology* 2: 69, 1956).

demonstrating bacteriophage multiplication within living *Escherichia coli* cells. The procedure consists of irradiating the infected leaf cells with an appropriate dose of ultraviolet light; that is, one which kills the infectious agents but causes no undue harm to the host.

The ultraviolet (UV) survival curves for TMV-infected leaf cells suggest that a regular sequence of events preparatory to multiplication takes place within the cell. The data from a number of experiments carried out at increasing times after inoculation are shown in figure 1. They describe the survival of TMV as a function of UV dose, both before and after cellular attachment. The *in vitro* inactivation is steep and exponential; characteristic of a single target or 'one-hit' type of curve. Irradiating the leaves immediately after the union of virus and cell cytoplasm shows only a slight difference in the survival of what are now the infectious centers. This similarity in the inactivation of infectious centers

and extracellular virus is maintained for about 2 hours, after which the host-virus complex becomes more and more resistant to the effects of UV.

The change in slope 2 hours after infection may reflect the time necessary for deeper cellular penetration of the virus particle, where it then becomes partly shielded from the full dose of UV by other UV-absorbing materials, or, prior to multiplication, the intracellular virus may undergo some change in form which is more resistant to inactivation by ultraviolet light. As the time between inoculation and irradiation is further increased, the shape of the inactivation curve becomes 'multi-target' in nature. The first indication of virus multiplication is apparent after 7 hours of host-virus association. The 7-hour curve is a theoretical survival curve for three particles within a cell, while the points which fit the line are experimental. Nine hours after infection the curves suggest that between five and eight particles, each independently capable of maintaining the infection, are now present.

On closer examination, it was found that the infectious unit passed through three clearly defined phases of UV sensitivity before multiplication occurred. *N. glutinosa* leaves were irradiated with a constant dose of UV (90 sec.) at different times after virus inoculation and the survival of the infectious centers ascertained. The experiments were carried out at two different temperatures, the results of which are recorded in figure 2.

Four distinct step-like phases in the UV sensitivity of infectious centers are represented. The first three phases are the following: an initial phase of constant UV sensitivity; a second phase, showing a gradual increase in resistance to UV damage, and a third phase, marked by a plateau of little changing sensitivity. Up to and including this third phase, the slopes of the survival curves as seen in figure 1 are exponential, so that whatever these changes in UV sensitivity reflect, they are still expressions of only a single infectious unit. The fourth phase displays a rapid rise in UV resistance which is correlated with the appearance of the 'multi-target' curve. Such resistance depends primarily on the number of particles present in each infectious center. The timing of the phases is highly temperature dependent. It takes almost twice the time to reach the fourth or multiplicative phase with plants kept at 20°C than it does with the same material at 30°C. The qualitative features of the curves nonetheless remain unchanged.

The meaning of the first three preparatory phases is obscure, but some additional information, obtained from other TMV strains in radiation studies, suggest that the changes in UV resistance are related directly to the virus state, rather than associated with any change in the sensitivity of the host cell. Consequently, the final slopes of the 'multi-target' curves (see figure 1) assume an added significance. Their shallowness as compared to the *in vitro* and first 2-hour *in vivo* curves suggests that the intracellular reproducing particles may be different in some way from the *in vitro* rod. This notion concerning a dif-

ference in properties between intracellular and extracellular virus will be brought up again with reference to some other observations.

In these experiments, a susceptible cell was presented with but a single infectious unit. Nevertheless, when susceptible cells were simultaneously presented with more than one particle, the survival curve for infected centers still retained its exponential features. Even with as many as seven infectious particles per susceptible site, no multiplicity of infection was observed. Consequently, one is led to the conclusion that the initial host-virus complex is a highly specific association; for either the host cell contains only a single attachment site for the virus, or an exclusion mechanism operates to keep other particles

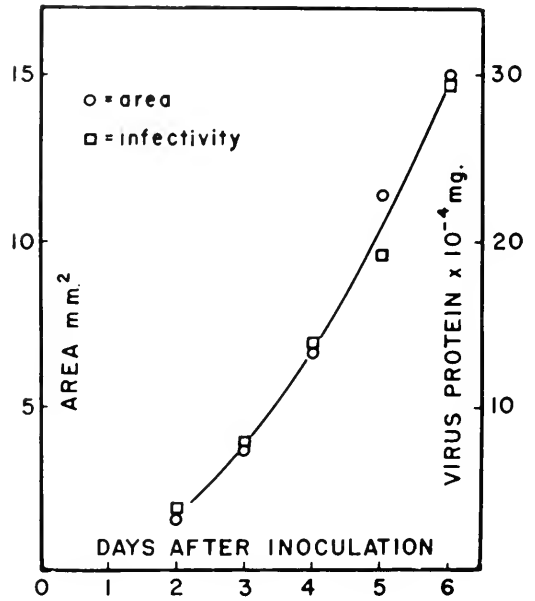


FIG. 3. Increase in lesion area and infectivity with time after inoculation.

out. Thus, one and only one virus particle initiates the infection, and then undergoes three phases of development before it begins to multiply.

There is a limitation imposed on the further exploitation of the Luria-Laterjet technique to the investigation of the plant virus system because of the spread of the virus to adjacent cells. After further multiplication of the virus it would be difficult to tell whether one were inactivating many particles in a single cell, or destroying virus particles now contained in numerous cells. However, additional information concerning TMV reproduction has been obtained from studying the kinetics of lesion spread.

By the time a lesion is apparent to the naked eye, hundreds of leaf cells have been invaded, have produced their complement of virus particles, and have died. The necrotic area, in the form of an ellipse, grows with time in an orderly

fashion, and correlated with it is a proportionate increase in the amount of virus which can be extracted from the dead cells. A graph showing the increase in lesion area and associated infectivity is shown in figure 3. Beginning 2 days after inoculation, and continuing for 5 days, both the lesion area and the infectivity rose synchronously. Each cell produced on the average of 100,000–200,000 virus particles. The axial growth of the lesion during this interval was a linear function of time. The data for both semi-axes of the elliptical lesion are graphically represented in figure 4. Given this rate of axial growth, and an average cell size, it was calculated that a cell died and was added onto the periphery of the lesion approximately every 3 hours.

To the unaided eye, the lesion appears sharply demarcated, but microscopic observations reveal degenerative changes in approximately 3–4 cells beyond the necrosis. Assuming that these changes are associated with virus infection, one

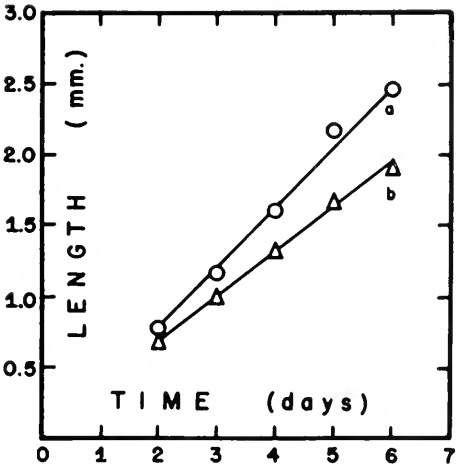


FIG. 4. Increase in axial length of lesion. Major semi-axis, *A*; minor semi-axis, *B*.

may say that between 12 and 16 hours elapse from the moment of infection to cell death. This 3–4 cell perimeter of degenerate cells has a further and more important bearing on our concept of the intracellular virus. Although large amounts of such tissue have been carefully dissected away from the necrotic area and tested for virus activity, little or no infectivity could be demonstrated. The suggestion from the radiation studies, that the intracellular reproducing unit may be different from the *in vitro* virus, finds its counterpart here in these degenerate cells. The disease is present, yet few or no particles capable of initiating a new infection can be recovered. It is as if the invasion of tissue and the spread of the necrosis to cells other than the inoculated cell was accomplished by some more elementary reproducing unit, and that later, perhaps just before cell death, the rods which typify extracellular TMV make their appearance. Conceivably, then, the characteristic *in vitro* rod may be but the end product of

TMV replication, its prime function being the transmission of the disease to a new host.

In considering a model for the continuous virus spread in *N. glutinosa* we are faced with the problem of the cell wall. When intact, it presents an impenetrable barrier both to extracellular as well as intracellular virus. In fact, the only method for extracting TMV involves destroying this cell wall. Nothing comparable to lysis or the gradual exodus of virus particles through a cell membrane has been observed in this plant system. In order for the virus to spread, then, from cell to cell, it most probably is obliged to use the only pathway available, and that is via the plasmodesmata. These fine protoplasmic strands that join each cell with its neighbor can serve as adequate bridges for the virus to cross. Thus a simple model to describe the continuous spread of necrosis is one based on the chance migration of infectious units to adjacent cells. The rate of spread would depend upon the quantity of infectious particles per cell, the velocity of cyclosis, and the number of available cell exits in the form of plasmodesmata. Admittedly, the model may be too simple, for it does not consider such possibilities as active transport of particles or other host responses.

Once the virus is extracted from its cellular environment, it is characterized by a very low infectivity. This low infectivity has been ascribed at least in part to the inefficiency of inoculation methods. Data from certain of our serological studies suggest that of equal importance is the fact that only a small fraction of the rods actually possess the necessary information for starting a new infection. We found that by mixing a series of low virus concentrations with antiserum, different sized aggregates formed. The infectivities associated with these clumped rods nevertheless remained alike. Part of the data bearing on this point appear in table 1.

Although the concentrations of serum and virus employed were varied, their ratios were maintained constant. After a suitable incubation period the mixtures were centrifuged moderately, at a speed which did not sediment single virus particles from normal serum controls. The upper portions from the centrifuged samples were assayed for virus activity along with the control uncentrifuged mixtures. The level of survivors before centrifugation showed little change, despite the fact that the average aggregate size increased. This is seen most clearly in the last column of table 1, where the percent of virus infectivity remaining unsedimented decreased markedly with increasing concentrations. Our interpretation of the data is simply that the number of infectious particles present initially is minute compared to the total number of rods in the population. Then, despite the increment in mean aggregate size, the probability of trapping more than one viable particle per clump, under these conditions, is very small.

The low infectivity of the virus inoculum is puzzling. We do not know whether most of the infectivity is destroyed while extracting the virus, or whether out of the hundreds of thousands of rods the cell makes, it impresses on

only a few the structure necessary to initiate a new infection. Should the latter case be correct, it would leave us in some doubt concerning the true nature of the infectious unit. Once an infection has been established, however, we are concerned only with particles which are independently capable of maintaining the infection. Any non-viable particles produced in conjunction with the viable ones would not be measured.

These investigations continue to give us a better perspective and understanding of certain aspects of TMV replication. To date, the data are provocative and lead to a good deal of speculation, which of course is useful in planning future experiments. In some cases, however, our data do not mesh as well as might be desired, particularly the time intervals for virus spread obtained by lesion growth kinetics as compared with the rate of multiplication derived from irradiation data. This may be only an expression of different kinds of observations. The irradiation experiments measure what we consider to be the events occur-

TABLE 1. VIRUS INACTIVATION AND AGGREGATE FORMATION AT DIFFERENT CONCENTRATIONS OF THE SAME VIRUS-ANTISERUM RATIO*

1 ANTISERUM CONC. $\times 10^{-5}$ ML/ML	2 VIRUS CONC., 10^{-4} MG/ML	3 % SURVIVAL	4 % SURVIVAL AFTER CENTRIFUGING†	5 % SURVIVORS UNCENTRIFUGED (4/3)
5.57	2.2	19.8	9.0	45
16.7	6.7	23.2	2.1	9.1
50.0	20.0	16.5	0.24	1.5

* *J. Immunol.* 74: 112, 1955.

† Top 5 ml.

ring within the first cell infected, whereas the lesion measurements are concerned primarily with virus spread through thousands of cells.

The primary or initial infection may be uniquely different from the subsequent cellular infection, for the following reasons. First, an injured cell, which is required to start the infection, may be in quite a different physiological state from an uninjured cell into which the virus spreads after multiplication. Second, if there is a time factor necessary for the penetration of the virus particle from outside to inside the cell, this would be of significance only for the first cell, since further spread of infection from cell to cell probably takes place by infectious units which never leave the cytoplasmic environment. Third, the spread of virus from the initially infected cell is continuous; consequently, adjacent cells become infected before the original cell has produced its total complement of virus particles.

SUMMARY

Taken as a whole, the data reveal the following about TMV infection in *N. glutinosa*: One and only one virus particle initiates the infection in a susceptible cell. The infectious unit then passes through three distinct phases of ultraviolet

(UV) sensitivity prior to entering a fourth, or reproductive, phase. Depending on the temperature, multiplication begins in about 6 hours.

Evidence from the UV and lesion data suggest that the replicating unit may be different from the *in vitro* rod in two properties—its UV sensitivity and its inability to initiate a new infection.

As multiplication continues, infectious units begin spreading to adjacent cells in a continuous fashion. The rate is governed by chance alone.This continuous spread of infectious units introduces virus into a new cell approximately every 3 hours. On the order of 12–16 hours elapse between infection and the subsequent death of the cell.

Shortly before the cell dies it produces approximately 200,000 unique, rod-shaped nucleoprotein particles. Of these, only a small fraction appear to possess the ability to initiate a new cycle of virus multiplication.

Mechanisms in Fertilization[1,2]

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FERTILIZATION LEADING to biparental inheritance is a well nigh universal phenomenon among animals and plants, both unicellular and multicellular. Wherever it is studied it is seen to consist of a series of morphological, cytological and physiological events, specific for the species, which under favorable conditions proceed in a very precise and orderly fashion. The orderly nature of these events suggests that they are interrelated and that they all proceed from a few or even a single 'trigger-like' reaction between the interacting cells. This presumed trigger reaction, frequently called the 'activation initiating reaction,' and the physiological events which proceed from it, have been the subject of intensive investigation over a period of many years. But in spite of this effort, relatively little positive information concerning the activation initiating reaction of fertilization is available. In fact some recent work indicates that the activation initiating mechanism may be more complicated than previously believed and that the concept of a simple trigger reaction may need revision in so far as it applies to metazoan fertilization.

This review will be confined to certain aspects of the recent work, namely fertilization in the ciliate, Paramecium; possible trigger action on the spermatozoan as a prerequisite for fertilization; and some features of the activation of the egg. A broader treatment of the subject of fertilization may be found in several recent reviews (15, 62, 85, 86, 97, 99, 115, 116, 119).

FERTILIZATION IN PARAMECIUM

The physiology of fertilization has been studied more thoroughly in Paramecium than in any other protozoan (see ref. 62 for detailed review). Indeed, some aspects of fertilization, particularly the activation initiating mechanism, are more thoroughly understood in this form than in any metazoan.

Mating or conjugation in Paramecium occurs only under certain physiological conditions and these conditions are specific for the species or even for particular varieties within a species. Furthermore, mating occurs only between definite sexes or mating types, and is characterized by a very high order of

[1] The writer's studies have been aided by grants from the National Institutes of Health, the American Cancer Society and the National Science Foundation.

[2] Contribution No. 72 from the Oceanographic Institute, Florida State University.

specificity (see refs. 10, 62, 107, 130 for detailed accounts of this breeding specificity). The process involves several types of union between mates and a variety of physiological and cytological changes in them.

The initial step in conjugation is an adhesion of potential mates upon random contact. Under appropriate conditions this initial adhesion assumes mass proportions, involves tens or even hundreds of paramecia and results in the agglutinative mating reaction. This initial adhesion or mating reaction is not mediated by diffusible, water-soluble sex substances present in the medium (45, 60, 61, 105, 106) but appears to result from interaction of substances that are attached to or built into the surfaces of the cilia. These substances, the 'mating type substances' (60) are presumably specific, complementary substances which interact in antigen-antibody-like fashion (61, 62). Subsequent to the mating reaction, conjugants unite more intimately (fig. 1), lose their mating reactivity and undergo a very precise series of nuclear changes. These include disintegration of the macronucleus and meiosis of the micronucleus or

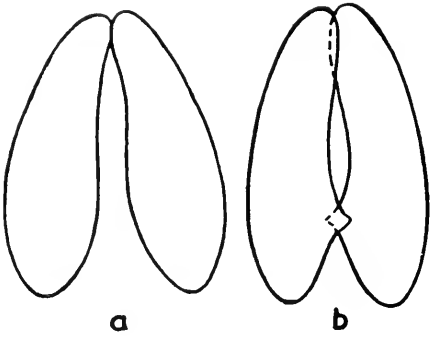

FIG. 1. Types of union in conjugation. *a*, Holdfast union. This is the secondary union that follows the initial agglutinative mating reaction. *b*, Holdfast and paroral cone union of more advanced conjugants. The gamete nuclei are exchanged across the bridge formed by the tertiary or paroral union.

micronuclei, followed by the formation of gamete nuclei. After reciprocal cross fertilization the mates separate and proceed to reconstitute the original nuclear apparatus (see refs. 107, 130 for detailed accounts of these nuclear changes).

Clearly this elaborate and precise series of events is induced in some way during sexual association, for it ordinarily fails to occur in the absence of conjugation. Furthermore, since culture fluids and filtrates are not involved in conjugation, the inducing or triggering mechanism would seem to depend upon contact of potential mates. The essential feature of such contact would appear to be an interaction of substances. This view received striking support from the discovery that dead paramecia can induce conjugation changes in living animals. Paramecia that have been killed and fixed by appropriate treatment with drastic agents such as formalin or picric acid (58 60) retain their mating reactivity. Such dead paramecia adhere to and under appropriate conditions agglutinate massively with living animals. This striking reaction is highly

specific. It occurs only between living and dead animals of complementary mating type, never between animals of the same type. Following adhesion to the dead animals and with the time schedule characteristic of normal conjugation, the living animals lose their mating reactivity, separate from the dead animals and undergo the morphological and cytological changes characteristic of normal conjugation (fig. 2). These changes include macronuclear breakdown

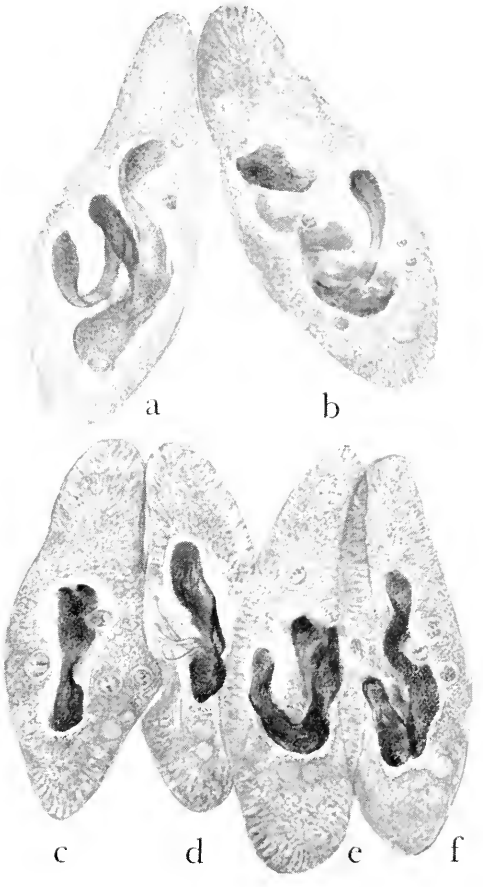

FIG. 2. Drawings of *Paramecium aurelia* activated by contact with formalin-killed animals of opposite mating type. The living animals were isolated as 'pseudo-selfing pairs' 248 minutes after mixing living and dead animals.

a, Micronuclei are in metaphase of second meiotic division; macronucleus is in 'skein' stage of breakdown. *b*, Second meiotic division completed; macronucleus in skein stage. *c*, Micronuclei are in metaphase of second meiotic division. Note paroral cone. *d*, Micronuclei are in telophase of second meiotic division. *e* and *f*, These paramecia are in same condition as in *a*.

and meiosis (59, 60). In brief, specific contact with dead animals of complementary type induces the primary physiological and cytological events of conjugation in living paramecia. Thus the dead paramecia activate living paramecia in the same sense that the spermatozoan activates the egg. This action of dead paramecia upon living animals of complementary mating type has been reported in *Paramecium aurelia* (60), *P. calkinsi* (61) and *P. caudatum* (39).

The dead paramecia described above may be regarded as a collection of substances adsorbed to or built into the surface of an inert carrier, the body of the dead animal. Evidently this collection of substances includes the specific mating type substances. Furthermore, it must include the substance or substances that interact to activate conjugating paramecia. The problem of central interest, then, is to identify and characterize these substances and describe the mechanism of their interaction and physiological action.

Two main lines of evidence indicate that the activating substances are the mating type substances, the substances which are located on the surfaces of the cilia and which, by definition, interact in the initial adhesive reaction, the mating reaction.

The first line of evidence follows from the nature of the reactive dead paramecia. These form only the mating reaction union with living mates. They fail to associate with the living animals in more intimate fashion or form the secondary or tertiary types of union described in figure 1. In the absence of these other types of union it may be assumed that any special substances responsible for such unions fail to interact. Clearly, then, interaction of the mating type substances must initiate activation, or some completely unknown and unsuspected substances must interact to initiate the series of activation changes. The first of these possibilities seems the more likely in view of the fact that neither the mating reactivity nor activating action of the dead paramecia is destroyed by such harsh and diverse agents as formalin and picric acid.

A second line of evidence supporting the view that mating type substance interaction initiates activation in Paramecium resulted from an analysis of a mutant stock of *Paramecium aurelia* (66, 67). These mutant, 'can't mate,' (CM) animals give the initial agglutinative mating reaction with both CM and normal animals of complementary mating type. However, they never undergo the subsequent changes of conjugation. They cannot be activated by sexual association with complementary CM or normal animals. Apparently some block, the CM block, prevents activation from proceeding beyond the intial stages in CM animals (fig. 3). Nevertheless, these mutant, blocked animals, whether alive or dead, can specifically activate normal, non-mutant animals of complementary type. Since the CM animals can activate normal animals, they clearly possess the activation initiating mechanism at least so far as their ability to activate normal mates is concerned.

Finally, since the CM animals possess the activation initiating mechanism and since they can unite with mates only in the mating reaction, initiation of activation must be related to the mating reaction. It follows from this that interaction of mating type substances initiates activation in conjugating paramecia.

The mutant CM animals are of further interest. Like non-mutant *P. aurelia*,

they regularly undergo natural autogamy. Natural autogamy includes loss of mating activity, formation of paroral cones (fig. 1), macronuclear breakdown and meiosis. It therefore includes the major activation changes of conjugation. Accordingly, it is reasonable to suppose that the same chain of reactions operates in autogamy and sexually induced activation. However, autogamy occurs spontaneously in single animals. It involves no sexual association with a mate. Since the CM animals can not be activated by sexual means but can nevertheless undergo natural autogamy, it follows that sexually induced activation and natural autogamy are initiated through separate routes or receptors. Furthermore, natural autogamy must be initiated at a point or site 'internal' to the 'CM block.' These relationships are presented schematically in figure 3.

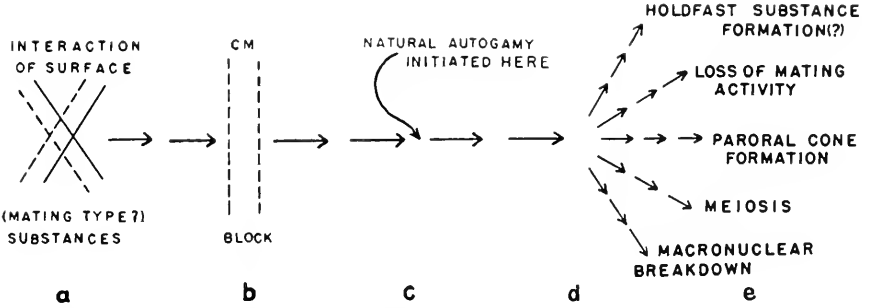

FIG. 3. Scheme for activation in *Paramecium*. *a*, Complementary surface substances, the mating type substances, interact to initiate activation. *b*, The CM block assumed to lie 'internal' to the activation initiating reaction. *c*, Natural autogamy initiated internal to the CM block. *d*, Breakup of main reaction chain into side reactions. These are assumed to arise independently from the main chain. (See ref. 62 for detailed discussion.)

These findings have interesting implications for parthenogenesis (62, 67). From the physiological viewpoint, natural autogamy in Paramecium may be considered analogous to natural parthenogenesis in Metazoa. The study of the CM animals has shown that sexually induced activation and autogamy are initiated through different routes in Paramecium. Similarly, it is possible that sexually induced and natural parthenogenetic activation of the egg may also be initiated through different routes in those forms with eggs that develop with or without fertilization by a spermatozoan. The comparison of conditions in Paramecium and Metazoa may even be extended to include artificial parthenogenesis. Certain agents (paramecins) are known to activate some stocks of paramecia (see ref. 62 for review and references). Accordingly, these agents may be regarded as artificial parthenogenetic agents for paramecia. The mode of action of these agents has not been investigated extensively, but Jacobson (43) found that the action of the agents is delayed as compared with normally conjugating controls. This delay is interpreted as the time required for the

agent to penetrate to a subsurface site of action. This site must, then, be different from and presumably 'internal' to the surface site of activation in conjugation. Similarly artificial parthenogenetic agents and the spermatozoa may initiate activation of the metazoan egg through different routes or receptors. In fact different parthenogenetic agents may activate the egg by action at different sites in a chain of reactions. Indeed each of several parthenogenetic agents may be specific for a particular site in such a chain (62, 67, 97).

According to the account presented above, the mating type substances play a primary role in the fertilization of Paramecium. These substances are proteins or closely associated with proteins (62, 65, 68). Although they are not antigenic, they appear to be closely associated with antigenic material (68). They are located on the cilia and possibly other parts of these ciliates. They probably interact in antigen-antibody-like fashion, they are responsible for the primary specificity of conjugation and for the initial adhesion or mating reaction. Finally, their interaction initiates or 'triggers' the subsequent events of conjugation. These relations are summarized in figure 3.

TRIGGER ACTION ON THE SPERMATOZOAN

Until recently the normal, motile spermatozoan was not generally considered to require any special preparation in anticipation of its union with and activation of the egg. Lillie (48), however, did suggest that the spermatozoan itself must be 'fertilized' before it can activate the egg. Evidence in support of such a view has come from recent studies of J. C. Dan, and these have been confirmed in part by Colwin and Colwin and by Metz and Morrill.

Examination of spermatozoa under high magnification, preferably at the electron microscope level, reveals that the morphology of the sperm acrosome region may vary. The variation ranges from acrosomes of compact to others of filamentous form. These extremes are the usual ones but intermediate conditions are sometimes found. Dan (22) considers the 'compact' condition of the acrosome the normal or original and the filamentous form a product of the normal or compact form. Conversion of the compact to the filamentous form constitutes the 'acrosome reaction.'

In the sea urchin (22) the acrosome reaction takes the form of a breakdown of the membrane surrounding the acrosome. Calcium ion is required for this reaction in the sea urchin (24). In the starfish (20, 23, 71) the reaction involves the appearance of a very long (*ca.* 25 μ), fine filament from the acrosome region (fig. 4). Dan suggests that this filament is discharged from the acrosome region. Very long filaments have also been observed in mollusc (25, 94), echinoid (94), holothurian (19) and enteropneust (18) sperm. In the annelid Nereis (71) the prominent cone-shaped acrosome may be replaced by a fine filament, possibly as a result of lysis or retraction of superficial acrosome material (fig. 5). Sabellaria sperms produce filaments of varying length (16).

The acrosome reaction has been reported to occur under a variety of conditions. These include the following: *a*) contact with glass, *b*) treatment with alkaline sea water, *c*) treatment with egg water, *d*) treatment with egg water plus an adjuvant, *e*) exposure to unfertilized eggs. Examination of the available data, (16, 18–20, 22–25, 71) shows that the acrosome reaction is a rather widespread phenomenon (24 species in 4 phyla). In 23 of the 24 species examined,

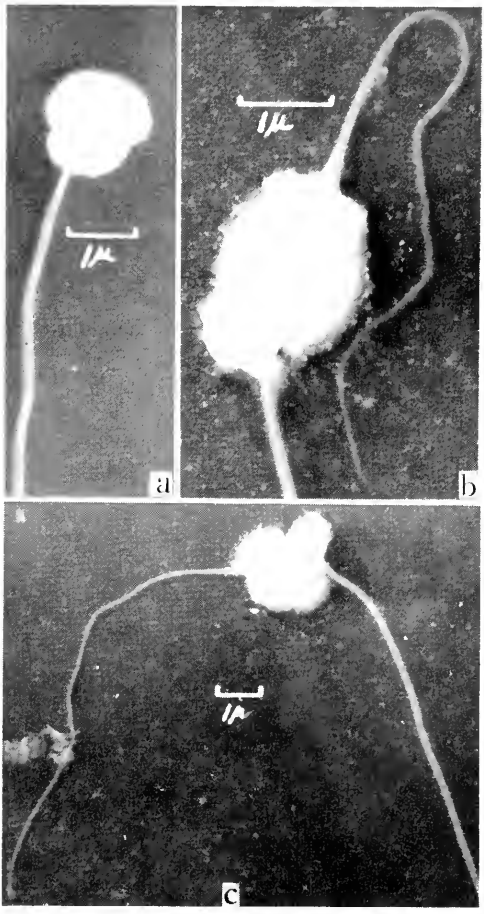

FIG. 4. Electron photomicrographs of *Astereis forbesii* sperm fixed in 5% formalin, dialyzed against distilled water, air dried to the collodion membrane and shadow cast with chromium.

a, Control sperm from sea water suspension. Sperm nucleus and middle piece are compact and closely applied to each other; no trace of acrosome filament. *b* and *c*, Spermatozoa with acrosome filaments from a suspension treated with 0.01M Versene and egg water. The long acrosome filament projects upward from the nucleus in *b*, and to the left in *c*. In both *b* and *c* the entire sperm head region appears to have undergone partial breakdown with 'loosening' and displacement of the midpiece.

an acrosome reaction resulted from egg water treatment (and adjuvant in starfish) or contact with eggs. Sperm of 4 species produced filaments in alkaline sea water and/or upon contact with glass. In the absence of negative reports in these data, it must be concluded that only a very few species have been examined exhaustively for filament formation under diverse conditions.

In a study of 5 species (3 molluscs, 2 echinoids) Rothschild and Tyler (94)

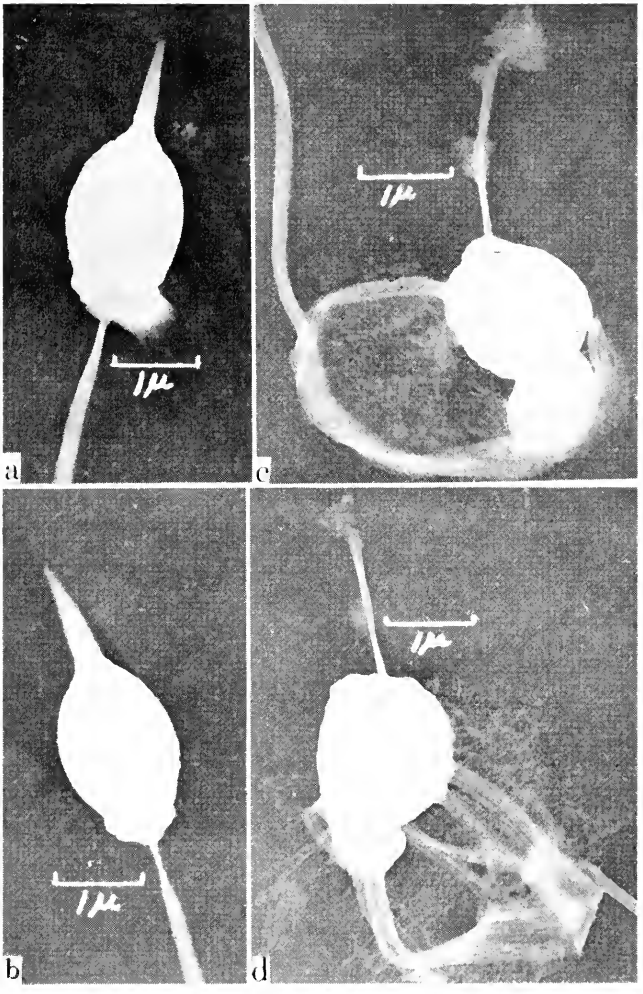

FIG. 5. Electron photomicrographs of *Nereis limbata* sperm fixed in 5% formalin, dialyzed against distilled water, air dried to the collodion membrane and shadow cast with chromium.

a and *b*, Control sperm from sea water suspension. In these the acrosome is a prominent cone with a rather broad base, the nucleus is compact and oval and the midpiece is closely applied to the nucleus. Popa's sensillae amoeboideae of the *Nereis* sperm midpiece are not evident in these preparations. However, structures answering Popa's description may be seen on living *Nereis* sperm with phase optics. *c* and *d*, Sperm with acrosome filaments from a suspension treated with egg water. The filaments are of constant diameter and appear to have undergone some disintegration at the tips. The nucleus in both *c* and *d* has rounded up, the midpiece has loosened and the base of the tail has looped about the head. All of these are constant characteristics of the 'reacted' *Nereis* spermatozoan.

were unable to confirm certain of these effects. They found very long acrosome filaments on all chiton sperms examined following dilution with sea water. Under similar conditions, 20 per cent of Mytilus sperm had long, fine filaments, whereas the remainder had shorter filaments with a proportionately larger basal acrosome region. These investigators report shorter knob-like acrosomes on living sea urchin (Echinocardium) sperm in contact with glass, but sperm with long filaments as well as others with knob-like acrosomes occurred in formalin-fixed material prepared for electron microscopy. Finally, Rothschild and Tyler found no increase in the proportion of sea urchin (*Stronglylocentrotus purpuratus*) sperm with filaments following treatment with egg water.

It is not immediately apparent why Dan, Colwin and Colwin, and Metz and Morrill on the one hand, and Rothschild and Tyler on the other, fail to agree on three major points, namely *1*) the relative numbers of sperm with acrosome filaments in sea water suspensions, *2*) induction of an acrosome reaction on contact with a surface and *3*) the effect of egg water. The two groups of investigators even examined the same species, *Mytilus edulis*, in one case although it must be admitted that the populations were probably not closely related (Misaki, Japan vs. Millport, Scotland). *Point 1* may result from subtle differences in preparation. Wide differences in result might be expected from relatively minor variations in technique if the acrosome reaction is very easily induced by contact with glass. Rothschild and Tyler examined but one species each with regard to *points 2* and *3*. These investigators prepared much of their material for electron microscopy by Anderson's (8) critical point method, which is the method least likely to induce desiccation artifacts. Unfortunately, Dan and Colwin and Colwin give no quantitative data in terms of actual numbers of reacted and unreacted spermatozoa in their experiments.

In their study, Metz and Morrill (71) examined sperm of Asterias and Nereis for acrosome filaments. The data are presented in table 1. In Asterias, agglutination of sperm by egg water ordinarily occurs only in the presence of an adjuvant (57) and the essential function of this last agent is a metal binding or chelating action (63). Following treatment with sea water or adjuvant (Versene) very few Asterias sperms (not over 2%) were found to have filaments (table 1). Spermatozoa of three other asteroids (*Luidia clathrata*, *Astropecten duplicatus*, *Henricia sanguinolenta*) also lacked acrosome filaments following sea water or Versene treatment. Likewise, Asterias sperm treated with egg water alone failed to produce filaments in appreciable numbers except in *experiment 2* (table 1). In this experiment the egg water sample was old and agglutinated sperm in the absence of Versene. Treatment with adjuvant and egg water, however, resulted in the appearance of appreciable numbers of sperms with filaments in *experiments 1*, *2* and possibly *4*. The role of the adjuvant in this effect is not immediately apparent, for starfish sperm can bind the fertilizin (sperm agglutinin) in egg water, at least in reduced amounts, even

in the absence of an adjuvant (57). Finally, these observations confuse the picture for normal fertilization in the starfish. The formation of a filament extending through the egg jelly from the sperm to the egg surface appears to be a normal, and perhaps essential, initial step in starfish fertilization (14, 20, 28, 40). A comparable situation appears to obtain in the enteropneust, Saccoglossus, for Colwin and Colwin (18) in a very thorough study have described and illustrated with photographs an acrosome filament extending from the sperm head through the egg membranes to the egg surface in this form. Dan (23) has suggested that the starfish filament is an acrosome filament formed in response to egg products. However the data presented in table 1 indicate that acrosome filament formation depends upon a metal-binding agent as well as the egg water. The presence of such an agent under natural conditions seems

TABLE 1. PERCENTAGE OF ASTERIAS FORBESI AND NEREIS LIMBATA SPERM WITH ACROSOME FILAMENTS FOLLOWING TREATMENT WITH EGG WATER

EXP. NO.	SPERM + VERSENE + EGG WATER	SPERM + VERSENE + SEA WATER	SPERM + SEA WATER + EGG WATER	SPERM + SEA WATER + EGG WATER
Asterias				
1	33 (30)*	0 (12)	0 (75)	0 (44)
2	64 (36)	2 (30)	60 (10)	
3	3 (37)	1 (54)	2 (41)	1 (32)
4	8 (52)			0 (30)

	SPERM + EGG WATER	SPERM + SEA WATER
Nereis†	85 (48)	30 (90)

* Figures in parentheses are the number of spermatozoa examined and recorded. Only sperm with acrosome region lying free and unobstructed upon the collodion membrane were recorded. This may have introduced a selective error in the data, especially in the case of fertilizin treated sperm, for it eliminates most cells in clumps and agglutinates. The agglutinates might be expected to contain a high percentage of sperm with filaments.

† In two other experiments no filaments were observed on egg water treated or control sperm. However, the egg water did not agglutinate the sperm in these two experiments

unlikely. Certainly fresh, normal egg water lacks the necessary chelating action. Possibly the acrosome reaction results from contact with the egg jelly (20).

Three experiments were performed on Nereis sperm. In two of these, no acrosome filaments appeared on either sea water or egg water treated sperm, but in a third experiment filaments were found on both (table 1). The egg water agglutinated the sperm strongly in the last experiment. Treatment with egg water appeared to effect a three-fold increase in the percentage of sperms with filaments. Evidently Nereis sperms possess acrosome filaments under certain conditions. These filaments may be formed in response to egg water under agglutinating conditions. They may also be produced under other conditions, perhaps as a result of contact with glass. It is of interest to note that the form of the Nereis sperms with filaments as seen with the electron microscope closely

resembles the figures of Lillie (47) for fixed and sectioned sperm in contact with the egg.

Information concerning the mechanism of acrosome filament formation might result from electron microscope examination of sectioned spermatozoa. Afzelius (1) has examined sectioned sperm of four species of sea urchins. In all of these the acrosome contains a terminal granule of osmiophilic material and a less osmiophilic basal 'cavity' projecting into the sperm nucleus. In one of the four species, namely *Echinocardium cordatum*, the terminal granule is located at the end of a stalk and this stalk appears to be filled with the same material as the basal cavity. However, in some preparations the stalk material appears to be oriented as longitudinal fibers or broken into flakes. Echinocardium is the only species that has been examined both for acrosome filaments and in sections. Comparison of the figures of Afzelius (1) and Rothschild and Tyler (94) indicates that Afzelius examined sperm that lack acrosome filaments. This is of further interest, for Afzelius prepared his material by dropping undiluted semen into his fixative. The fact that sperm prepared in this direct way lack filaments supports the view that the compact condition of the acrosome is the initial one.

Afzelius suggests that the acrosome particle of sea urchin sperm contributes to the dispersing material described by Dan (22) in discharged acrosomes and that the less opaque, often fibrillar material corresponds to the central core of the filaments. This may prove to be the case in certain instances, but as yet no substantial evidence is available to support this view. Furthermore, the Echinocardium filament figured by Rothschild and Tyler (94) is a very long, finely tapering structure and shows no appreciable dispersed material. Dan's suggestion that acrosome filaments may be discharged in trichocyst-like fashion at least has the merit that in both acrosomes and trichocysts there does not appear to be a highly organized, preformed structure in the undischarged organelle.

Aside from inducing acrosome filaments, treatment of sperm with egg water appears to have a second morphological effect, namely a loosening (23) or displacement of the sperm mid-piece (83, 94, 117). The significance of the mid-piece effect is not apparent. Moreover, the mechanism of this effect would seem to be complicated, for electron photomicrographs show that this structure is a single, continuous ring with a central canal for passage of the sperm tail (1)

These studies are clearly in an early stage of development. More organisms may need to be examined, more quantitative data obtained and a thorough series of specificity studies carried out with respect to the action of egg water. Finally, the active agent in egg water requires characterization. Its identification with or separation from the specific sperm isoagglutinin, fertilizin, is a problem of special interest. However, the data now available suggest the interesting possibility that the spermatozoan is subject to a trigger reaction

induced by a specific agent from eggs and that this triggering is a necessary preliminary to fertilization of the egg by the spermatozoan.

ASPECTS OF EGG ACTIVATION

The various aspects of fertilization of the egg have been considered in detail in a number of recent reviews (15, 62, 85, 86, 97, 99, 115, 116, 119). These should be consulted by readers interested in a complete coverage of the subject, for only two aspects of the fertilization of the egg need be considered here. These are the activation initiating mechanism of fertilization and the nature of the propagated responses of the egg upon fertilization.

a) Activation Initiating Mechanism. Although the activation of the egg has been the subject of investigation for many years, the mechanism of this reaction remains obscure. From morphological studies it is clear that the reaction requires only superficial union between the egg and the activating sperm. This is especially evident in Nereis (47), the starfish (14, 28, 40), Urechis (79, 111) and Saccoglossus (18), for in these forms the egg undergoes definite fertilization changes before the sperm nucleus is drawn into the egg cytoplasm. Furthermore, the activating sperm may be removed from the egg surface by microdissection (30) or by natural means in hybrid crosses (9, 51, 97). This clearly indicates that the sperm-egg union required for the activation reaction is not a very firm union. Finally, the area of contact would appear to be very small. In the starfish and Saccoglossus at least the initial stages of activation of the egg are apparently initiated by association of the sperm acrosome filament with the egg surface or cortex. If this association involves only contact with the egg surface, then the area of contact for Asterias would appear to be of the order of 0.002 μ^2. This value is calculated from the diameter of the acrosome filament near its tip (approximately 0.05 μ, assuming no flattening, in Fig. 4*B*).

Many investigators have considered the activation initiating mechanism to consist of an interaction of relatively specific egg and sperm substances. This view was first proposed in detailed form in Lillie's (48) 'fertilizin theory of fertilization.' The interaction of these substances is presumed to initiate a chain of events which culminates in the morphological, physiological and biochemical phenomena of egg activation just as interaction of the mating type substances initiates activation in Paramecium (see first section). Attention has been directed primarily to two substances, fertilizin from the egg and antifertilizin from sperm. In the sea urchin, fertilizin constitutes the jelly layer that surrounds the egg (113; fig. 6) and it or a similar agent may also reside within the egg (77, 78) although this has been disputed (13). On standing, the sea urchin egg jelly layer gradually dissolves and in so doing charges the surrounding sea water with fertilizin. Antifertilizin is located on the sperm surface (116) but may be obtained in solution by heating (29), freeze thawing (112) or acid extraction (127) of sperm.

Fertilizin and antifertilizin interact in antigen-antibody-like fashion (108, 115). The interaction is most strikingly manifested by agglutination of sperm and eggs by fertilizin and antifertilizin respectively, and these reactions are characterized by a very high order of specificity, an order of specificity, in fact, which compares favorably with the fertilization reaction itself (99, 115, 116). These properties suggest that fertilizin and antifertilizin play an important if not essential, role in fertilization. These substances evidently aid fertilization at least to the extent that their partial removal (113, 122, 123, 127) or blockage with the complementary substance (113, 127) or with specific antisera (114) reduces the fertilizing capacity of the gametes. These substances may function in the attachment of the sperm to the egg (115). Indeed, their interaction may yet be found to constitute the activation initiating reaction. However, there is no substantial evidence to support such a view, but neither are there unequivocal data to rule it out. In spite of this rather unsatisfactory state of affairs, this approach to the problem appears at present likely to be the most rewarding one (see refs. 72, 97, 119).

The approach through the medium of artificial parthenogenesis once seemed to hold great promise for a clear understanding of the activation initiating mechanism, and a number of interesting theories have been advanced to explain the action of parthenogenetic agents. However, no common factor has yet been found to unify the great body of data on parthenogenesis or to relate it directly to the action of the sperm in the activation reaction.

Other avenues of attack upon the activation problem may yet be found. Interesting recent leads in this direction have been provided by the demonstration of a lipoprotein splitting action of sperm (54) and the possibility of relating this action to Loeb's (51) cytolysis theory of activation. Likewise, the rather striking activating action of dead paramecia upon living mates (62) raises the possibility of comparable action of dead sperm on eggs. Such action has not been clearly demonstrated, but a step in this direction has been attained by the discovery that dead sperm will agglutinate with fertilizin (57, 70).

b) Deviations From the All-or-None Principle. The simplified concept of activation given in the introduction, attractive though it may be, probably requires some modification. Under optimal conditions the egg may appear to display all-or-none, trigger-like behavior at activation, but under other conditions deviations from the normal pattern do occur. Three possible sources of such deviations should be recognized, namely *1*) the activation initiating mechanism itself, *2*) the system for propagation of the egg responses and *3*) the final reactions which produce the morphological and physiological end effects of activation. A partial failure or abnormal operation of any of these three mechanisms might be expected to produce recognizable and measurable deviations from the optimum expression of activation. A clear distinction can not always be made among these three possibilities, since in most cases only the end effects of activation can be measured. Two deviations that may relate

to the activation initiating mechanism directly will be considered here. These are the reversal of fertilization and variations in the normal fertilization response that may be attributed to the condition of the gametes.

Reversibility of fertilization. Complete reversal of fertilization might be considered to require expulsion of the fertilizing sperm from the egg cytoplasm, reversal of meiosis in some forms, and return to the morphological and physiological conditions of the unfertilized egg. Such reversal has not been obtained, but an approximation of it has been described by Tyler and Schultz (129) for early stages in the fertilization of the echiuroid, Urechis.

The unfertilized egg of Urechis is indented. Upon insemination the activating spermatozoan attaches to the egg surface. Three minutes later the indentation of the egg disappears. Subsequently, the spermatozoan enters the egg and the fertilization membrane forms. If the egg is transferred to acidified sea water within 3 minutes following insemination, the egg rounds out and the sperm enters as in normal fertilization. However, in the acid sea water further development ceases, the indentation of the egg reappears and the egg returns to the prefertilization condition. If such an egg is transferred to normal sea water, development is not resumed and the sperm remains passively in the egg cytoplasm. If, now, the egg is reinseminated, a second sperm attaches to the egg, the egg again rounds out, the second sperm enters and development proceeds, but the development is now dispermic for both the first and the second sperms contribute to the division figure. Trispermic development may be obtained if the egg is given a second acid treatment and a third insemination.

Evidently the development of the Urechis egg is not only blocked by the acid treatment but certain of the initial activation changes are actually reversed. Notable among these are the rounding out of the egg and the block to polyspermy. Blocked and reversed eggs can be reactivated by treatment with an appropriate parthenogenetic agent (121, 129) as well as by a second insemination. Attempts to duplicate this reversal of fertilization in the sea urchin have failed (91).

Unfortunately this remarkable experiment has so far served mainly to demonstrate reversibility of activation. A further examination of the reversed eggs, employing methods recently developed for the sea urchin, might now be in order. One point of particular interest would be an attempt to extend the period between the initial insemination and the acid treatment. Possibly pretreatment with trypsin or other agents which prevent elevation of the fertilization membrane in the sea urchin would be effective.

Effects resulting from the condition of the gametes. It has long been known (49) that even under favorable conditions insemination does not always result in optimal fertilization and development of the egg. Variations from the accepted normal are usually ascribed to a suboptimal condition of the gametes, especially the egg. Deviations from the normal pattern include partial or complete failure

to elevate the fertilization membrane, a high incidence of polyspermy, abnormal cleavage and complete failure of eggs to respond to insemination.

It is not yet clear in most cases whether these variations result from suboptimal activation initiating reactions or from defects in subsequent activation phenomena. However, in certain cases the effects seem to be related rather closely to the initial steps in the activation of the egg. Since these appear to bear upon the nature of the activation initiating mechanism, they will be considered in this section. The most interesting of the recent work concerns the effect of metal binding agents in improving fertilization. A number of studies have shown that proteins, amino acids, Versene and other agents with metal binding or chelating action have a number of effects on echinoderm sperm. These effects include an increase in motility (57, 64, 69, 93, 118, 120, 131, 133) with or without an appreciable increase in respiration, prolongation of the life span of the sperm (93, 120, 133) and an increase in fertilizin binding capacity (57). Tyler and Rothschild (128) first suggested that certain of these effects may result from a metal binding or chelating action of these agents. Substantial evidence to support this view has been presented by Tyler (118) and Rothschild and Tyler (93).

One or more of these effects could readily account for the observed (57, 93, 120, 133) improvement in the fertilizing capacity of a treated sperm suspension when this is measured quantitatively in terms of the concentration of sperm required to yield a given percentage of fertilized eggs. However, these effects do not so readily account for a qualitative improvement in fertilization in terms of the degree of elevation of the fertilization membrane, etc. Nevertheless, such qualitative improvement has been reported by several investigators.

Runnstrom and his associates have described a number of deviations from the normal fertilization reaction in several sea urchin species (11, 34, 97, 99). These deviations are attributed to variations in the degree of 'cytoplasmic maturity' of eggs that have completed nuclear maturation. 'Underripe' eggs fail to respond to insemination by any manifestation of fertilization, or give low percentages of fertilization and produce poor fertilization membranes (blister-like membranes, tight membranes, incomplete incorporation of cortical granules into the fertilization membrane). Upon attaining cytoplasmic maturity the eggs give the optimal fertilization reaction. 'Overripe' eggs respond to insemination by imperfect or complete failure of membrane formation and abnormal cleavage or complete refractoriness to the inseminating sperm. This cycle of events is reported to be seasonal among individuals of a population (97). Early in the breeding season the sea urchins yield underripe eggs, later ripe eggs appear and finally toward the end of the breeding season the individuals produce overripe eggs. In some cases, at least, underripe sea urchin eggs pass through the ripening cycle on standing in sea water (34, 96).

The suboptimal fertilization of underripe and overripe eggs can be substantially corrected by fertilization of the eggs in solutions of proteins, amino acids or Versene (11, 34, 103). These agents presumably act by binding metals which have an inhibitory action on fertilization.

An analysis of the mechanism of action in this fertilization improving effect might properly begin with the question whether the chelating agent acts upon the sperm, the egg or both gametes. The chelating agents clearly affect unfertilized eggs. The effects include a swelling of the jelly, a rounding of eggs, an increased stretching in the centrifugal field (11, 46, 99), and an increase in the rate of smoothing of the wrinkles produced by hypertonic sea water treatment (132). Runnstrom and his colleagues appear to regard the fertilization-improving action of chelating agents as mainly an action on the eggs. However, before accepting this view, a more direct study of the problem would appear to be in order.

The writer puts a somewhat different interpretation on conditions observed in *Arbacia punctulata* from Florida waters. The animals in the local population definitely contain gametes from September until June. They have not been examined thoroughly during the summer months. From late January until October (with the possible exception of the summer) the Arbacia yield quantities of gametes. The eggs fertilize readily in sea water, form good fertilization membranes and cleave normally. However, beginning in October or November and continuing through December the animals yield decreasing amounts of gametes, and these become progressively more difficult to fertilize. The eggs that do fertilize are frequently polyspermic and fail to elevate normal membranes. A few tests have shown that these 'winter eggs' fertilize normally in Versene-sea water. Although no serious study of this annual cycle in the Florida Arbacia has yet been made, the writer hazards the view that the animals actively produce gametes from February until September or October at which time gametogenesis ceases. The gametes remaining in the gonads beyond this date are neither resorbed nor spawned. These 'stored' gametes become progressively less fertilizable. In view of the correcting effect of Versene on these 'stored' gametes it appears likely that the storage is associated with an accumulation of metals which inhibit fertilization. It will be interesting in this case also to determine whether the action of Versene is upon the sperm, the egg or both gametes.

A thorough examination of both the Swedish and Florida material in this regard seems especially desirable in view of the findings of Tyler and Atkinson (120) and Tyler (118). These investigators find that Lytechinus eggs form poor fertilization membranes when inseminated with aged sperm (see also ref. 98). This effect is corrected by treatment of the sperm alone with Versene or other chelating agents. On the other hand, no improvement results when eggs alone are treated with Versene, washed in sea water and inseminated in

sea water. Similar results are obtained when jellyless eggs are employed in the experiment. However, it must be granted that the last two experiments do not rule out the possibility of an improving action of Versene on eggs, for such action might reverse readily upon washing the eggs in sea water (118). Such reversal does occur with starfish sperm (57).

Another significant observation from this work is the finding that the quality of fertilization is a function of the sperm concentration both in the presence or absence of a chelating agent. High sperm concentrations produced good fertilization membranes whereas more dilute sperm from the same suspension yielded poor membranes.

In Tyler's material the chelating agents evidently act upon the sperm to improve fertilization, but the mechanism of this action is obscure. Tyler (118, p. 231) interprets his experiments in terms of an impairment of the sperms upon aging "in such a way that, while still capable of fertilization, they cannot elicit a normal response on the part of the egg." This impairment evidently involves the action of metals, but the precise nature of this action is unknown. Nevertheless, the information so far available points up the complexity of the problem of the activation initiating mechanism.

Graded responses on the part of the egg have long been recognized in parthenogenetic activation. However, the 'site' of action of parthenogenetic agents is unknown. There is certainly no clear evidence that any of the agents initiate activation through the same route or receptor that operates in normal fertilization (62, 67, 97). Until some clear understanding of the site of action of parthenogenetic agents is available, it would not appear profitable to undertake a comparison of graded responses in the two types of activation.

The fact that certain graded responses in fertilization can be ascribed to the condition of the sperm argues strongly against a simple all-or-none, trigger-like activation initiating reaction.

c) Propagated Responses of Egg at Fertilization. Upon reaction with the fertilizing spermatozoan, a variety of changes occurs in the egg. Certain of these clearly arise at the site of sperm union and spread in wave-like fashion over the egg surface to meet at the pole opposite the point of sperm attachment. They have been studied most intensively in the sea urchin egg, although other material has been examined recently (17, 21, 73). Most studies on propagated responses of the egg have been concerned with the cortical reaction. However, the effects of fertilization are not confined to the superficial regions of the egg. For example, fertilization induces the egg nucleus to begin or to complete maturation in many forms. Recently Allen and Rowe (7) have observed changes in the endoplasm of stretched sea urchin eggs at fertilization. These changes proceed from the region of sperm attachment and appear to keep pace with the cortical changes. A more thorough study of such endoplasmic responses would be desirable.

Structure of sea urchin egg surface. Before considering the cortical reaction, the structure of the sea urchin egg surface must be understood. This subject has long been the source of some dispute. The mature, unfertilized egg is enclosed in a layer of gelatinous material which is identical with fertilizin (115, 116). Below the jelly the egg is surrounded by a membrane, the vitelline membrane (see ref. 101), according to most accounts. Most investigators agree that this membrane is closely applied to the plasma membrane which bounds the egg surface. The cortical region of the egg contains a layer of hyaline material and the cortical granule layer (36, 37, 50, 74). The cortical granules of the Arbacia egg are approximately 0.8 μ (74) in diameter and form a single layer in the mature egg. A number of workers (27, 74, 76, 97) find that the granules lie in the cortical cytoplasm beneath the plasma membrane (fig. 6*A*). However, Parpart and Laris (80, 81) conclude from permeability studies that the granules lie between the vitelline membrane and the plasma membrane and, therefore, external to the plasma membrane (fig. 6*B*). The properties of

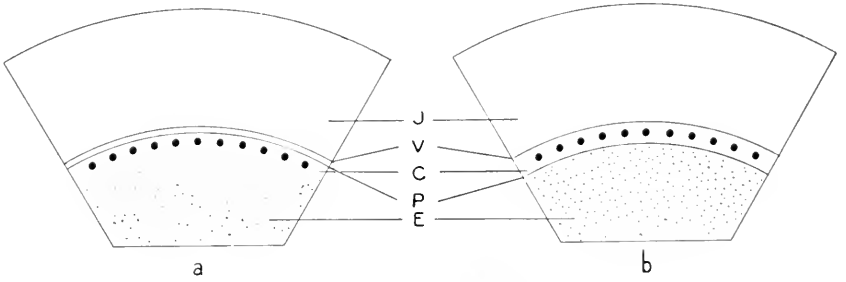

FIG. 6. Structure of the sea urchin egg surface. *a*, Modified from Runnstrom. *b*, According to Parpart and Laris. *J*, egg jelly layer; *V*, vitelline membrane; *C*, egg cortex containing the cortical granules; *P*, plasma membrane; *E*, endoplasm.

the isolated egg cortex and the cortex-free egg surface may also be interpreted to support this view (3, 5). Furthermore, this construction is supported by the fact that a single rather than a double membrane appears external to the granules in electron photomicrographs of sectioned eggs (38, 56). However, electron photomicrographs have not yet revealed a membrane (plasma membrane of Parpart) internal to the cortical granules. Irrespective of their position in the mature egg, the cortical granules are dispersed throughout the interior cytoplasm of the oocyte. During maturation they move peripherally to occupy their surface position in the mature egg (55, 76, 102).

The cortical reaction of the sea urchin egg includes the following well established phenomena: changes in the optical properties of the cortex as seen under dark field illumination (74, 89, 95), a wrinkling of the egg surface (74), breakdown of the cortical granules, elevation of the vitelline membrane and its conversion into the fertilization membrane and an increase in the thickness of the hyaline layer.

These cortical changes proceed across the egg surface in wave-like fashion from the point of sperm attachment. The time required for the most rapid change, namely the dark field change, is 20 seconds at 18°C in the material of Rothschild and Swann (89). By direct observation, Allen and Hagstrom (5) find both the dark field change and granule breakdown to occur simultaneously. The wrinkling of the egg surface is also temporally related to cortical granule breakdown (74). Elevation of the vitelline membrane and the increase in the hyaline layer follow some seconds later.

This series of activation phenomena and the wave-like manner of their passage over the egg surface raise several interesting questions. These include the following: Are the various features of the cortical response interdependent or can they occur independently? Does the propagative mechanism for these responses pass through the cortex, the endoplasm or both parts of the egg? What is the mechanism of propagation? Answers to these questions will be sought in the paragraphs below.

Interrelation of visible cortical responses. Several of the visible cortical events can occur independently of one or more of the others. In fact, it is not unlikely that the egg can at least cleave in the absence of any of the visible changes. Thus eggs inseminated following butyric acid treatment can cleave, although they fail to elevate membranes or undergo granule breakdown (76). However, formation of a substantial hyaline layer is evidently necessary to cement the blastomeres together. As is well known, the blastomeres separate and normal development fails in the absence of the hyaline layer. Substantial elevation of the membrane depends upon discharge of the cortical granules (81). Likewise, hardening of this membrane to form the fertilization membrane depends upon incorporation of cortical granule material into the vitelline membrane (27, 76, 97). The marked birefringence of the fully formed fertilization membrane also depends upon this incorporation of cortical granule material (42, 97). The dark field color change of the egg cortex and granule breakdown can occur independently in suboptimal material (5, 97) or following treatment with antifertilizin or basic proteins (97). However, the dark field changes, cortical wrinkling and hyaline layer thickening do appear to be closely related to granule breakdown for these responses occur together in graded fashion and in proportion to the degree of stimulation in eggs treated with an alternating current (6).

Cortical vs. endoplasmic propagation. The wave-like passage of the visible responses over the egg surface suggests that the response is propagated in the egg cortex. Runnstrom and Kriszat (100), Allen (2) and Allen and Hagstrom (5) subscribe to this view. Rothschild (84, 88) suggests from a consideration of the rate of propagation that the impulse may pass through the endoplasm of the egg. In support of the first view, Allen (3) showed that fragments of the egg cortex could be isolated, and that such fragments would respond to insemination by formation of a thin fertilization membrane and by cortical

granule breakdown. Allen further showed that the effect was specific when reciprocal crosses were attempted between two species of sea urchins. This result strengthens the view that the sperm activated the isolated cortex by the normal fertilization mechanism. However, since the external surfaces of the isolated fragments were attached to glass in many cases, it is not immediately clear how the activating sperm reached the normal site of activation. The experiment raises the interesting possibility that the sperm can activate by contact with either side of the cortex. Since fertilization membrane formation and cortical granule breakdown result from insemination of the cortical fragments, the normal propagative response probably operates in these fragments. This result would indicate that the cortex alone can propagate the response. However, it may be argued that a small amount of endoplasm adheres to the isolated cortex. The force of this argument will depend to a considerable extent on the interpretation given to the structure of the egg surface (see earlier section).

The evidence for a cortical transmission of the response advanced by Runnstrom and Kriszat (100) has been challenged by Rothschild (88). The former investigators found that areas of jellyless eggs adhering to glass did not undergo the cortical reaction. They reasoned that the adhering region is altered sufficiently to prevent propagation in the cortex. However, as Rothschild (88) points out, it may be argued with equal force that the alteration may prevent a response of the cortex to an endoplasmically propagated stimulus.

In further support of the endoplasmic transmission hypothesis, Rothschild (88) cites experiments of Horstadius and Runnstrom (41). These workers inseminated eggs constricted in capillary tubes. The eggs frequently elevated membranes only on the end opposite that of insemination. This result is most readily explained by assuming that an endoplasmic stimulus reaches the egg surface only at the far end of the stretched egg. However, this interpretation must be treated with caution, for the physical properties of the two ends of eggs constricted in capillaries are evidently different (26, 41). These physical differences might well reflect differences in susceptibility to a propagated cortical response. Furthermore, the cortex of constricted eggs showed dark field cortical changes in the elongated area following insemination, even though membranes did not form in this region.

Mechanism of propagation. The nature of the transmission mechanism of the propagated cortical response is even more obscure than the region of the egg through which it passes. In accord with many earlier workers, Sugiyama (110) believes that an invisible cortical reaction precedes the first visible evidence of cortical change. Sugiyama's view is based on the observation that certain agents induce local, non-propagative cortical granule breakdown. Accordingly, Sugiyama reasons that breakdown of one cortical granule does

not induce breakdown of neighboring granules. Therefore some invisible mechanism must operate in the propagative reaction.

Two views concerning the mechanism of propagation of the cortical responses have been advanced. The first view considers the reaction to be a self-propagating chain reaction. Such a chain reaction system was formulated by Lillie (48) in his fertilizin theory. Lillie visualized a chain reaction involving combination of the fertilizin and antifertilizin of the egg, and it must be granted that the possibility of some such reaction between fertilizin and antifertilizin has not been ruled out. A number of other hypothetical schemes for a self-propagating chain reaction may be formulated. For example, an essential proenzyme might be distributed throughout the egg cortex or even the entire egg. If this proenzyme were converted locally to the active form by the sperm and if the enzyme autocatalytically convert more proenzyme into the active material, a relatively simple chain reaction might result. The conversion of chymotrypsinogen to chymotrypsin by action of the latter (12) is one of a number of examples of this type of proenzyme-enzyme relationship. In this connection it is of interest that proteolytic enzymes are activated in the egg at fertilization (53). Others (72, 97) have proposed somewhat similar but more complex mechanisms for egg activation and propagation of the cortical response.

Several investigators have considered the possibility that the passage of the cortical response might be associated with the propagation of an action potential similar to that of muscle and nerve. Rothschild and Swann (89) have reviewed the earlier studies. Although neither a resting potential across the egg surface nor an action potential at fertilization had been demonstrated in any egg at that time, more recent studies have revealed both. Scheer *et al.* (104) recorded transient egg potentials associated with fertilization. These investigators were unable to detect a resting potential in the sea urchin egg. More recently, however, Grundfest *et al.* (32) have demonstrated a resting potential of 40–60 millivolts across the surface of the Asterias egg, and Tyler *et al.* (125) have observed a drop of 5–10 millivolt in this potential 15–30 seconds following insemination. After about 30 seconds the potential rose to the resting level, and later surpassed it. Evidently, then, the echinoderm egg does exhibit a resting potential and a potential change occurs during the activation of the egg. Apparently the difficulty in recording these phenomena in the past has resulted from failure of the electrode to penetrate the plasma membrane of the egg (52, 124). A closer correlation of the electrical changes with other activation phenomena of the egg may shortly be expected to result from the efforts of these investigators.

A second possible mechanism of propagation is the diffusion of some substance from the sperm. Rothschild (84) has emphasized that the rate of propagation of the cortical reaction is not incompatible with such a mechanism. Local reactions in the neighborhood of the penetrating sperm that might well result from such diffusion have been demonstrated in nematode eggs by cyto-

chemical methods (82). However, other positive evidence in support of the view is lacking. Finally, the fact that the propagated response can apparently be elicited by pricking with a fine needle (75) casts doubt on the necessity of a substance from the sperm.

Block to polyspermy. In eggs in general and marine eggs in particular, one spermatozoan, and only one, ordinarily participates in the development of the egg. This is true even though many sperms may be present in the vicinity of the egg at the time of fertilization. Clearly some mechanism prevents more than one sperm from entering the egg. This mechanism, the block to polyspermy, has been the subject of extensive study. Earlier investigations led to the view that the block does not depend upon the formation of the fertilization membrane but to a change of some sort in the constitution of the egg surface. This change is generally assumed to arise at the site of union with the activating sperm and to spread over the egg surface. Just's (44) frequently quoted statement describes the change as a 'wave of negativity.'

On the basis of subjective observation most investigators concluded that the block to polyspermy sweeps over the egg surface very rapidly. Gray (31), for example, estimated that the time required was of the order of 10^{-5} seconds. However, recent investigations have considerably altered the earlier views concerning both the time required for development of the block and the nature of the block itself.

Rothschild and Swann (88, 89, 91, 92) undertook to measure the time necessary for the development of the block to polyspermy in the egg of *Psammechinus miliaris*. They began their study by determining the rate of propagation of the first visible change in the egg cortex, namely, the dark field color change. From motion picture records they found that the propagation time of this response was about 20 seconds at 18°C. In an attempt to relate this visible cortical change to the physiological block to polyspermy, these workers performed a number of elaborate experiments and subjected them to a searching mathematical analysis. They first calculated the number of sperm-egg collisions that would occur in the unaffected part of the egg during the passage of the dark field change. At sperm densities of 10^5, 10^6, and 10^7 per milliliter the number of such collisions was estimated to be 1.6, 16 and 160 respectively. However, no appreciable polyspermy was observed at these sperm concentrations in spite of the number of collisions. This absence of polyspermy would be explained if the block swept over the egg much more rapidly than the visible change, but it could also result if sufficiently few of the sperm-egg collisions succeeded in fertilizing the egg. The studies of Rothschild and Swann indicate that both of these factors contribute to the prevention of polyspermy.

Several lines of evidence indicate that the number of successful sperm-egg collisions constitutes only a fraction of the total collisions. Thus, the number of blebs formed on immature eggs (oocytes) was far lower than the calculated

number of sperm-oocyte collisions (less than 1 bleb for every 45 collisions). Accordingly, if the oocytes are unable to propagate a block to polyspermy and the blebs faithfully record successful sperm-egg collisions, the latter must be but a small proportion of the total collisions. Using mature eggs, this view was further confirmed. Eggs were inseminated with sperm suspensions of known densities and at intervals the excess sperms were killed (by hypotonic sea water treatment); the proportion of fertilized eggs was then determined and the number of sperm-egg collisions calculated. From this information the probability of a successful collision, p, was calculated for a number of sperm densities. Aside from the interesting observation that increasing the sperm density decreased the value of p, presumably because of sperm-sperm interaction, the calculations again show that only a small proportion (1.5% of the collisions at sperm density 10^7/ml) of the collisions are successful in fertilizing the egg. Since the probability of a successful sperm-egg collision appears to be low, the possibility of a rather slow block to polyspermy—of the order of seconds rather than fractions of a second—must be entertained. The calculations suggesting this are based on a number of assumptions, including the assumption that a sperm suspension may be treated as a collection of gas molecules. Rothschild and Swann justify their assumptions insofar as available data permits. Nevertheless, confirmation of the rate of propagation of the block to polyspermy by an independent method would seem to be desirable. Direct measurements of the time required for the completion of the block to polyspermy were accordingly made. The experimental procedure was designed to fit the following premise: If all the eggs in a batch are fertilized at time $t = 0$ and the time required to complete the block to polyspermy is 5 seconds, then there will never be more polyspermic eggs than at 5 seconds. Furthermore, if the sperm and eggs are separated (killing the sperm but not the eggs with hypotonic sea water) at intervals between $t = 0$ and $t = 5$ seconds, an increasing proportion of polyspermic eggs should be found as one approaches $t = 5$ seconds. Conversely, the time required to just produce the complete block to polyspermy will be the time required to just produce the maximum number of polyspermic eggs. Using this method, Rothschild and Swann (92) obtained values for the conduction time of the complete block to polyspermy ranging from 17 to 94 seconds in a series of 14 experiments. The mean value for all the experiments was 63 seconds.

This astonishing value for the conduction time of the block is far in excess of the time (20 seconds) required for propagation of the visible dark field response of the egg cortex. Evidently, then, the visible dark field color change is not the block to polyspermy. In fact, the 63-second value compares favorably with the time required for initiation of fertilization membrane formation.

The 63-second value appears unrealistic from a subjective point of view. Therefore, the absence of polyspermy at reasonably high sperm concentrations

still demands explanation. This is provided in part by the small proportion of successful sperm-egg collisions. Calculating from the data of Rothschild and Swann (89) at a sperm density of 10^7 per milliliter, 1000 sperm-egg collisions should occur in a 63-second interval. On the average, 500 of these should occur on the unblocked surface of the egg. Of these 500 collisions, 1.5 per cent, or 7.5, should be successful.

This figure would admit of some polyspermy, but the low percentage of successful collisions clearly helps to account for the absence of polyspermy when 63 seconds are required for development of the complete block. A second factor may also be involved in preventing polyspermy, namely a high speed, but partial block. Evidence for the existence of such a block was derived from consideration of the 'fertilization parameter,' α. The fertilization parameter is a measure of the proportion of sperm-egg collisions that will succeed in fertilizing the egg. It is, therefore, a measure of the 'fertilizability' of the gametes. Comparison of α for unfertilized and fertilized eggs during the 63-second interval when the block to polyspermy is developing shows that α falls to about 1/20 of its prefertilization value following the first fertilization. In other words, only 1/20 of the successful prefertilization collisions are successful following the first fertilization. This suggests that a high speed but partial block sweeps over the egg surface, followed by a slower, complete block. The high speed block is estimated to require less than 2 seconds for completion, whereas the low speed block is not complete until the end of the 63-second interval. This postulated high speed, partial block, combined with the relatively low probability of a collision being successful, would seem to account for the absence of polyspermy at fertilization.

It should be noted that Allen and Hagstrom (4, 5) deny the existence of the high speed block to polyspermy. Their argument is based on the observation that sperm readily activate the unfertilized region of partially fertilized eggs. However, in the absence of quantitative data it is impossible to evaluate the position of Allen and Hagstrom. Furthermore, in partially fertilized eggs, propagation of the cortical reaction and the complete block to polyspermy is interrupted. This writer sees no reason why the postulated high speed block may not also be interrupted by the treatment. In fact, it may be argued that the experiments of Allen and Hagstrom support the high speed block. A high speed block would be required to prevent polyspermy in material which normally fertilizes so readily.

The studies of Rothschild and Swann clearly show that the complete block to polyspermy develops slowly in the normal egg. The means of propagation of the block, and especially the rapid partial block postulated by these workers, is unknown but some information concerning the mechanism of the block has been obtained in recent years.

It has long been known that treatment of eggs with agents such as nicotine, chloroform and chloral hydrate facilitates polyspermy. With the exception of

nicotine, the mechanism of action of the agents has not been examined and is unknown. Nicotine does not increase the speed of the sperm, the rate of propagation of the dark field change in the egg cortex, or the time of appearence of the fertilization membrane (33, 87, 90). Therefore, the action of nicotine is probably not upon the sperm or the slow speed block to polyspermy. Likewise, nicotine does not increase the receptivity of the egg surface sufficiently to account for the facts of polyspermy. Therefore Rothschild (87) concludes that nicotine induces polyspermy by abolishing the high speed block to polyspermy.

The studies of Sugiyama (109) and Hagstrom and Hagstrom (35) have shown that fertilized sea urchin eggs can be 'refertilized' following appropriate treatment. In these experiments the fertilization membranes were removed or their formation prevented by mechanical or chemical (trypsin, chymotrypsin, urea) treatment. Subsequently the membraneless, fertilized eggs were treated with Ca- and/or Mg-free sea water or urea, returned to sea water and reinseminated. In some of the experiments over 90 per cent of such reinseminated eggs became polyspermic. According to these investigators even the blastomeres of early cleavage stages can be refertilized. These studies have been confirmed and extended by Tyler, Monroy and Metz (126) in *Lytechinus pictus* and *L. variegatus*. The eggs of these sea urchins will refertilize following simple mechanical removal of the fertilization membrane. The membranes were removed by squirting the eggs through a syringe 1–2 minutes after the initial insemination. Following reinsemination, 100 per cent polyspermy was obtained in some tests. Refertilization of the membraneless eggs was obtained up to 40 minutes after the first insemination. From these results it is clear that no absolute cytoplasmic block to polyspermy develops within the first 40 minutes following fertilization in the Lytechinus egg. In Lytechinus, and probably in other sea urchins as well, the fertilization membrane apparently constitutes the final, low speed block. In the species examined by Sugiyama and by Hagstrom and Hagstrom the hyaline plasma layer may also constitute a block to polyspermy. This layer is removed by the urea or Ca-Mg-free sea water treatment. Since this treatment appears to be necessary for refertilization in these forms, it is assumed that removal of the hyaline layer is required for refertilization.

In most of the experiments cited, the high speed, partial block (92) should have developed before treatment of the eggs. Evidently, then, the eggs refertilize in spite of any such partial block, or the block is not effective under the conditions of the experiments. This interesting point will require further study.

CONCLUSIONS

The orderly nature of the early events of development suggests that these proceed through a branching system of 'chain reactions' from a few or possibly a single reaction between the interacting cells. This primary activating re-

action of fertilization would appear to be a chemical reaction between the interacting cells. The high order specificity of fertilization may reside in the structure of the substances that interact in this reaction.

These views have been entertained for many years, but until recently no substantial, positive experimental evidence has been produced to support them. Many 'sex substances' have been found in the most diverse plant and animal groups and a number of these have been shown to play important or essential roles in fertilization, but in no single instance has a specific organic substance been isolated, characterized and demonstrated to participate directly in the activation initiating reaction of fertilization. The closest approximation to such concrete evidence is found in the studies on Paramecium.

In Paramecium, highly specific surface proteins, the mating type substances, interact in antigen-antibody-like fashion to initiate the morphological, physiological and cytological changes of fertilization. Evidence for this role of the mating type substances has been obtained primarily from studies on the specific activation of living paramecia by dead animals of opposite mating type. As yet the mating type substances have not been extracted from living or dead animals in active form.

Among Metazoa positive evidence for a triggering action at fertilization is wanting. However, the formation of an acrosome filament following treatment of sperm with egg water may prove to be a trigger-like response to a specific agent from eggs. Although the activation of the egg frequently appears to show all-or-none behavior, graded responses at fertilization have long been known. In the most thoroughly analyzed cases the suboptimal quality of the fertilization can be attributed to the condition of the sperm. Metal ions seem to be involved in this effect. The fact that the degree of response of the egg can be determined by the condition of the sperm argues strongly against a simple, all-or-none trigger mechanism. Further examination of these graded responses, and a serious effort to relate them to the graded responses of the egg following parthenogenetic activation, should contribute substantially to an understanding of the activation initiating mechanism of the egg.

Following the activating reaction a series of changes occurs in both the cortex and endoplasm of the egg. These changes are clearly propagated from the site of sperm union, but the nature of the primary propagative reaction and the region or regions of the egg through which it passes have not yet been clearly established. One promising approach would appear to result from the discovery of a resting potential across the egg surface and the potential change associated with the activation of the egg. Further studies may shortly relate the propagative system of the egg to that of muscle and nerve.

REFERENCES

1. Afzelius, B. A. *Z. Zellforsch. u. mikroscop. Anat.* 42: 134, 1955.
2. Allen, R. D. *Exptl. Cell Research* 6: 412, 1951.

3. Allen, R. D. *Exptl. Cell Research* 8: 397, 1955.
4. Allen, R. D. and B. Hagstrom. *Exptl. Cell Research* 9: 157, 1955.
5. Allen, R. D. and B. E. Hagstrom. *Exptl. Cell Research Suppl.* 3: 1, 1955.
6. Allen, R. D., A. Lundberg and J. Runnstrom. *Exptl. Cell. Research* 9: 174, 1955.
7. Allen, R. D. and E. C. Rowe. *Biol. Bull.* 109: 344, 1955.
8. Anderson, T. F. *Am. Naturalist* 86: 91, 1952.
9. Bataillon, E. *Compt. rend. soc. biol.* 142: 1351, 1906.
10. Beale, G. H. *Genetics of Paramecium Aurelia.* London: Cambridge, 1954.
11. Borei, H. G. and U. Bjorklund. *Exptl. Cell Research* 5: 216, 1953.
12. Breyer, W. J. and H. Neurath. *J. Biol. Chem.* 217: 527, 1955.
13. Byers, H. L. *Biol. Bull.* 101: 218, 1951.
14. Chambers, R. *Biol. Bull.* 58: 344, 1930.
15. Chang, M. C. and G. Pincus. *Physiol. Rev.* 31: 1, 1948.
16. Colwin, A. L. and L. H. Colwin. *Biol. Bull.* 109: 357, 1955.
17. Colwin, L. H. and A. L. Colwin. *J. Morphol.* 95: 1, 1954.
18. Colwin, L. H. and A. L. Colwin. *J. Morphol.* 95: 351, 1954.
19. Colwin, L. H. and A. L. Colwin. *Biol. Bull.* 109: 357, 1955.
20. Colwin, L. H. and A. L. Colwin. *Biol. Bull.* 109: 357, 1955.
21. Costello, D. P. *J. Gen. Physiol.* 32: 351, 1949.
22. Dan, J. C. *Biol. Bull.* 103: 54, 1952.
23. Dan, J. C. *Biol. Bull.* 107: 203, 1954.
24. Dan, J. C. *Biol. Bull.* 107: 335, 1954.
25. Dan, J. C. and S. K. Wada. *Biol. Bull.* 109: 40, 1955.
26. Dan, K. and K. Okazaki. *J. Cell. & Comp. Physiol.* 38: 427, 1951.
27. Endo, Y. *Exptl. Cell Research* 3: 406, 1952.
28. Fol, H. *Mem. Soc. Phys. Hist. Nat. Geneve.* 26: 89, 1879.
29. Frank, J. A. *Biol. Bull.* 76: 190, 1939.
30. Goodrich, H. B. *Biol. Bull.* 38: 196, 1920.
31. Gray, J. *A Text-Book of Experimental Cytology.* London: Cambridge, 1931.
32. Grundfest, H., C. Y. Kao, A. Monroy and A. Tyler. *Biol. Bull.* 109: 346, 1955.
33. Hagstrom, B. E. *Exptl. Cell Res.* 9: 407, 1955.
34. Hagstrom, B. *Exptl. Cell Res.* 9: 313, 1955.
35. Hagstrom, B. and B. Hagstrom. *Exp. Cell Res.* 6: 491, 1954.
36. Harvey, E. N. *J. Exptl. Zool.* 10: 507, 1911.
37. Hendee, E. C. *Papers Tortugas Lab., Carnegie Inst. Wash.* 27: 101, 1931.
38. Hillier, J., A. I. Lansing and T. B. Rosenthal. *Biol. Bull.* 103: 293, 1952.
39. Hiwatashi, K. *Sci. Rep. Tohoku Univ., Biol.* 18: 137, 1949.
40. Horstadius, S. *Pubbl. staz. zool. Napoli* 17: 222, 1939.
41. Horstadius, S. and J. Runnstrom. *Exptl. Cell Research* 4: 468, 1953.
42. Inoué, S. and K. Dan, *J. Morphol.* 89: 423, 1951.
43. Jacobson, W. E. *Anat. Rec.* 101: 708, 1948.
44. Just, E. E. *Biol. Bull.* 36: 1, 1919.
45. Kimball, R. F. *Quart. Rev. Biol.* 18: 30, 1943.
46. Kriszat, G. *Exptl. Cell Research* 7: 103, 1954.
47. Lillie, F. R. *J. Morphol.* 22: 361, 1911.
48. Lillie, F. R. *J. Exptl. Zool.* 16: 523, 1914.
49. Lillie, F. R. *Problems of Fertilization.* Chicago: Univ. Chicago Press, 1919.
50. Lindahl, P. E. *Protoplasma* 16: 378, 1932.
51. Loeb, J. *Artificial Parthenogenesis and Fertilization.* Chicago: Univ. Chicago Press, 1913.
52. Lundberg, A. *Exptl. Cell Research* 9: 393, 1955.
53. Lundblad, G. *Arkiv Kemi* 7: 127, 1954.

54. Maggio, R. and A. Monroy. *Exptl. Cell Research* 8: 240, 1955.
55. McCullock, D. *Exptl. Cell Research* 3: 605, 1952.
56. McCullock, D. *J. Exptl. Zool.* 119: 47, 1952.
57. Metz, C. B. *Biol. Bull.* 89: 84, 1945.
58. Metz, C. B. *Anat. Rec.* 94: 347, 1946.
59. Metz, C. B. *Anat. Rec.* 96: 531, 1946.
60. Metz, C. B. *J. Exptl. Zool.* 105: 115, 1947.
61. Metz, C. B. *Am. Naturalist* 82: 85, 1948.
62. Metz, C. B. *Sex in Microorganisms.* Am. Assoc. Advan. Sci. Symp., 1954.
63. Metz, C. B. *Biol. Bull.* 107: 317, 1954.
64. Metz, C. B. and C. W. Birky, Jr. *Biol. Bull.* 109: 365, 1955.
65. Metz, C. B. and W. Butterfield. *Biol. Bull.* 101: 99, 1951.
66. Metz, C. B. and M. T. Foley. *Biol. Bull.* 93: 210, 1947.
67. Metz, C. B. and M. T. Foley. *J. Exptl. Zool.* 112: 505, 1949.
68. Metz, C. B. and E. M. Fusco. *Anat. Rec.* 101: 654, 1948.
69. Metz, C. B. and J. E. Donovan. *Science* 112: 2921, 1950.
70. Metz, C. B. and J. E. Donovan. *Biol. Bull.* 101: 202, 1951.
71. Metz, C. B. and J. B. Morrill, Jr. *Biol. Bull.* 109: 349, 1955.
72. Monroy, A. *Arch. neerl. zool.* 10: Suppl. 1, 18, 1953.
73. Monroy, A. *Pubbl. staz. zool. Napoli* 25: 188, 1954.
74. Moser, F. *J. Exptl. Zool.* 80: 423, 1939.
75. Moser, F. *J. Exptl. Zool.* 80: 447, 1939.
76. Motomura, I. *Sci. Rep. Tohoku Imp. Univ., Ser. 4,* 16: 345, 1941.
77. Motomura, J. *Sci. Rep. Tohoku Univ. Ser. 4* 18: 554, 1950.
78. Motomura, J. *Sci. Rep. Tohoku Univ. Ser. 4* 20: 93, 1953.
79. Newby, W. W. *Mem. Am. Phil. Soc.* 18, 1940.
80. Parpart, A. K. and P. C. Laris. *Biol. Bull.* 107: 301, 1954.
81. Parpart, A. K. and P. C. Laris. *Biol. Bull.* 109: 350, 1955.
82. Pasteels, J. *Arch. Biol.* 59: 405, 1948.
83. Popa, G. T. *Biol. Bull.* 52: 238, 1927.
84. Rothschild, Lord. *J. Exptl. Biol.* 26: 177, 1949.
85. Rothschild, Lord. *Biochem. Soc. Symp.* 7: 40, 1951.
86. Rothschild, Lord. *Biol. Rev.* 26: 1, 1951.
87. Rothschild, Lord. *J. Exptl. Biol.* 30: 57, 1953.
88. Rothschild, Lord. *Quart. Rev. Biol.* 29: 332, 1954.
89. Rothschild, Lord and M. M. Swann. *J. Exptl. Biol.* 26: 164, 1949.
90. Rothschild, Lord and M. M. Swann. *J. Exptl. Biol.* 27: 400, 1950.
91. Rothschild, Lord and M. M. Swann. *J. Exptl. Biol.* 28: 403, 1951.
92. Rothschild, Lord, and M. M. Swann. *J. Exptl. Biol.* 29: 469, 1952.
93. Rothschild, Lord and A. Tyler. *J. Exptl. Biol.* 31: 252, 1954.
94. Rothschild, Lord and A. Tyler. *Exptl. Cell Research Suppl.* 3: 304, 1955.
95. Runnstrom, J. *Protoplasma* 4: 388, 1928.
96. Runnstrom, J. *Arkiv. Zool.* 40A; No. 19, 1948.
97. Runnstrom, J. *Advances Enzymol.* 9: 241, 1949.
98. Runnstrom, J. *Exptl. Cell Research* 1: 304, 1950.
99. Runnstrom, J. *Symp. Soc. Exptl. Biol.* 6: 39, 1952.
100. Runnstrom, J. and G. Kriszat. *Exptl. Cell Research* 3: 419, 1952.
101. Runnstrom, J., L. Monné and L. Broman. *Arkiv. Zool.* 35A; No. 3, 1943.
102. Runnstrom, J. and L. Monné. *Arkiv Zool.* 36A: No. 18, 1945.
103. Runnstrom, J., F. Monné and E. Wicklund. *J. Colloid Sci.* 1: 421, 1946.

104. Scheer, B. T., A. Monroy, M. Santangelo and G. Riccobono. *Exptl. Cell Research* 7: 284, 1954.
105. Sonneborn, T. M. *Proc. Natl. Acad. Sci. U. S.* 23: 378, 1937.
106. Sonneborn, T. M. *Collecting Net* 14: 1, 1939.
107. Sonneborn, T. M. *Advances Genetics* 1: 263, 1947.
108. Spikes, J. D. *Biol. Bull.* 97: 95, 1949.
109. Sugiyama, M. *Biol. Bull.* 101: 335, 1951.
110. Sugiyama, M. *Biol. Bull.* 104: 210, 1953.
111. Tyler, A. *Biol. Bull.* 60: 187, 1931.
112. Tyler, A. *Proc. Nat. Acad. Sci. U. S.* 26: 317, 1939.
113. Tyler, A. *Biol. Bull.* 81: 190, 1941.
114. Tyler, A. *Proc. Soc. Exper. Biol. & Med.* 62: 107, 1946.
115. Tyler, A. *Physiol. Rev.* 28: 180, 1948.
116. Tyler, A. *Am. Naturalist* 83: 195, 1949.
117. Tyler, A. *Anat. Rec.* 113: 525, 1952.
118. Tyler, A. *Biol. Bull.* 104: 224, 1953.
119. Tyler, A. Gametogenesis, fertilization and parthenogensis. In: *Analysis of Development*, edited by B. H. Willier, P. A. Weiss and V. Hamburger. Philadelphia: Saunders, 1955, Sec. V, Ch. 1.
120. Tyler, A. and E. Atkinson. *Science* 112: 783, 1950.
121. Tyler, A. and H. Bauer. *Biol. Bull.* 73: 164, 1937.
122. Tyler, A. and C. B. Metz. *Biol. Bull.* 107: 321, 1954.
123. Tyler, A. and C. B. Metz. *Pubbl. staz. zool. Napoli.* 27: 128, 1955.
124. Tyler, A. and A. Monroy. *Biol. Bull.* 109: 370, 1955.
125. Tyler, A., A. Monroy, C. Y. Kao and H. Grundfest. *Biol. Bull.* 109: 352, 1955.
126. Tyler, A., A. Monroy and C. B. Metz. *Biol. Bull.* 110: 184, 1956.
127. Tyler, A. and K. O'Melveny. *Biol. Bull.* 81: 364, 1941.
128. Tyler, A. and Lord Rothschild. *Proc. Soc. Exper. Biol. & Med.* 76: 52, 1951.
129. Tyler, A. and J. Schultz. *J. Exptl. Zool.* 63: 500, 1932.
130. Wichterman, R. *Biology of Paramecium.* New York: Blakiston, 1953.
131. Wicklund, E. *Arkiv. Zool.* 42A: No. 11, 1949.
132. Wicklund, E. *Arkiv. Zool.* 7, Ser. 2, 97, 1954.
133. Wicklund, E. *Arkiv. Zool.* 7, Ser. 2, 117, 1954.

Onset and Termination of Insect Diapause[1]

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IN MANY INSECTS at a specific stage in their life history, development suddenly ceases and the insect enters a state of dormancy, called diapause, which enables it to over-winter or survive unfavorable conditions. The particular stage at which diapause occurs is characteristic for a species but may vary even in closely related forms. Whenever it intervenes during embryogenesis, larval or pupal life, it results in developmental arrest, and growth and molting cease. When it occurs in adult life, activity and the maturing of gametes are brought to a standstill. The end of diapause is signaled by the onset of development. Thus the diapausing insect possesses a mechanism for turning off and on the normal processes of growth and presents to the experimenter an exquisite example of natural growth regulation. Teleologically, the adaptive value of diapause is manifest; physiologically, its mechanism is still in doubt.

Although the end result of diapause in all cases is the cessation of growth, the physiological events that bring about embryonic diapause are very likely different from those acting in post-embryonic life. The notion of a unitary theory to explain all diapause appears wishful (but cf. 4, 18). In the present discussion we shall consider primarily post-embryonic diapause, more particularly the larval and pupal diapause of Hymenoptera and Lepidoptera. These forms are especially amenable to physiological and biochemical analysis and provide insight into general mechanisms underlying post-embryonic diapause. In these insects diapause is controlled by neurosecretory cells in the brain which release a tropic factor stimulating the prothoracic glands to secrete a growth hormone, PGH (48, 9). Normal nervous connections are unnecessary for neurosecretory activity, since implantation of isolated brain fragments containing neurosecretory cells is effective (50). When neurosecretion ceases, growth soon stops and diapause supervenes.

Three principal problems are outstanding in the study of diapause: *1*) what shuts off the neurosecretory activity of the insect's brain? *2*) what turns it on again? *3*) How does PGH react with the tissues to terminate diapause and initiate growth and molting? These questions bear directly on the more general questions of triggering of endocrine activity and of mitosis and the biochemical

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mechanism of a hormone action, subjects which are considered by Dr. Szego and Dr. Sawyer in this symposium.

Two insects have proven especially useful in attacking these problems the giant Cecropia silkworm, *Platysamia cecropia*, and the diminutive parasitic chalcid wasp *Mormoniella vitripennis*. In Cecropia, diapause is obligatory and occurs in each generation immediately after pupation. *Mormoniella*, by contrast, has a facultative larval diapause which occurs at the end of the last larval instar in occasional individuals.

Turning to the first question, what turns off neurosecretion in the brain and triggers the onset of diapause? We find a wealth of specific details but few generalizations. For example, in insects with facultative diapause, inactivation of the brain may be triggered by temperature, absolute or changing photoperiod, humidity, nutrition, etc. Thus in the parasitic wasp, *Tritneptis klugii*,[2] exposure of larvae to low temperature causes the larvae to enter diapause (19), while in the tomato moth, *Diataraxia oleracea*, short photoperiods during the last larval instar result in pupal diapause (45). Perhaps it is simplest to believe that an insect like Cecropia, which enters diapause in every generation, possesses a facultative diapause which is triggered by stimuli which are always present in the environment.

In some cases of diapause, notably where photoperiod is the stimulus, it is clear that the stimuli reach the brain via sensory pathways and in some manner influence neurosecretion (45, 46). In the obligatory diapause of Cecropia, however, there is evidence that nervous connections are unnecessary for inactivation of the brain after pupation (54, 44). We should note in this connection the important observation of Van der Kloot that in Cecropia the cessation of neurosecretion is accompanied by the disappearance of electrical activity in the entire brain, a finding which suggests that the factors that turn neurosecretion on and off are ". . . the same (factors) which regulate the activity of neurons" (43). Recognizing this fact, increasing attention is being given to the role of the rest of the nervous system in triggering neurosecretion.

It is not difficult to comprehend how various triggering stimuli might inhibit neurosecretory cells and cause diapause. But the true dimensions of this problem first reveal themselves when we recognize that in most insects the triggering stimulus acts at an early stage in the life cycle while diapause is not manifest until much later. For example, in *Mormoniella* exposure of female wasps to low temperature during oögenesis causes diapause in their offspring at the close of the last larval instar (29). There are numerous other examples in the literature (3). In cases where diapause appears in the final larval instar after having been triggered at an earlier stage it is clear that whatever the mechanism which inactivates the neurosecretory cells in the last instar, it has not interfered with

[2] Kindly supplied by Dr. A. Wilkes and Dr. J. C. Martin, Entomology Laboratory, Belleville, Ontario, Canada.

the normal functioning of these very same cells during the intermediate larval molts.

In only one case do we have a clue to how environmental stimuli cause delayed-action diapause, and this is in the embryonic diapause of the commercial silkworm. In a remarkable series of experiments Fukuda (11–15) and Hasegawa (17) showed that the subesophageal ganglion of the moth produces a 'diapause hormone' which causes the eggs in the ovaries to become 'diapause eggs,' i.e., embryos developing from these eggs enter diapause at the very young germ band stage. The production or liberation of this hormone by the subesophageal ganglion can be inhibited by the brain as long as the subesophageal connectives to the brain are intact. But it is important to note that whether the brain does so inhibit the subesophageal ganglion depends on the photoperiod and temperature experienced by the mother when she was an embryo. Thus we are back where we began: the brain is the key and in this case its activity in adult life is influenced by very specific happenings in embryonic life. The sins of the mothers are visited upon the offspring at least until the first generation.

Although this diapause factor for silkworm eggs has been found in the sub esophageal ganglion of many Lepidoptera, contrary to the opinion of Hinton (18) there is no real evidence that it induces diapause in anything except silkworm eggs and the mechanism may be peculiar to egg diapause. A preliminary report that this factor delayed the termination of pupal diapause in Cecropia was later withdrawn (20). However, the subesophageal ganglion may play some role in triggering neurosecretory activity for Harker (16) has just made the intriguing observation that diurnal activity rhythms of the cockroach are eliminated by removing the subesophageal ganglion and restored by implanting it.

In summary it seems fair to state that we understand little how triggering stimuli acting early in development have a delayed action in producing diapause. All we know with certainty is that in all cases that have been studied in detail the brain plays a central role.

The second question, what turns on the brain and thus terminates diapause, has proven more amenable to experimental analysis. In most insects diapause can be terminated by exposing the insect to low temperature for a period of weeks and then returning it to room temperature, whereupon the insect resumes its development (3). In the Lepidoptera and Hymenoptera there is cogent evidence that low temperature acts by rendering the insect's brain competent to secrete the hormone that stimulates the prothoracic glands (48, 52, 9). This action of low temperature on the brain is independent of connections between the brain and the rest of the central nervous system; for if an isolated brain is implanted into a pupa, and the pupa chilled, the implanted brain is rendered competent for neurosecretion (54). The biochemical meaning of the term 'renders competent' has not been determined, and to attack this question the following rather simple experiments were performed with diapausing larvae

of the wasp *Mormoniella*.[3] At temperatures above 15°C, these larvae will remain in permanent diapause until death ensues after a year or more. However, if at any time the larvae are exposed to temperatures below 15°C for several months, and then returned to higher temperatures, diapause ends and the larval-pupal and pupal-adult transformations ensue. As has been observed in many other species (27, 3), as the period of exposure to low temperature increases, upon return to higher temperature diapause terminates more rapidly and a larger percentage of the chilled larvae eventually develop. The most effective chilling temperature is about 5°C; lower and higher temperatures are less effective and, as already noted, temperatures above 15°C are totally ineffective.

The first series of experiments were of the type familiar to an earlier generation of plant physiologists studying vernalization. *Mormoniella* larvae, 2–4 weeks after they had entered diapause, were exposed to alternating periods at 5°C and 25°C according to the following schedule:

a) 5°C continuously
b) 5°C for 7 days, 25° for 1 day, 5° for 7 days, etc.
c) 5°C for 7 days, 25° for 2 days, 5° for 7 days, etc.
d) 5°C for 7 days, 25° for 4 days, 5° for 7 days, etc.
e) 5°C for 7 days, 25° for 7 days, 5° for 7 days, etc.
f) 5°C for 2 days, 25° for 2 days, 5° for 2 days, etc.

After a cumulative exposure to 5° for *n* weeks, 60 animals were removed from each set of conditions, placed at 25° and their development observed. The results, summarized in figure 1, reveal that the effects of sub-threshold chilling in terminating diapause can be undone by warming. Two days at 25°C undid a great deal of the effects of 7 days at 5°, while 7 days at 25° completely undid the effects of 7 days at 5° (31).

These data are consistent with the hypothesis that at low temperature the insect's brain synthesizes and accumulates a substance which, when it reaches threshold concentration, enables the brain when returned to high temperature to function as an endocrine organ and release the brain hormone. This substance is apparently broken down at high temperature, and it may be the brain hormone itself. Chilling at temperatures above 15°C never terminates diapause, and we may conclude that at temperatures above 15° the rate of the breakdown reaction exceeds the rate of the synthetic reaction, so that the substance never accumulates to threshold concentrations; below 15° the synthetic reaction outstrips the breakdown reaction and the substance accumulates to threshold levels. In other words, the temperature coefficient (Q_{10}) of the synthetic reaction is less than that of the breakdown reaction, and at about 15°C the rates of the two reactions are equal (cf. 2, 3, 27, 48; 41, pp. 191–196).

If this hypothesis is correct, and low temperature acts by effectively pro-

[3] Kindly supplied by Professor Phineas T. Whiting of the University of Pennsylvania.

moting some synthetic reaction in the brain, this synthetic reaction—presumably requiring energy—ought to be inhibited when the insect's metabolism is decreased. An effective method of inhibiting metabolism during low temperature exposure is to chill animals anaerobically. To this end, groups of 40 diapausing *Mormoniella* larvae were enclosed in chambers containing pure nitrogen and an oxygen absorbent (10) and were maintained in this anaerobic state at 5°C for periods up to 16 weeks. Parallel control groups were maintained in air at 5°C. After treatment, the larvae were returned to air and placed at 25°C along with the controls.

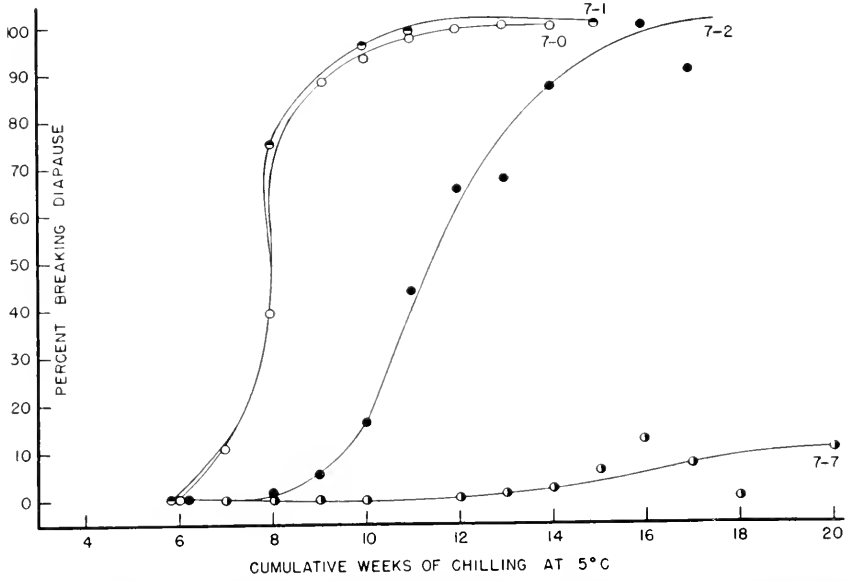

FIG. 1. Effects of alternating periods of chilling and warming in terminating larval diapause in *Mormoniella*. Data for *7-4* are substantially the same as those for *7-2*; data for *2-2* are substantially the same as those for *7-7*. For further details see text.

Prolonged anaerobiosis at low temperature had little effect on the viability of the animals, and within a day of being placed in air at 25°C they regained their motility. Although they remained alive and apparently healthy for many months, not one of the anaerobically-chilled larvae ever pupated, while the air-chilled control groups broke diapause and developed normally. Thus anaerobiosis prevented chilling from terminating diapause. In terms of our hypothesis, the simplest explanation is that the synthetic reaction occurring at low temperature requires oxidative metabolism. There was no permanent damage to the endocrine system of the anaerobic animals for, when subsequently rechilled aerobically, they initiated development and developed normally.

These results do not tell us the nature of the synthetic reaction in the insect's brain, nor of the breakdown reaction. However, they do indicate that low temperature renders the brain competent to secrete the brain hormone by slowing down some breakdown reaction within the brain, thereby enabling the brain to accumulate a substance which it synthesizes via oxidative metabolism. This substance is necessary for the production of the brain hormone or is, perhaps, itself the brain hormone. Possibly the substance synthesized is the cholinergic material which Van der Kloot (43) reports increases in the Cecropia pupal brain after chilling; and perhaps the breakdown reaction is the hydrolysis of this material.

Recently Andrewartha (3), and Andrewartha and Birch, (4) proposed that low temperature acted by rendering intractable food reserves laid down in the fat body or egg yolk available to the insect, and that this was a necessary step in triggering neurosecretion and represented the action of low temperature in terminating diapause. The experimental results just presented are not consistent with this suggestion, since the effects of chilling are reversed by warming.

A more direct approach to the action of low temperature on the brain would be to chill brains *in vitro*, and unequivocally demonstrate a direct action of low temperature on the brain itself. This prospect is now feasible, for we have recently succeeded in maintaining isolated brains of pupal silkworms alive in hanging drop preparations at 5°C for as long as 2 months, using a modification of the tissue culture medium devised by Morgan, Morton and Parker (23). Whether or not brains chilled in this manner are rendered competent for neurosecretion remains to be seen.

Let us now turn to the final question: the mechanism of action of PGH. In all insects that have thus far been studied, PGH triggers cell enlargement, mitosis and secretion of a new cuticle. Although its action in eggs and embryos is yet to be demonstrated, it very likely is involved in embryogenesis (cf. 49). Exposing a diapausing insect to the hormone effects the immediate termination of diapause and initiation of development. The biochemical mechanism whereby PGH triggers this complex biological end-result has been examined in greatest detail in the Cecropia silkworm by comparing diapausing pupae with developing adults whose tissues have been exposed to PGH.

The diapausing pupa exhibits two striking physiological characteristics; namely, the complete absence of cell division and an exceedingly low metabolic rate (12 μl/gm/hr. at 25°C). Not only is mitosis normally absent, but the diapausing cells do not divide even when repairing extensive injury. Thus, although pupae heal completely following excision of as much as one-fourth of their epidermis, no mitosis occurs. Healing is accomplished solely by cell enlargement and migration. In the absence of adequate concentration of PGH the pupa spreads itself thin to heal its wounds (39).

The exceedingly low metabolism of the diapausing pupa (less than $\frac{1}{10}$ that of the mature larva and $\frac{1}{20}$ that of the adult moth), has led to the frequent

suggestion that diapause is caused by a quantitative deficiency in energy-yielding enzyme systems, a deficiency which is repaired in some way by PGH. Convincing evidence to the contrary is provided by the curious phenomenon of injury metabolism (33, 34). The simple insertion and withdrawal of a fine hypodermic needle in the diapausing pupa evokes a doubling or tripling of the oxygen consumption within 24 hours. After more extensive integumentary injury, metabolism increases 6- to 14-fold and requires more than 2½ months to return to initial levels. Injury metabolism is unattributable to enhanced muscular activity, or to reactions requiring the participation of either the central nervous system or the insect's endocrine system as presently defined, for surgically isolated anterior and posterior halves of pupae, and pupae from which the entire central nervous system has been extirpated exhibit an injury respiration. Nor can injury metabolism be correlated with localized repair of the wound, for the high metabolism persists for months after repair is apparently completed. It seems to be triggered by substances released at the site of injury, for injury to one member of a parabiotic pair causes an almost equal rise in the metabolism of both animals (30). The injury metabolism is peculiar to the diapausing pupa. It cannot be demonstrated in freshly pupated animals, in animals after the initiation of adult development or in non-diapausing species like the bee moth *Galleria melonella* at any stage (34, 40).

For the present discussion it is of special importance that, though the metabolism of the diapausing pupa can be increased by injury to levels characteristic of the post-diapausing insect midway in adult development, no development takes place. Thus the diapausing pupa, while capable of respiring at rates that characterize the growing insect, fails to grow in the absence of the proper hormonal stimulus of the prothoracic glands and the proper quality of metabolism. It appears that the increment of respiration induced by injury cannot be coupled to morphogenesis. The injury-stimulated respiration gives assurance that the absence of morphogenesis during diapause is not attributable to a simple *quantitative* deficiency in the dehydrogenase enzymes which release hydrogen from substrate or the redox enzymes which transmit the hydrogen to oxygen. Nor is it due to an inability to form sufficient high energy bonds, for Telfer and Williams (42) have recently shown that the incorporation of C^{14}-labeled glycine into pupal proteins is stimulated by injury to about the same degree as respiration. Since the incorporation of amino acids into pupal proteins most likely requires the same kinds of high energy phosphate and sulfur bonds as are used in adult development, this finding reemphasizes the qualitative nature of the energetic defect in the diapausing pupa.

Since it appears to be quality rather than quantity of metabolism that distinguishes the diapausing pupa from the developing adult, let us examine more closely the metabolic differences between the two. It has long been recognized that the over-all gas exchange of diapausing pupae is peculiar in that exceed-

ingly low respiratory quotients (R.Q.) are commonly recorded, especially at low temperatures. Indeed, in our own experience, continuous measurements of oxygen uptake and carbon dioxide output of Cecropia pupae at 10°C over as long as 4 days often yielded R.Q.'s as low as 0.1. These low R.Q.'s have formed the basis of various theories of diapause, most recently the theory of Agrell (1), which held that diapause was due to a defect in the enzyme systems responsible for decarboxylation, more particularly a deficiency of thiamine necessary for cocarboxylase. Under this view, PGH repaired the defect in the decarboxylase system.

In our efforts to understand these low R.Q's we soon became aware of their remarkable origin. While the pupa consumes oxygen continuously, it stores most of its metabolic carbon dioxide and releases it in brief 'bursts' which in Cecropia at 10°C occur every 3 to 7 days (25, 28, 33, 37). When this discontinuous release of carbon dioxide is taken into account in respiratory measurements, the R.Q. of the pupa regains normal proportions, namely 0.78.

Since the over-all pattern of gas exchange of the diapausing insect reveals no qualitative peculiarities, let us examine the pathways of intermediary metabolism. It had been observed more than 20 years ago by Bodine and Boell (6, 7, 26) that in the eggs of the grasshopper *Melanoplus differentialis* pronounced alterations in the cytochrome system occurred in synchrony with the termination of diapause. For this reason attention focuses on the role of terminal oxidases in relation to the action of PGH (cf. 47, 51).

The principal terminal oxidases which have been demonstrated in animals are cytochrome *c* oxidase, cytochromes of the *b* type, flavoproteins and tyrosinase. An effective method of detecting the participation of the various terminal oxidases in respiration and in physiological processes like growth is the use of metabolic inhibitors, especially cyanide, phenylthiourea and carbon monoxide. Cyanide inhibits cytochrome oxidase, catalase, peroxidase and tyrosinase, while phenylthiourea inhibits only tyrosinase. In insects where hemoglobins are absent, carbon monoxide inhibits both cytochrome oxidase and tyrosinase but apparently fails to inhibit any other enzymes or substrates. Carbon monoxide's inhibition of cytochrome oxidase is reversed by light, its inhibition of tyrosinase is not, and it therefore affords a remarkably specific tool for tracking the participation of the cytochrome oxidase system in biological reactions.

The effects of carbon monoxide on cytochrome oxidase are reversed by oxygen, that is, inhibition depends on the CO/O_2 ratio. A ratio of 16:1 inhibits about 70 per cent, while 25:1 inhibits 85 per cent. To achieve such ratios without lowering the oxygen pressure to a point where it limits respiration, respiratory measurements were performed at positive pressures. As Paul Bert (5) long ago showed, gaseous pressure per se is without conspicuous effects on insects unless it is exceedingly high. Animals were enclosed in a specially designed high

pressure respirometer and their oxygen uptake recorded in air upon which 5 atmospheres of CO had been superimposed. Occasional experiments were performed at atmospheric pressure.

From a large number of inhibition experiments we learned that the respiration of the diapausing pupa is completely insensitive to phenylthiourea, and is inhibited less than 15 per cent by high concentrations of carbon monoxide ($CO/O_2 = 25:1$) or cyanide (10^{-3} M). This minor inhibition was accounted for in terms of the cyanide- and carbon monoxide-sensitivity of the intersegmental muscles of the pupal abdomen. The other tissues of the insect, or at any rate those that contribute more than a trivial amount to the overall respiration, were not inhibited (35). Indeed, simultaneous exposure of pupae to both 10^{-3} M cyanide and 16:1 CO/O_2 had only trivial effects on respiration although this maneuver certainly left less than 5 per cent of the cytochrome *c* oxidase system functioning (29).

The situation in the developing adult is quite different. The termination of the pupal diapause and the progress of adult development are accompanied by a marked increase in respiratory sensitivity to cyanide and carbon monoxide. The effect of these agents becomes no longer limited to the muscular tissue but extends to the insect as a whole. Cyanide and carbon monoxide appear to act exclusively on the extra metabolism accompanying development and reduce the over-all metabolism to the old diapausing level (35).

The onset of diapause immediately after pupation is accompanied by a reversal of the above events: 6 hours after pupation, 30–40 per cent of the respiration is inhibited by 16:1 CO/O_2; 48 hours after, 20 per cent is inhibited, as the insect gradually loses the carbon monoxide- and cyanide-sensitive system.

The insensitivity to carbon monoxide and cyanide in most tissues of the diapausing pupa argues in favor of the presence and utilization of a terminal oxidase other than cytochrome oxidase or tyrosinase. Cytochrome oxidase is the principal terminal oxidase of the somatic musculature of the diapausing pupa. Months later, with the termination of the pupal diapause, cytochrome oxidase becomes the principal terminal oxidase of the growing post-diapausing insect as a whole. Cytochrome oxidase is likewise the principal terminal oxidase of the growing larva and prepupa and gradually disappears from most tissues of the insect after pupation and the onset of diapause.

These qualitative changes in the insect's metabolism from cytochrome oxidase to non-cytochrome oxidase and back to cytochrome oxidase are synchronized with the secretion of PGH. When PGH titer falls at the time of pupation the cytochrome oxidase system disappears within a week. When PGH titer rises at the onset of adult development the system reappears. However, these observations in themselves provide only circumstantial evidence that the coupling of metabolism to cytochrome function is causally related to PGH action and its biological end result, the termination of diapause and the initiation of devel-

opment. Part of this problem is morphogenetic in character and demands solution in morphological terms. Is the change in terminal oxidase coincidental, or is there an obligatory coupling between the function of the cytochrome system and the actual development of the insect? A partial answer to this question is provided by observations on the effects of carbon monoxide on the growth of Cecropia.

As in the respiration experiments, carbon monoxide's effects on growth were appraised at positive pressures in transparent pressure chambers. The results revealed that embryos, larvae and adults were killed by less than 5 days' exposure to 20:1 CO/O_2. Diapausing pupae, by contrast, survived at least 21 days' exposure to CO/O_2 as high as 33:1. This was not simply due to the pupa's ability to withstand anoxia, since the maximum oxygen debt that the pupa could sustain was about 1000 μl/gm live weight, and more than 3 days' exposure to pure nitrogen was lethal. Nor was this CO-resistance characteristic of the pupal stage generally, for non-diapausing pupae like *Galleria* were killed by carbon monoxide (32, 36, 40).

The ability to survive in the presence of carbon monoxide persists throughout the early stages of adult development. When a developing postdiapausing Cecropia was exposed to suitable pressures of carbon monoxide, development immediately ceased, but the animal continued to live. Carbon monoxide, in effect, enforced an artificial diapause. As soon as the animal was removed from carbon monoxide it resumed its development where it had left off and produced an essentially normal adult moth. From this experiment we learn that carbon monoxide inhibits an enzyme necessary for growth but apparently not for the maintenance of the insect. This carbon monoxide-sensitive enzyme could be cytochrome oxidase or tyrosinase.

Conclusive proof that cytochrome oxidase is the target of carbon monoxide was provided by reversing its inhibition of adult development with light (36). Animals that had just initiated adult development had the opaque cuticle overlying the anlagen of the genitalia removed and replaced by transparent plastic windows. They were then compressed with carbon monoxide (CO/O_2 = 20:1) and half the animals were maintained in darkness while half had their windows illuminated. After 5 days the animals were compared with controls kept in air. The illuminated genitalia had begun developing into adult organs, while the unilluminated animals had not progressed. This difference was particularly striking at the anterior and posterior ends of the illuminated animals: the illuminated gentalia showed considerable progress in development, whereas the unilluminated facial region showed no morphological advance (cf. 55).

These experiments assure us that the target of carbon monoxide in our studies is cytochrome oxidase and that the absence of growth is due to specific inhibition of cytochrome oxidase. Moreover, the experiments show that carbon monoxide and light afford a remarkable tool for the student of growth. With a

beam of light one can turn on growth in a group of cells, while unilluminated neighboring cells are prevented from growing.

The data just considered amplify the respiration studies and reveal systematic shifts from a cytochrome oxidase system to a non-cytochrome oxidase system during development. The loss or inactivation of the carbon monoxide-sensitive cytochrome oxidase system at the time of pupation is compensated by the development or activation of a carbon monoxide- and cyanide-stable respiratory system capable of underwriting the maintenance requirements of the diapausing insect. The identity and intracellular location of the terminal oxidase in the diapausing pupa is still uncertain. The most likely candidates are an autoxidizable flavoprotein transferring electrons from reduced pyridine nucleotides to oxygen or an autoxidizable cytochrome of the *b* type (35, 24).

The reappearance of a functioning cytochrome oxidase system at the outset of development is necessary for the continuation of development because this enzyme system plays an obligatory role in the energetics of the insect's development. The absence of all but a trace of a complete cytochrome oxidase system in the diapausing pupa therefore assumes special significance. Since the presence and function of this system appear to be prerequisite for adult development, its virtual absence in the dormant pupa can in itself account for the developmental standstill of diapause. These facts persuade us that the reactivation of the cytochrome oxidase system is among the biochemical changes set in motion by PGH, changes which couple endocrine action to the termination of the pupal diapause.

How does PGH reactivate the cytochrome oxidase system in the pupal tissues? Recently Shappirio (38) has demonstrated by sensitive spectro-photometric techniques that the absence of a functional cytochrome oxidase system within the epidermis of the diapausing pupa is due to the virtual absence within the diapausing cells of cytochromes *b* and *c*, a finding which narrows down the defective cytochrome oxidase system of the diapausing pupa to the absence of these two molecules (though of course other biochemical defects, as yet undefined, may exist). In some way PGH effects the resynthesis of cytochromes *b* and *c*.

It appears unlikely that we shall get answers to this problem until extensive studies are made with the pure hormone on isolated tissues, cell fractions and enzyme preparations. This objective is now at hand, since Karlson and Butenandt at the Max Planck Institute in Tübingen have recently isolated and crystallized PGH (8, 20). The hormone has the elementary formula $C_{18}H_{30}O_4$ and its spectrum suggests that it may be an unsaturated ketone. Injection of this material to a final concentration of 10 parts per million induces development in isolated pupal abdomens of Cecropia (53, 20). The material is still available only in microgram quantities, but already attempts have been made to demonstrate an *in vitro* effect (20–22). Thus far, like most attempts with other hormones, they have failed. However, it is clear that it is from this point that future work will proceed.

In summary, then, we see that while none of the problems of the triggering of diapause are solved, some of them at least have been pursued to the molecular level. And it is of course for explanations on the molecular level that general physiology seeks.

ADDENDUM

Since this paper was submitted for publication there have appeared a significant monographic treatment of diapause (22a) and a detailed review of the chemistry of insect hormones (20a). Also, Van der Kloot has described striking electrophysiological and biochemical differences between the brains of diapausing and non-diapausing pupal silkworms (44a), and Jones has shown that PGH causes embryonic molting in Orthoptera (19a). In addition, further experimental analysis of the 'synthesis-breakdown' hypothesis of diapause termination by low temperature has shown that both the synthetic reaction and the breakdown reaction are aerobic (20a).

REFERENCES

1. Agrell, I. Pupal diapause caused by a vitamin deficiency. *Nature* 167: 283, 1951.
2. Andrewartha, H. G. Diapause in eggs of Austroicetes cruciata Sauss., with special reference to the influence of temperature on elimination of diapause. *Bull. Entomol. Research* 34: 1–17, 1943.
3. Andrewartha, H. G. Diapause in relation to the ecology of insects. *Biol. Rev.* 27: 50–107, 1952.
4. Andrewartha, H. G. and L. C. Birch. *Distribution and Abundance of Animals.* Chicago: Univ. Chicago Press, 1954.
5. Bert, P. *La Pression Barometrique: Recherches de Physiologie Experimentale* (translated by M. A. and F. A. Hitchcock). Columbus, Ohio: College Book Co., 1943.
6. Bodine, J. H. and E. J. Boell. Carbon monoxide and respiration. Action of carbon monoxide on respiration of normal and blocked embryonic cells (Orthoptera). *J. Cell & Comp. Physiol.* 4: 475–482, 1934.
7. Bodine, J. H. and E. J. Boell. Respiratory mechanisms of normally developing and blocked embryonic cells (Orthoptera). *J. Cell. & Comp. Physiol.* 5: 97–113, 1934.
8. Butenandt, A. and P. Karlson. Uber die Isolierung eines Metamorphosehormons der Insekten in kristalliesierter Form. *Z. Naturforsch.* 9b: 389–391, 1954.
9. Church, N. S. Hormones and the termination and reinduction of diapause in *Cephus cinctus* Nort. *Canad. J. Zool.* 33: 339–369, 1955.
10. Fieser, L. A new absorbant for oxygen in gas analysis. *J. Am. Chem. Soc.* 46: 2639–2647, 1924.
11. Fukuda, S. Production of the diapause eggs by transplanting the subesophageal ganglion in the silkworm. *Proc. Japan. Acad.* 27: 672–677, 1951.
12. Fukuda, S. Function of pupal brain and subesophageal ganglion in the production of non-diapause and diapause eggs in the silkworm. *Annot. Zool. Jap.* 25: 149–155, 1952.
13. Fukuda, S. Determination of voltinism in the univoltine silkworm. *Proc. Japan Acad.* 29: 381–384, 1953.
14. Fukuda, S. Determination of voltinism in the multivoltine silkworm. *Proc. Japan Acad.* 29: 385–388, 1953.
15. Fukuda, S. Alteration of voltinism in the silkworm following transection of pupal oesophageal connectives. *Proc. Japan Acad.* 29: 389–391, 1953.
16. Harker, J. E. Control of diurnal rhythms of activity in Periplaneta americana L. *Nature* 175: 733, 1955.
17. Hasegawa, K. Studies in voltinism in the sikworm, *Bombyx mori* L., with special refer-

ence to the organs concerning determination of voltinism (a preliminary note). *Proc. Japan Acad.* 27: 667–671, 1951.

18. Hinton, H. E. The initiation, maintenance and rupture of diapause: a new theory. *The Entomologist* 86: 279–291, 1953.
19. Horwitz, J. and H. A. Schneiderman. Unpublished observations.
19a. Jones, B. M. Endocrine activity during insect embryogenesis. Function of the ventral head glands in locust embryos (*Locustana pardalina* and *Locusta migratoria*, Orthoptera). *J. Exper. Biol.* 33: 174–185, 1956.
20. Karlson, P. Biochemische Probleme der Insektenmetamorphose. *Verhandl. deut. Zool. Tubingen* 68–85, 1954.
20a. Karlson, P. Biochemical studies on insect hormones. *Vitamins and Hormones* 14: 228–266, 1956.
21. Karlson, P. and H. Schmid. Über die Tyrosinase der Calliphora-Larven. *Hoppe-Seyler's Z. physiol. Chemie* 300: 35–41, 1955.
22. Karlson, P. and E. Wecker. Die Tyrosinaseaktivität der Puppariumbildung von Calliphora erythrocephala. *Hoppe-Seyler's Z. physiol. Chemie* 300: 42–48, 1955.
22a. Lees, A. D. *The Physiology of Diapause in Arthropods.* London: Cambridge, 1955.
23. Morgan, J. F., H. J. Morton and R. C. Parker. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. *Proc. Soc. Exper. Biol. & Med.* 73: 1–8, 1950.
24. Pappenheimer, A. M., Jr. and C. M. Williams. Cytochrome b_5 and the dihydrocoenzyme I-oxidase system in the Cecropia silkworm. *J. Biol. Chem.* 209: 915–929, 1954.
25. Punt, A. The respiration of insects. *Physiol. Comparata et Oecol.* 2: 59–74, 1950.
26. Robbie, W., E. J. Boell and J. H. Bodine. A study of the mechanism of cyanide inhibition. I. Effect of concentration on egg of *Melanophus differentialis*. *Physiol. Zool.* 11: 54–62, 1938.
27. Salt, R. W. Some effects of temperature on the production and elimination of diapause in Cephus cinctus. *Canad. J. Res., D* 25: 66–86, 1947.
28. Schneiderman, H. A. Discontinuous release of carbon dioxide by diapausing pupal insects. *Anat. Rec.* 117: 540, 1953.
29. Schneiderman, H. A. Unpublished observations.
29a. Schneiderman, H. A., J. Horwitz and C. Kurland. An analysis of the action of low temperature in terminating the diapause of Mormoniella. *Anat. Rec.* 125: 557, 1956.
30. Schneiderman, H. A. and A. Jankowitz. Unpublished observations.
31. Schneiderman, H. A. and R. W. Risebrough. Unpublished observations.
32. Schneiderman, H. A. and C. M. Williams. Terminal oxidases in diapausing and non-diapausing insects. *Anat. Rec.* 113: 55–56, 1952.
33. Schneiderman, H. A. and C. M. Williams. Respiratory metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.* 105: 320–334, 1953.
34. Schneiderman, H. A. and C. M. Williams. Metabolic effects of localized injury to the integument of the Cecropia silkworm. *Anat. Rec.* 117: 640–641, 1953.
35. Schneiderman, H. A. and C. M. Williams. Qualitative changes in the metabolism of the Cecropia silkworm during diapause and development. *Biol. Bull.* 106: 210–229, 1954.
36. Schneiderman, H. A. and C. M. Williams. The cytochrome oxidase system in relation to the diapause and development of the Cecropia silkworm. *Biol. Bul.* 106: 238–252, 1954.
37. Schneiderman, H. A. and C. M. Williams. Experimental analysis of the discontinuous respiration of the Cecropia silkworm. *Biol. Bull.* 109: 123–143, 1955.
38. Shappirio, D. G. Cytochrome system of the Cecropia silkworm in relation to diapause and adult development. *Anat. Rec.* 120: 731–732, 1954.

39. SMITH, R. D. AND H. A. SCHNEIDERMAN. Healing of epidermal wounds in diapausing pupae of the silkworm. *Anat. Rec.* 120: 724–725, 1954.
40. SMITH, R. D. AND H. A. SCHNEIDERMAN. Unpublished observations.
41. SONNEBORN, T. M. Beyond the gene—two years later. *Science in Progress* 7: 167–202, 1951.
42. TELFER, W. H. AND C. M. WILLIAMS. Incorporation of radioactive glycine into the blood proteins of the Cecropia silkworm. *Anat. Rec.* 122: 441–442, 1955.
43. VAN DER KLOOT, W. G. Neurosecretion and brain activity in the Cecropia silkworm. *Anat. Rec.* 120: 718–719, 1954.
44. VAN DER KLOOT, W. G. Unpublished observations.
44a. VAN DER KLOOT, W. G. Control of neurosecretion and diapause by physiological changes in the brain of the Cecropia silkworm. *Biol. Bull.* 109: 276–294, 1955.
45. WAY, M. J. AND B. A. HOPKINS. Influence of photoperiod and temperature on the induction of diapause in Diataraxia oleracea L. (Lepidoptera). *J. Exper. Biol.* 27: 365–376, 1950.
46. WIGGLESWORTH, V. B. *The Physiology of Insect Metamorphosis.* London: Cambridge, 1954.
47. WILLIAMS, C. M. The endocrinology of diapause. *Bull. biol. France et Belg.* Suppl. 33: 52–56, 1947.
48. WILLIAMS, C. M. Physiology of insect diapause. II. Interaction between the pupal brain and prothoracic glands in the metamorphosis of the giant silkworm, *Platysamia cecropia*. *Biol. Bull.* 93: 89–98, 1947b.
49. WILLIAMS, C. M. Physiology of insect diapause. III. The prothoracic glands in the Cecropia silkworm, with special reference to their significance in embryonic and post-embryonic development. *Biol. Bull.* 94: 60–65, 1948.
50. WILLIAMS, C. M. Extrinsic control of morphogenesis as illustrated in the metamorphosis of insects. *Growth Symp.* 12: 61–74, 1948.
51. WILLIAMS, C. M. Biochemical mechanisms in insect growth and metamorphosis. *Federation Proc.* 10: 546–552, 1951.
52. WILLIAMS, C. M. Physiology of insect diapause. IV. The brain and prothoracic glands as an endocrine system in the Cecropia silkworm. *Biol. Bull.* 103: 120–138, 1952.
53. WILLIAMS, C. M. Isolation and identification of the prothoracic gland hormone of insects. *Anat. Rec.* 120: 743, 1954.
54. WILLIAMS, C. M. Unpublished observations.
55. WOLSKY, A. Production of local depressions in the development of Drosophila pupae. *Nature* 139: 1069–1070, 1937.

Initiation of Nerve Impulses in Cochlea and Other Mechano-Receptors[1]

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FROM THE POINT OF VIEW of a neurophysiologist who is particularly interested in sense organs, the term 'trigger action' might apply to two or three rather different aspects of the activity of the cochlea. It might apply to the release of patterns of activity of the organism as a whole which are 'triggered' by some particular sound. Specific vigorous reactions to sensory stimulation by sight, sound or smell are certainly commonplace in biology, and certainly the sense organs are the pathway whereby the information that sets off the specific reactions reaches the central nervous system from the external world.

At a much simpler level, the level of general physiology, the term 'trigger action' applies very aptly to the stimulation of a nerve impulse by electrical, chemical, mechanical or any other means. Here, too, a response that involves the liberation of energy by the stimulated tissue runs a well-defined, predetermined course. It is initiated by a stimulus, or 'trigger.' In this particular instance, the reaction is highly stereotyped. Also, many different stimuli can be used, although all of them share the requirement that a certain threshold value of stimulation must be produced if triggering is to occur.

This type of 'trigger action' occurs in sense organs, and we shall examine one of them in some detail to see how mechanical vibrations can serve to set off nerve impulses. We shall be dealing here with trigger action at the level of the cell rather than at the level of the organism. Both levels of trigger action have in common the release by the organism or cell of more energy than was provided by the stimulus. A stereotyped pattern ensues, and the sequence of events is then determined by conditions other than those which constituted the original trigger.

The term 'trigger action' certainly implies, although perhaps it does not require, that the amount of energy involved in the response shall be large relative to the amount of energy required to pull the trigger. Such a relation is very clear in the contraction of a muscle when stimulated by either a motor nerve impulse or an electric shock. It is rather less evident in the case of a single

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sensory nerve impulse, although it becomes more apparent if the stimulus to the nerve is near threshold and if the nerve impulse sets off nervous reactions beyond the first synapse.

In the sense organs the amount of energy in the original stimulus may be very small, and of the same order of magnitude as the work done in exciting the sensory nerve fibers. We know that each sense organ provides a differential sensitivity to a particular form of energy, and usually the absolute sensitivity at threshold is very great. As long as sufficient energy is available in the stimulus, as in the stretch receptors of our muscles, the only question seems to be just how the energy is applied to the nerve in a way that will excite without causing in-

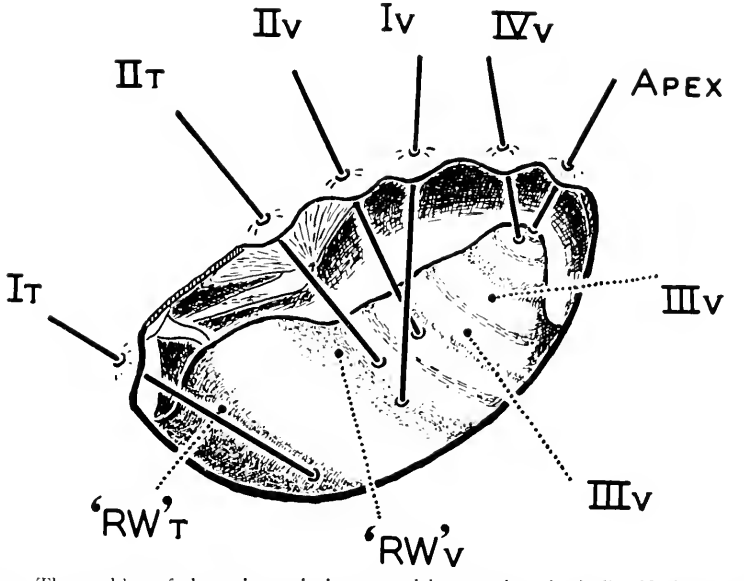

FIG. 1. The cochlea of the guinea pig is exposed by opening the bulla. Various positions for intracochlear electrodes are shown. The wires are attached to the edge of the bone with dental cement. The positions IT and Iv are shown in the cross section drawing in figure 3.

jury. The case becomes rather more interesting, however, if it can be shown that the amount of energy available in the stimulus is probably inadequate to do the physical work necessary to excite a nerve impulse. In the case of the eye, the ear and probably the organs of chemical sense, the stimuli near threshold are so weak that an additional second-order trigger action of some sort can safely be inferred. By this, we mean that a mechanism of some sort must be interposed between the physical or chemical stimulus and the nerve fiber to amplify the energy of the stimulus to make possible stimulation of the nerve. We shall examine the inner ear with reference to this possibility as an example of a particularly delicate trigger mechanism.

The cochlea represents the most sensitive of the general class of mechanoreceptors. At the same time, its anatomy makes it accessible, although with some difficulty, for the study of its mechanical, electrical and neural events. In the guinea pig we are particularly fortunate in finding the cochlea of the inner ear projecting into a large tympanic bulla with only a thin covering of bone, like a snail's shell, between the empty space of the bulla and the inner sense organ itself. Figure 1 shows how holes may be drilled through this bony shell. One or more pairs of electrodes in the form of very fine nichrome steel wires, insulated except at the tips, may be introduced into the various parts of the inner ear

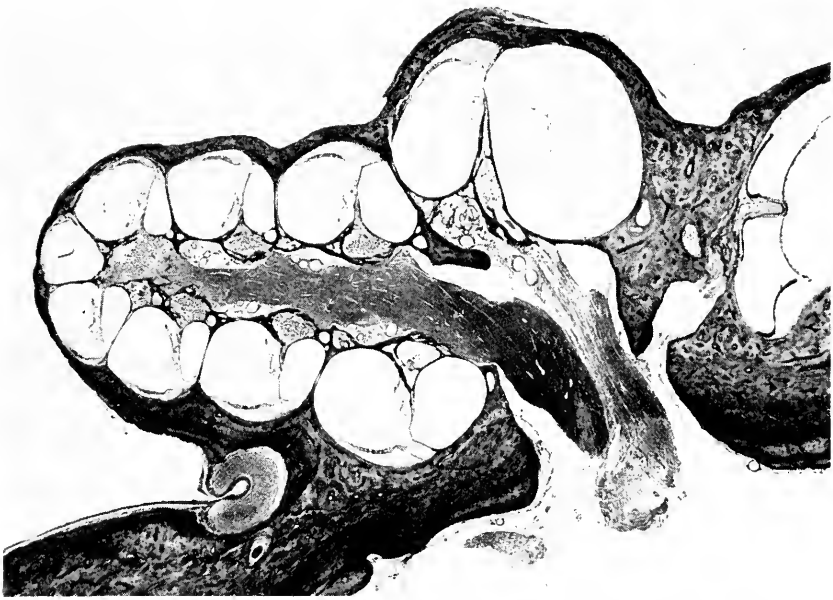

FIG. 2. Mid-modiolar section of a guinea pig cochlea, showing the auditory nerve. In this particular specimen the nerve cells of the spiral ganglion and the organ of Corti in Turn I have degenerated.

Larger holes can also be drilled for the insertion of micropipettes with which to withdraw or inject fluids or to measure DC potentials. It is even possible to introduce hyperfine pipette-electrodes into the cells of the sense organ themselves, and record their electrical potentials with very little damage to the tissue. Figure 2 shows an actual cross section of the cochlea, and figure 3 shows diagrammatically the introduction of two wire electrodes and one pipette electrode into the scala vestibuli, the scala tympani and scala media, respectively, of the first turn and the placement of a reference electrode on the tissues of the neck. It also shows how the AC potential known as the 'cochlear microphonic' appears across the organ of Corti between scala media and scala tympani, and

also shows that the less well known 'summating potential' appears in the scala media. Both of these potentials occur in response to stimulation by sound.

Let us first dismiss the summating potential. This response is best described as a change in the resting DC potential of the scala media. In its amplitude and time course it looks like a full-wave rectification and running integration of the acoustic stimulus. It appears only at relatively high sound levels and it continues to increase in amplitude at levels well above that at which the cochlear microphonic reaches its maximum. The summating potential is thus a high-intensity response. It may have an origin and function broadly similar to the

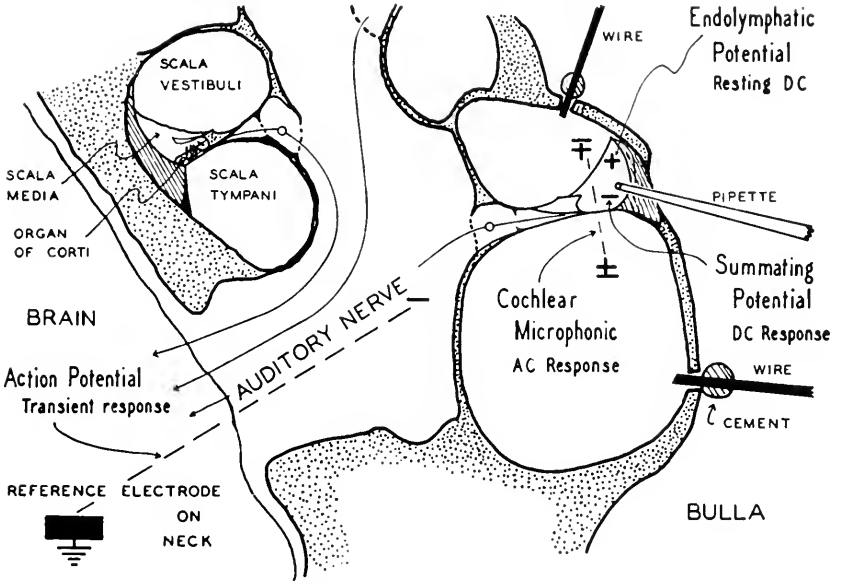

FIG. 3. Position of electrodes and orientation of potentials in the first turn of the guinea pig cochlea. The small droplets of cement on the electrodes prevent the wires from slipping too far through the holes drilled through the bony wall. The pipette is introduced through the spiral ligament by means of a micromanipulator.

cochlear microphonic, but, from the point of view of trigger action, it is less interesting than the microphonic and we shall not consider it further.

Neither shall we be concerned with the way in which the middle ear conducts sound to the inner ear or with how the inner ear performs its mechanical acoustic analysis. The result of this acoustic 'analysis' is that low-frequency sounds agitate the whole length of the basilar membrane of the cochlea, and particularly its apical end, while high-frequency sounds cause movement only in the basal turn. The movements are, however, fundamentally the same regardless of the part of the cochlea in which they occur. It is with these typical move-

ments in a short segment of the cochlea that we are concerned. How do they initiate nerve impulses?

A cross-section diagram of the cochlear canal (fig. 4) shows the structure of the sense organ and allows a visualization of the essential movement. The scala vestibuli and the scala tympani, both filled with perilymph, are separated by the 'cochlear partition.' This partition consists of Reissner's membrane, the tectorial membrane, the organ of Corti and the basilar membrane as flexible or movable parts, and the spiral ligament, the lamina spiralis and the limbus as

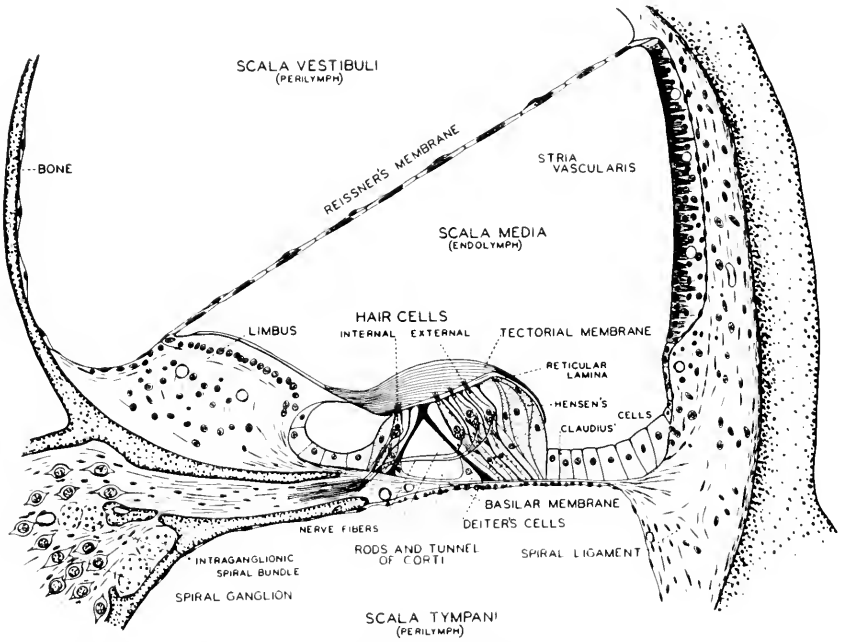

FIG. 4. Diagram of the structures of the cochlear partition, including the organ of Corti, based on fixed and stained sections of the second turn of the guinea pig cochlea. Details of the tectorial membrane are based on descriptions of the microdissection of fresh specimens.

supporting structures. The elasticity of the basilar membrane and the stiffness of the structures attached to it allow it to be displaced in both directions from its position of rest by the pressure of sound waves delivered to the perilymph. The basilar membrane is not under static tension, as was formerly supposed; but the whole structure does have some elasticity, so that it returns, after displacement, to a mid position. Parts of it are stiffer than others. The rods of Corti are quite stiff and so is the plate-like structure, the reticular lamina, that is supported by the rods of Corti, Deiters' cells and the cells of Hensen. We know, from direct micromanipulation under the microscope (3) and from the

way in which the organ of Corti breaks under the stress of very intense sound, that the reticular lamina is like a strong rigid plate lying on a more flexible cushion. The rods of Corti serve to pivot its motion around the edge of the bony spiral lamina, near which the foot of the inner rod is attached. The amplitude of movement is greatest near the spiral ligament, well out from the axis of rotation (fig. 5). This rocking of the organ of Corti as a whole while the basilar membrane bulges up and down seems well established as a basic form of movement of the cochlear partition.

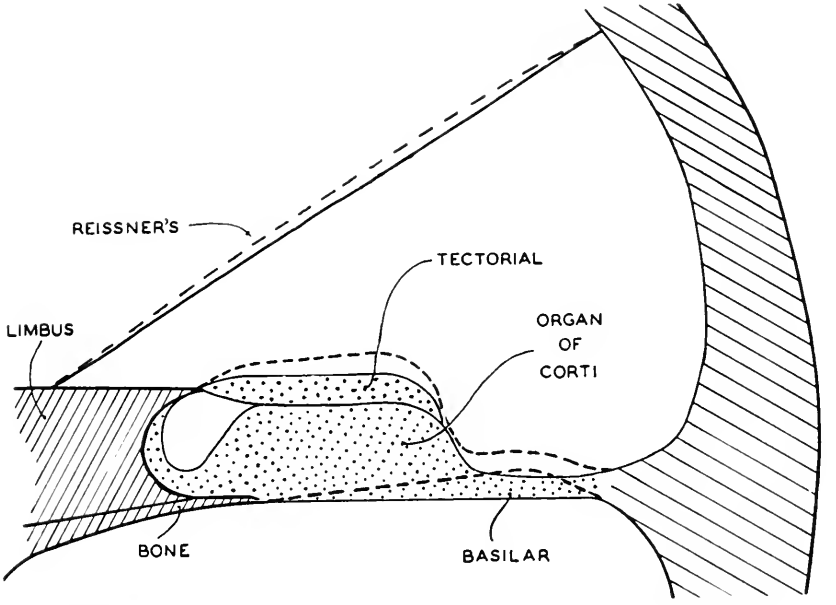

FIG. 5. Movements of the cochlear partition, based on description by von Békésy (3).

The sensory cells of the inner ear, the hair cells, are located in the organ of Corti. Their 'upper' ends are inserted in the reticular lamina to form a continuous plate. The bodies of the outer hair cells beneath the lamina lie exposed to the fluid in free spaces within the organ of Corti. Their 'lower' ends are supported by cup-like portions of Deiters' cells. The nerve endings of the auditory nerve are closely applied to these lower ends, and lie between the hair cells and their supports. Under the electron microscope (10) the endings appear as structures of some substance, well supplied with mitochondria. The outer hair cells, to which the greatest sensitivity is usually attributed, are thus fixed firmly at their upper and lower ends, but are remarkably free mechanically between these supports. The inner hair cells differ in many details from the outer hair cells, but their general character and situation are similar.

The tectorial membrane contains a system of fibers as well as the more familiar jelly-like substance. It is described by Békésy (3) as stiff and resistant to quick vibrations, although yielding easily to slow movements. We now know that it is attached to the organ of Corti near its inner border (fig. 4) and, by a web-like extension, along the outer border also. The cilia or 'hairs' of the hair cells are firmly imbedded in it. Its inner edge is hinged like the leaf of a book along the projecting edge of the rigid limbus. The tectorial membrane is a very significant mechanical structure. Its axis of rotation lies considerably above the axis of rotation of the organ of Corti beneath it. We have already noted that the latter apparently swings up and down as a relatively rigid structure.

The net result of this mechanical arrangement seems to be a sliding or shearing movement between the tectorial membrane and the reticular lamina, and the hairs are bent from side to side as a result (fig. 6). Békésy (3) points out that this arrangement serves to deliver the force of the movement of the cochlear

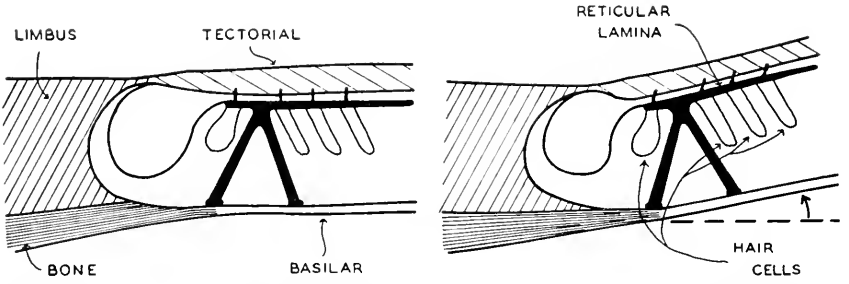

FIG. 6. Movement of the organ of Corti and the tectorial membrane, based on description by von Békésy (3). The shearing action between these two stiff structures bends the hairs of the hair cells.

partition very efficiently to the hair cells. It also seems obvious that the attachments of the tectorial membrane to the organ of Corti must serve to limit the amplitude of this movement and thus protect the hairs against further stress when a certain amplitude has been reached.

We may sum up this interpretation of the basic mechanical movements of the cochlear partition with the inference that the final critical event is a bending of the hairs of the hair cells. It is here, in the hairs or in the cells from which they arise, that we should look for a trigger action if such an action is interposed between the last mechanical event and the initiation of impulses in the nerve fibers.

The amplitudes of the mechanical movements in the cochlea are extremely small, particularly for faint sounds near the threshold of hearing. The greatest conceivable amplitudes are only of the order of a fraction of a millimeter even at the ear drum, and it seems that the movements we have described in the cochlear partition are less than those of the drum membrane, rather than greater. The best estimates of the amplitude of movement of the drum membrane at

threshold as a function of frequency are shown in figure 7 (4). We need not describe the methods by which these amplitudes have been measured and calculated, but we must note that their order of magnitude is of atomic and even sub-atomic dimensions! And the corresponding calculations of the amount of energy involved point to an energy level that is of the same order of magnitude

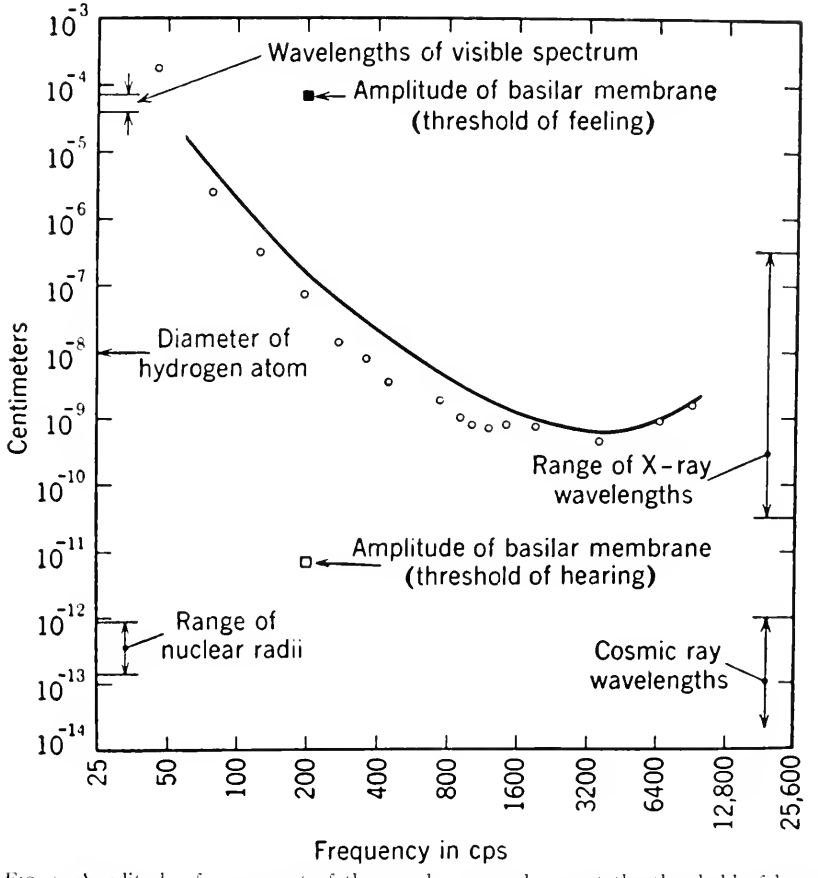

FIG. 7. Amplitude of movement of the ear drum membrane at the threshold of human hearing. *Open circles* are extrapolated from measurements of actual movement by Wilska; *solid line* is derived from calculations based on measurements of threshold sound pressures. From von Békésy and Rosenblith (4), by permission.

as that of the thermal energy that is responsible for Brownian movements. Such minute amounts of energy delivered to one end of a hair cell can hardly suffice to initiate nerve impulses at the other end without the interposition of a trigger mechanism to amplify the available energy, and even this trigger itself must be very delicately poised!

The requirement of a 'biological amplifier' suggests that we should seek a pool

of readily available energy (1) that can somehow be valved or modulated to do the work of exciting the nerve fibers or endings. Such a mechanism was suggested several years ago by the writer as a working hypothesis (6, 7) and, although some of the details of that hypothesis have been shown by experiment to be untenable or at least very improbable, the general outline is still plausible enough to warrant repeating it once more.

The amplifier hypothesis states that the immediate stimulus that excites the nerve fibers (or endings) is electrical, and specifically that it is the so-called 'cochlear microphonic.' The electrical energy is supposed to be derived from a pre-existing electrical potential known as the 'endolymphatic potential' by virtue of a change in the electrical resis ance across the upper surface of the hair cells in the reticular lamina. The change of electrical resistance is supposed to be caused by the movement or bending of the hair cells where they arise from this upper surface. The change in resistance varies the current flow through the hair cells, and the part of this current that flows from the hair cell outward through the nerve ending and fiber is assumed to be the stimulus to the nerve.

Now let us consider the electrical phenomena in more detail. The cochlear microphonic has been recognized for about 25 years. It appears as an alternating electric potential that reflects faithfully, within limits, the pattern and amplitude of the mechanical movements of the cochlear partition. It is symmetrical and it shows no true threshold, no all-or-none character and no refactory period. It appears across the basilar membrane (cf. fig. 3), and a variety of experiments associate it c'early with the hair cells (5). Even more specifically, it is associated with the 'upper' end of the hair cell, because when a hyperfine micropipette is pushed through the organ of Corti in this region from the scala tympani the phase of the cochlear microphonic is reversed just at the point where the pipette enters the endolymphatic space (11). We have, then, as a matter of experimental observation, an electric counterpart of the mechanical movement located anatomically at just the point to which we were led by our analysis of the mechanical events in the cochlear partition. It is not the locus but the mechanism and the assumed function of the cochlear microphonic that are hypothetical.

We may note in passing that it had been widely assumed, ever since the microphonic was first identified, that the microphonic stimulates the nerve. The present writer was for many years inclined to be skeptical and to regard the microphonic as probably an epiphenomenon. The chief reason for this skepticism was the rather long latency, about 1 millisecond, between the microphonic and the first clearly recorded action potential of nerve. It now appears, however, that the main action potential is recorded as the impulses pass through the internal auditory meatus (cf. fig. 3), and its latency can easily be explained as conduction time. Furthermore, an electrode placed close beneath the spiral lamina, where the nerve fibers enter the bony structure of the central core of the cochlea, reveals an early electric potential that seems to signal the start of nerve

impulses in the peripheral endings of the fibers. The latency of the 'foot' of the action potential is so brief that it argues for rather than against direct electrical excitation of the nerve by the microphonic (9).

The present hypothesis, as we have said, designates the 'endolymphatic potential' as the source of the energy of the cochlear microphonic. The endolymphatic potential was discovered by Békésy in 1952 (2). A micropipette introduced into the scala media reveals a resting DC potential of about +80 millivolts (in the guinea pig) relative to the perilymph of scala tympani or scala vestibuli or to the tissues of the neck. The distribution of this positive potential

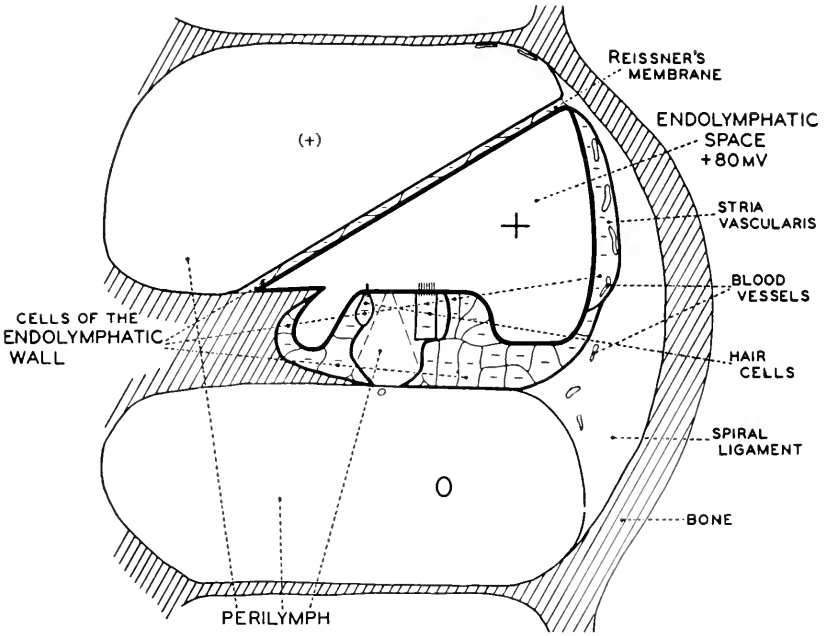

FIG. 8. Endolymphatic space and distribution of the DC potentials of the cochlear partition (11).

is shown in figure 8. In this figure is also indicated the familiar negative intracellular potential, like that within nerve and muscle cells, that is encountered when the tip of the electrode lies *within* cells of the cochlear partition. The endolymphatic potential is of about the same or greater magnitude relative to our reference electrode, but it is positive, not negative. There is, then, a potential difference of about 150 millivolts across the 'membrane' of the hair cells from which the hairs protrude.

The cochlear microphonic and the endolymphatic potential are both immediately dependent on oxidative metabolism. They fall almost to zero if the oxygen supply is cut off or if small quantities of cyanide or azide are injected into the cochlea. The parallelism of these changes, which are reversible if not

too extreme or prolonged, is consistent with the hypothesis that the cochlear microphonic depends upon the endolymphatic potential. (We here neglect a small residual anaerobic cochlear microphonic that persists after death and which is evidently generated by some other mechanism.) Our present hypothesis specifically attributes the changes in potential, i.e. the cochlear microphonic, to changes in current flow through the hair cells as a result of passive changes in

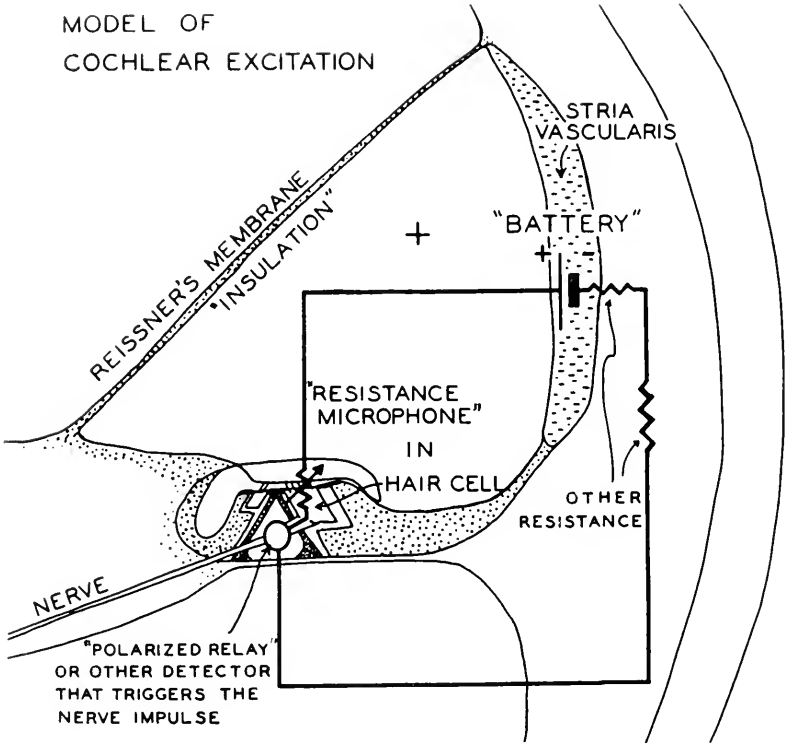

FIG. 9. The 'resistance microphone' theory of the origin of the cochlear microphonic. In this diagram the 'battery' is hypothetically located in the stria vascularis, as originally proposed (6, 7).

electrical resistance. The observed changes in potential are supposed to be due to changes in the 'IR drop' across the upper ends of the hair cells (7).

The source of the endolymphatic potential has not been identified. In the first formulation of the present hypothesis, the writer located it tentatively in the stria vascularis, which forms part of the external wall of the scala media, as shown in figure 9. The chief reason for this guess was that here is the richest blood supply, and the endolymphatic potential is very closely dependent on oxidative metabolism. Other evidence, not yet conclusive, seems to point to the hair cells themselves as the source of the endolymphatic potential. If this proves

to be the case, it may be simpler to speak only of a direct modulation of the potential of the 'battery' by mechanical movement, rather than of a battery of constant EMF and an anatomically distinct 'variable resistance microphone.' The final result, however, would be much the same wherever the 'battery' is located. There would still be a variation in current flow through the base of the hair cells and on through the nerve endings and fibers, that could serve as the stimulus to initiate nerve impulses. Either concept provides a biological amplifier which utilizes a pre-existing store of readily available energy. This electrical energy is valved or modulated by the microscopic or sub-microscopic mechanical action and the changes in current flow stimulate the nerve. In either case, it is a 'trigger action.'

This hypothesis is based on electrical phenomena that occur in the cochlea. Some counterparts of these phenomena have been described for other sensitive mechano-receptors, notably the non-auditory labyrinth and the lateral line organs. We do not claim that the hypothesis necessarily applies to all mechano-receptors. It may be unnecessary for those where plenty of energy is available in the original stimulus. The electrical trigger action that we suggest may be a specialization that makes possible the extreme sensitivity of the inner ear. Also there is no doubt that the hypothesis as outlined is oversimplified. Further experiments continue to reveal additional complexities in the action of all of these sense organs; but perhaps some parts of the framework of the mechanism here suggested will survive the test of time.

REFERENCES

1. VON BÉKÉSY, G. DC potentials and energy balance of the cochlear partition. *J. Acoust. Soc. Amer.* 23: 576–582, 1951.
2. VON BÉKÉSY, G. DC resting potentials inside the cochlear partition. *J. acoust. Soc. Amer.* 24: 72–76, 1952.
3. VON BÉKÉSY, G. Description of some mechanical properties of the organ of Corti. *J. acoust. Soc. Amer.* 25: 770–785, 1953.
4. VON BÉKÉSY, G. AND W. A. ROSENBLITH. The mechanical properties of the ear. In: *Handbook of Experimental Psychology*, edited by S. S. STEVENS. New York: Wiley, 1951, Ch. 27.
5. DAVIS, H. Psychophysiology of hearing and deafness. In: *Handbook of Experimental Psychology*, edited by S. S. STEVENS. New York: Wiley, 1951, Ch. 28.
6. DAVIS, H. Energy into nerve impulses: hearing. *Med. Bull. St. Louis Univ.* 3: 43–48, 1953.
7. DAVIS, H. Mechanism of hearing. In: *Transactions of the Fourth Conference on the Nerve Impulse*. New York: Macy, 1954.
8. DAVIS, H. The biophysics and neurophysiology of the inner ear. *Physiol. Rev.* In press.
9. DAVIS, H., I. TASAKI AND R. GOLDSTEIN. The peripheral origin of activity, with reference to the ear. *Cold Spring Harbor Symp. Quant. Biol.* 18: 143–154, 1952.
10. SMITH, CATHERINE A. Electron microscopic studies of the organ of Corti. *Anat. Rec.* 121: 451, 1955.
11. TASAKI, I., H. DAVIS AND D. H. ELDREDGE. Exploration of cochlear potentials in guinea pig with a microelectrode. *J. Acoust. Soc. Amer.* 26: 765–773, 1954.

Triggering of Insect Spiracular Valves

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IN MOST INSECTS the spiracles, or external openings of the respiratory system, are provided with valves. The anatomy of the valves differs greatly in different species, but the basic closing mechanism usually involves a chitinous lever which, in one position, holds one lip of a valve across the opening or else flattens a tubular chamber leading inward from the external opening of the spiracle (9). A common arrangement for moving the lever involves an elastic ligamentary opener and a muscular closer. Thus the insect must do work to keep the spiracles closed, whereas the opening is a passive process involving relaxation of the muscle. In accord with this fact, the spiracles are ordinarily found open in anesthetized, hypoxic or dead specimens.

Oxygen lack or carbon dioxide excess in the atmosphere surrounding an insect stimulates spiracular opening, and each gas modifies the other's effect in a definite and predictable fashion (10, 16, 6, 14). It therefore seems reasonable to infer that these gases could be involved in the normal control of the spiracles. The rapid and readily reversible responses to carbon dioxide and oxygen suggest that at least their immediate effects are due to direct local action at the spiracles rather than to general systemic alterations such as tissue hypoxia, narcosis, etc. (6). However, it has not been shown directly that the valves respond to normal changes in CO_2 and O_2 concentrations in the internal gaseous environment as well as to abnormal changes in the external gas phase.

In point of fact, the mechanism of even the external gas effects is obscure. Central innervation of the spiracles has been described in various insects, and in some spiracles the tonic contraction of the closer muscle is maintained by neural stimulation and its relaxation brought about by diminution of nervous activity (ref. 8 and personal communication). In species which normally have a marked ventilatory rhythm it seems clear that the observed synchronous and coordinated activity of the dozen or more spiracles must be centrally controlled (cf. also ref. 8). Even in such species, however, the valves may also act individually under certain conditions, a single spiracle, for example, responding to a localized jet of CO_2 (10). In still more striking experiments it has been shown that denervated spiracles close in response to increase in ambient CO_2 concentration (10, 16, 17, 8, 1). The mechanism of the CO_2 effect is itself unclear, quite aside from the question of whether it is direct or indirect. Spiracular

opening has been linked to an increase in hydrogen ion concentration (16, 14) but Case (7) has recently shown rather convincingly that the action of CO_2 is independent of hydrogen ion concentration.

By altering ambient O_2 and CO_2 concentrations Case (6) has shown that spiracular movement in adult flies can be graded. Sustained intermediate valve positions are also seen in various insects in atmospheric air, particularly during and after exercise. However, in many species at rest there appears to exist a rhythm of spiracular activity involving relatively long periods of closure alternating with brief periods of wide opening. This is seen well in adult lampyrid fireflies, for example, and has been described also in fleas (16, 11). The possible all-or-none nature of this activity is obviously of interest in connection with triggered-type responses, but it has been little studied in adult insects

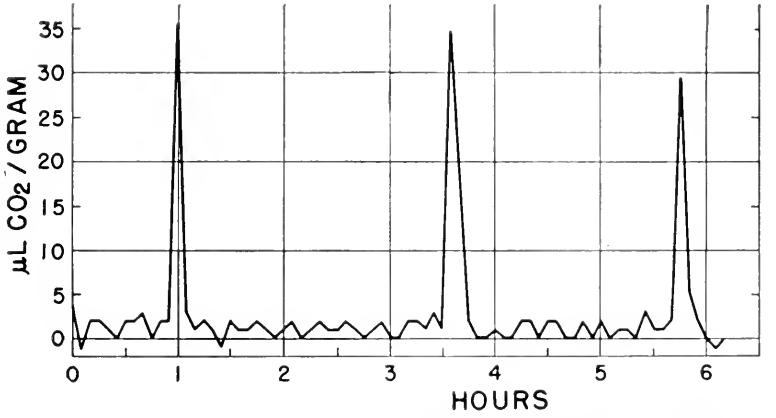

FIG. 1. CO_2 release pattern in diapausing *Agapema* pupae. (Buck and Keister, 1955, fig. 1, p. 146.)

under reasonably normal conditions. There is, however, a considerable amount of quantitative information on possibly analogous behavior in certain diapausing moth pupae, forms which offer special advantages because of their bodily inactivity, large size and relative insensitivity to handling (12, 15, 4).

The phenomenon in question is seen very dramatically in Warburg measurements of CO_2 release rate by the pupae (fig. 1), and appears also in continuous recordings by a method with much better time resolution (fig. 2). For technical reasons it has not been possible to study the spiracles during actual respirometry, but a direct causal connection between spiracular opening and the CO_2 'bursts' can hardly be doubted in view of the facts that *a*) all pupal gas exchange occurs via the spiracles (15, 4), *b*) there are no relevant ventilatory or other body movements (15, 4), *c*) the discontinuity of the CO_2 release cycle is reversibly abolished by intubating some of the spiracles so that the tracheal

gas is continuous with the ambient atmosphere (4), *d*) the spiracles show a cycle of long closed and brief open periods corresponding to the timing of the CO_2 release cycle (13, 14), *e*) the frequency and size of the bursts are influenced predictably by ambient CO_2 and O_2 concentrations (15, 4).

In the present connection two facets of the CO_2 release cycle are of special interest: *1*) Is it a triggered response, and if so what is the trigger?; and *2*) what is the physiological significance of this system in which diffusion appears to be controlled by alternating extremes rather than by maintained intermediacy?

THE CO_2 BURST CYCLE IN RELATION TO TRIGGERING

Superficially considered, the CO_2 burst appears to resemble certain triggered events. First, the burst takes place in the absence of any environmental cue or obvious abrupt physiological change within the pupa. Second, the start of the burst, considered either volumetrically (fig. 1) or from the standpoint of

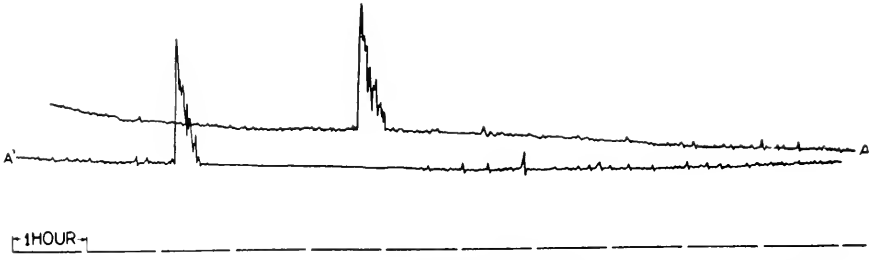

FIG. 2. CO_2 release pattern in pupa of *Papilio machaon*. Time course from left to right, with top trace continuous at *A* with bottom trace at *A'*. (Punt, 1950, fig. 12, p. 68.)

the duration of the actual period between the start of opening and the fully open position of the tracheal valve (fig. 3), is very sudden in comparison with the interburst period of near closure which may last 20–1000 times as long (15, 4). Third, the burst shows a rather marked constancy in volume (4). However, as has become apparent upon closer study of many ostensibly all-or-none responses, the 'event' which occurs at the start of the burst of CO_2 release is considerably more complex than at first appears. In some records of spiracular valve movement (fig. 3), it is clear that the valve is not totally closed throughout the entire interburst period, nor does the change which occurs at the start of the burst consist either of a simple shift from a closed to an open position or of repeated full oscillations between the two positions, as in the chattering of a relay. Neither, however, does it show a smooth transition through intermediate rest positions as in a graded response. Rather, we see wide-amplitude oscillations from the 'closed' position giving way rather abruptly to similar oscillations from or around the open position. Spiracular opening thus differs from irreversible events, such as spore activation or infection. Although it is

unclear whether or not the more prolonged oscillations which occur at the end of the burst can be considered to be basically the reverse of the spiracular opening, the response as a whole seemingly differs also from reversible or self-restoring events like the muscle twitch and nerve impulse. Because of the necessarily arduous type of recording, Scheiderman and his co-workers quite naturally chose for their observations pupae with high frequencies of burst production (fig. 3). Judging from our manometric records on another species, such pupae tend to have a more prolonged and 'smeary' type of burst than long-period pupae. It is, therefore, possible that both start and end of a normal burst may often involve sharper transitions between closed and open valve positions. 'Sharper' bursts are in fact seen when the ambient O_2 concentration is increased (fig. 4). Furthermore, though the spiracular flutter prior to full opening is a reasonable explanation of similar perturbations in continuous respirometer records (fig. 2), the possibility of such flutter being abnormally exaggerated should not be excluded in view of the fact that the valves have to be exposed surgically before direct observation is possible.

The mean respiratory quotient for an entire pupal CO_2 release cycle is a conventional 0.78, but during the interburst period it is 0.3 or less (15, 4). This indicates that although metabolic CO_2 is being *produced* steadily it is mainly impounded during the interburst periods and released in the intervening bursts. This suggests that tracheal and tissue CO_2 concentration must rise during the interburst period and that the eventual occurrence of a burst may mark the attainment of a critical value or threshold. For present purposes it does not matter whether the ultimate trigger is molecular CO_2, bicarbonate ion or hydrogen ion.

There is, however, no indication that such a critical concentration could involve any sort of biochemical discontinuity. On the contrary, all available evidence suggests that if attainment of a particular CO_2 concentration is the stimulus for spiracular opening, the actual trigger is simply one additional regular increment—the 'final straw,' so to speak. Thus it has been found (5) by equilibration of normal *Agapema* pupae with various CO_2–N_2 mixtures of constant O_2 concentration that the average internal CO_2 concentration is of the order of 6 per cent, and that the change during the interburst period does not exceed 20 per cent of that value (i.e., a range from 5.5 to 6.5%). This fits well with the observed steady 20 per cent rise in CO_2 release rate over the interburst period. Moreover, equilibration of drawn blood with similar gas mixtures has confirmed both the absolute values of the internal CO_2 concentration range and the expectation that the gas is taken up linearly with ambient CO_2 concentration over a wide range (2, 3). Hence there is every reason to believe that, whatever the absolute value of the tracheal or tissue CO_2 concentration at the time of the burst, the increase which ultimately sets off the burst is small. Actually, considering the fact that the concentration rises only 1 per cent over

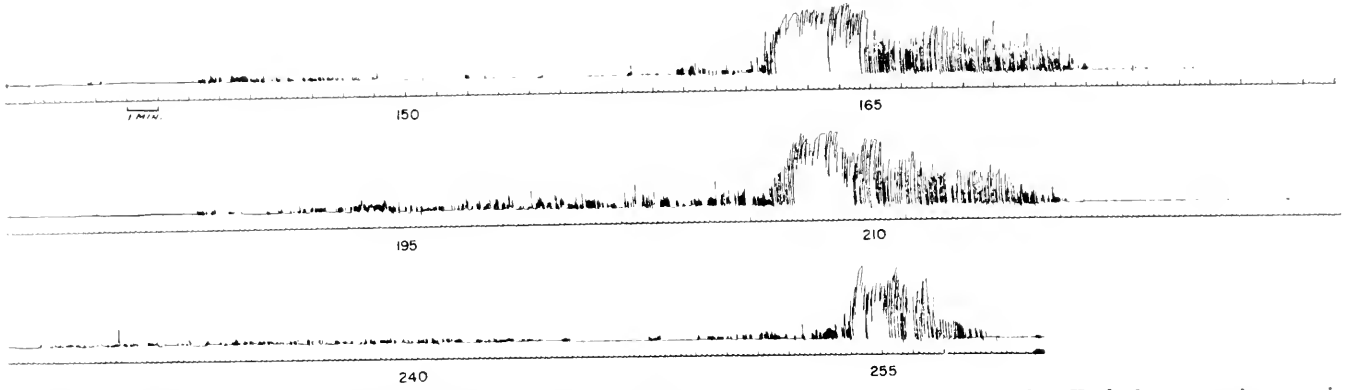

FIG. 3. Continuous record of spiracular valve movements during three consecutive cycles in diapausing *Hyalophora cecropia* pupa in 21% O_2. Time course from left to right. (Schneiderman, 1956, fig. 1, p. 1170.)

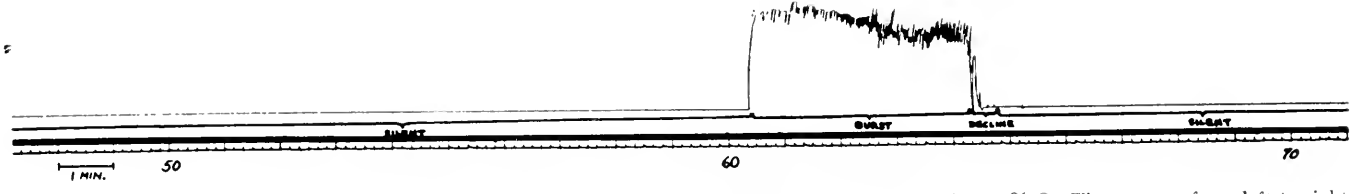

FIG. 4. Continuous record of spiracular valve movements in diapausing *Hyalophora cecropia* pupa in 100% O_2. Time course from left to right. (Schneiderman, 1956, fig. 2, p. 1171.)

the entire interburst period (which may last several hours) and that the spiracle performs its opening within about a minute (fig. 3), the triggering change must be of the order of hundredths of a per cent. The blood equilibration experiments show likewise that the hydrogen ion concentration changes linearly with ambient CO_2 concentration and to the extent of only about 0.02 pH unit per 1 per cent change in CO_2 concentration. The fact that the spiracles, once open, remain so until the internal CO_2 concentration has fallen many times the amount of the postulated critical increment, provides another point of similarity with triggered responses.

POSSIBLE RATIONALE OF CYCLIC REGULATION

Almost all insects are highly vulnerable to desiccation, and it has long been known that one of the major—perhaps the principal—function of the spiracular valves is to reduce evaporatory water loss from the tracheal system. Spiracular aperture must therefore always be a compromise between the necessity for minimizing transpiration and the necessity for permitting entrance of adequate O_2 and escape of adequate CO_2. In most insects the rate of O_2 uptake is not limited by ambient O_2 until its concentration has fallen very markedly below atmospheric. This means that respiration can be maintained with only a low percentage of O_2 in the tracheae and that the trans-spiracular O_2 gradient can be very steep. A comparably steep CO_2 gradient in the opposite direction could not, presumably, be established because the necessary tracheal CO_2 concentration would be narcotic. At any rate, the spiracles of most insects open when the external CO_2 concentration reaches 3–7 per cent, and almost certainly open also when the internal concentration reaches such levels, thus preventing buildup above that tracheal concentration. Consequently the fact which limits the reduction in spiracular valve aperture is tracheal CO_2 concentration: O_2 limitation is never a problem.

A priori, it might appear that the relative rates of loss of water vapor and CO_2 would be the same for all possible spiracular areas. This is presumably true for all conditions in which CO_2 escapes as fast as it is formed. When CO_2 is actually impounded, however, tracheal P_{CO_2} rises, while tracheal P_{H_2O} remains constant, being a function only of the temperature. This means that when tracheal P_{CO_2} is relatively high, CO_2 will escape faster, in proportion to the escape rate of water vapor through the same spiracle, than when no CO_2 is being impounded and tracheal P_{CO_2} is lower. In other words, restricting the spiracles below the area which will permit CO_2 to bleed off as fast as it is formed will reduce water loss not only absolutely, but also relative to CO_2 loss.

However, CO_2 accumulation cannot continue indefinitely, and when the spiracles are finally forced to open the situation is reversed; the internal CO_2 concentration will fall and the relative rate of water loss will increase. It should therefore be advantageous to the insect, as far as water conservation is con-

cerned, to prolong the period of restricted spiracular valves (interburst) and shorten the period of spiracular opening necessary to relieve CO_2 accumulation (burst).

Whether the retention phase could be made so extreme as to more than compensate for the exaggerated rate of water loss during the burst period is difficult to predict. Computations using known and estimated parameters of the CO_2 burst cycle in the moth *Agapema* indicate that only half as much water escapes during the observed alternating periods of CO_2 retention and release as would be lost if the spiracles were held steadily in a partly open position just sufficient to allow the metabolic CO_2 to escape as fast as formed; but the uncertainties of the measurements are sufficient that a larger differential would be desirable before the case could be considered proved. The possibility is, however, attractive, since it would on the one hand provide a neat example of adaptation for water conservation, and on the other a rationale for what appears to be an unusual or even illogical type of respiratory behavior. Actually, this type of regulation may not turn out to be as unusual as it appears. Conceivably the normal spiracular behavior of many resting insects, involving ostensibly the same sorts of relatively long periods of valve closure and short intervals of opening, may prove to be a much speeded-up version of the same behavior. In any case it shares the same characteristic of 'regulation by averaged extremes,' or 'grading by alternating all-or-none responses.'

SUMMARY

The CO_2 burst cycle in diapausing moth pupae and its underlying cycle of spiracular opening and closure fail in many respects to measure up to an ideal example of a triggered response. Not only is the response itself rather variable, but the possible trigger is identified only indirectly. Furthermore, the valve opening appears to involve muscular relaxation rather than contraction, as would ordinarily be expected in a rapid response. In two respects, however, the system has yielded quantitative evidence for triggering. First, it appears that the triggering entity—probably CO_2 concentration—increases very slowly and uniformly up to the 'ignition point,' and second, the response, once started, proceeds independently of the trigger. The cyclical nature of CO_2 retention and release, while not directly relevant to the trigger problem, is interesting in that it suggests a possible physiological advantage obtainable from an exaggerated alternation of overshoot and undershoot as compared with a steady state sort of regulation.

I am much indebted to Drs. Schneiderman, Beckel and Case for permission to refer to unpublished data, and for suggestions.

REFERENCES

1. Beckel, W. E. and H. A. Schneiderman. The spiracle of the cecropia moth as an independent effector. *Anat. Rec.* 125: 559–560, 1956.

2. Buck, John. Possible mechanism and rationale of cyclic CO_2 retention by insects. *Proc. Xth Int. Congr. Entomol.* In press.
3. Buck, John and Stanley Friedman. Carbon dioxide transport in diapausing pupae. *Anat. Rec.* 125: 556, 1956.
4. Buck, John and Margaret Keister. Cyclic CO_2 release in diapausing Agapema pupae. *Biol. Bull.* 109: 144–163, 1955.
5. Buck, John and Margaret Keister. Unpublished observations.
6. Case, J. F. Carbon dioxide and oxygen effects on the spiracles of flies. *Physiol. Zool.* 29: 163–171, 1956.
7. Case, J. F. Differentiation of the effects of pH and CO_2 on spiracular function. *J. Cell. & Comp. Physiol.* In press.
8. Case, J. F. Neural basis of insect spiracular function. *Anat. Rec.* 124: 270, 1956.
9. Hassan, A. A. G. The structure and mechanism of the spiracular regulatory apparatus in adult Diptera and certain other groups of insects. *Trans. Roy. Ent. Soc.* 94: 103–153, 1944.
10. Hazelhoff, E. H. On a new form of breathing regulation (regulation of diffusion) in insects and Arachnida. *Proc. Sect. Sci. Koninkl. Akad. Wetensch. Amsterdam* 29: 492–496, 1926.
11. Herford, G. M. Tracheal pulsation in the flea. *J. Exper. Biol.* 15: 327–338, 1938.
12. Punt, A. The respiration of insects. *Physiol. Comp. Oecol.* 2: 59–74, 1950.
13. Schneiderman, H. A. and W. E. Beckel. The coordination and control of spiracular valves in relation to discontinuous release of carbon dioxide by diapausing pupal insects. *Anat. Rec.* 120: 69, 1954.
14. Schneiderman, H. A. Spiracular control of discontinuous respiration in insects. *Nature* 177: 1169–1171, 1956.
15. Schneiderman, H. A. and C. M. Williams. An experimental analysis of the discontinuous respiration of the cecropia silkworm. *Biol. Bull.* 109: 123–143, 1955.
16. Wigglesworth, V. B. The regulation of respiration in the flea, Xenopsylla cheopsis, Roths. (Pulicidae). *Proc. Roy. Soc. London, s. B* 118: 397–419, 1935.
17. Wigglesworth, V. B. The effect of pyrethrum on the spiracular mechanism of insects. *Proc. Roy. Ent. Soc. London, s. A* 16: 11–14, 1941.

Initiation and Control of Firefly Luminescence

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NUMEROUS STUDIES HAVE CLEARLY ESTABLISHED that the firefly flash is initiated by way of a nerve impulse (3, 4). There remains some question however, as to the precise mechanism involved. Two general theories, previously discussed in detail by Buck (2), have been proposed. One, which may be called the oxygen theory, proposes nervous control of a valve-like mechanism (the tracheal end cell) in the tracheolar supply to the luminous organ. Since the luminescent reaction requires oxygen, the firefly would presumably be able to initiate or extinguish luminescence by the control of oxygen availability. The second theory proposes that the nerve impulse stimulates the photogenic cell directly. Recent studies with cell free extracts have suggested that the luminous material (i.e., luciferase, luciferin and ATP and oxygen) is available, but luminescence is prevented because of an inhibition of the enzyme (4). Presumably the nerve impulse reverses the inhibition by splitting the inhibitory complex, thus giving rise to active enzyme and a flash of luminescence. Evidence has recently been obtained concerning this latter idea.

FIREFLY FLASHES IN VIVO

It is possible to obtain two types of flashes in the intact firefly. The flash which occurs normally (fig. 1) has a duration of not longer than 0.2 second ($t_{\frac{1}{2}}$ rise time about 0.05 sec.). This type of flash may also be induced by electrical stimulation (1). The 'pseudo-flash,' reproduced in figure 2, occurs when oxygen is readmitted to a firefly which has been placed temporarily under partially anaerobic conditions (ca. $\frac{1}{2}$% O_2). In contrast to the normal flash, the duration of the pseudo-flash is about 2 seconds ($t_{\frac{1}{2}}$ rise time about 0.5 sec.).

The fact that the pseudo-flash is approximately ten times slower than the normal flash is not necessarily against the oxygen theory. It is possible that the tracheal system immediately connected to the luminous gland has the ability to rapidly change the oxygen tension locally. By placing the whole firefly under anaerobic conditions, all of the tracheal system would lose oxygen and considerable time might be required for recovery when the organism is placed under aerobic conditions. Unfortunately, there is no information available concerning the physiology of the tracheal system and the special tracheal end cells.

LUMINESCENT FLASHES IN VITRO

Flashes have also been observed in the *in vitro* system. The requirements for light emission, as previously reported (7), are as follows: luciferase, luciferin, Mg^{++}, adenosine triphosphate (ATP) and oxygen. If a reaction mixture containing these components is made anaerobic by bubbling hydrogen the luminescence ceases. When oxygen is readmitted a bright flash, which was called the 'oxygen' flash, occurs (5). This is the result of the accumulation under anaerobic conditions of an intermediate which, when oxygen is admitted, is

FIG. 1. Normal flash of *Photinus pyralis*. Sweep time, 0.5 second.

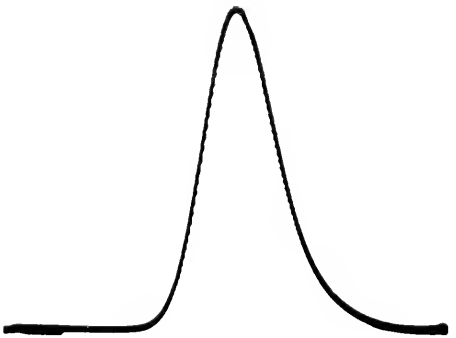

FIG. 2. Pseudoflash in *Photinus pyralis*. The firefly was placed in $^1/_2\%$ oxygen and after 2 minutes air was admitted. The resulting flash was recorded as shown. Sweep time, 4 seconds.

rapidly oxidized with light emission. A recording of an oxygen flash is shown in figure 3. It is evident that this flash is comparable to the pseudoflash of the intact firefly. Moreover, both occur under essentially comparable conditions. Although the oxygen flash provides a model mechanism for normal flashing, the duration of the oxygen flash is much longer than the normal flash. We have recently attempted to experimentally change the duration of the oxygen flash. Variations in the concentrations of luciferase, luciferin, ATP and Mg^{++} have no effect upon the duration. The substitution of Mn^{++} for Mg^{++} results in a flash having a slightly longer duration. In going from the anaerobic to the aerobic condition, the use of air in place of oxygen also results in a flash of a longer duration, particularly with respect to the rise time. When oxygen con-

centrations which are below saturation for the luminescent reaction are used to initiate the oxygen flash, the duration of the flash is also greatly increased, but mostly as a result of the longer half-time for decay. In these experiments, therefore, the rise time was dependent primarily upon the rate of mixing and the decay time upon the oxygen concentration at values below saturation for luminescence. These results indicate that the intermediate which accumulates under anaerobic conditions, and which is oxidized with light emission, either contains enzyme as a component of a complex or is non-enzymatically oxidized. Further, the intermediate is a complex in which metal ions (Mg^{++} or Mn^{++}) serve as binding agents. The Mg^{++} and Mn^{++} complexes have slightly different velocity constants in the oxidative reaction. These conclusions are in accord with the finding that oxygen is the only compound whose concentration has any

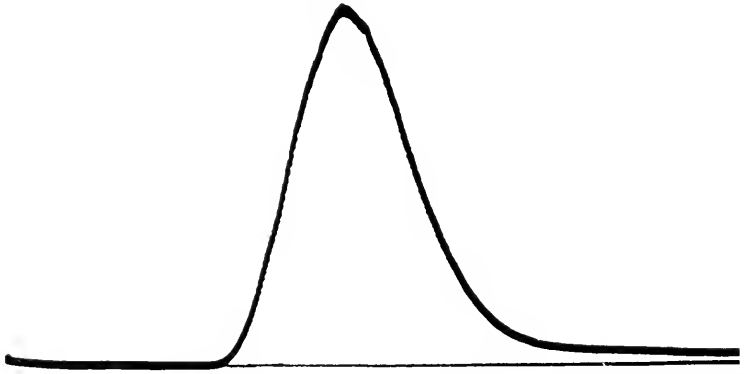

FIG. 3. Oxygen flash *in vitro*. The reaction mixture was placed under anaerobic conditions for 2 minutes. The resulting flash upon the admission of air was recorded. Sweep time, 5 seconds.

effect upon the duration of the flash. Since the velocity constant for the oxidation of the intermediate is too large to account for the rate of extinction of light in the normal flash, the normal flash cannot occur by the mechanism suggested by the oxygen flash *in vitro*.

An alternative mechanism might involve the removal of oxygen from the cell at the rate of extinction of the normal flash. This is difficult to believe, however, in view of the precision in the duration of flashes. It is even more difficult to believe that oxygen could be physically introduced into the liquid phase of the cell from the tracheolar gas phase at a rate sufficient to result in the rapid rise time observed in the normal flash.

A second type of flash may be obtained *in vitro* by the temporary reversal of inactivated enzyme. When all the components required for light emission are mixed together there is an initial flash of light ($t_{1/2}$ rise time less than 0.1 sec. at 25°C.) whose intensity rapidly decreases within a few seconds to a low baseline

level (7). If crystalline inorganic pyrophosphatase is added to the reaction mixture the baseline light intensity is greatly depressed. The rate of decay to the baseline level is likewise greater. Such a reaction, containing pyrophosphatase, is shown in figure 4. The decline of luminescence after its initiation with ATP to the low baseline level is due to a reversible inactivation or inhibition of luciferase. As shown in figure 4, additional luciferase added secondarily to the reaction mixture gives a response very similar to the initial reaction, indicating the pres-

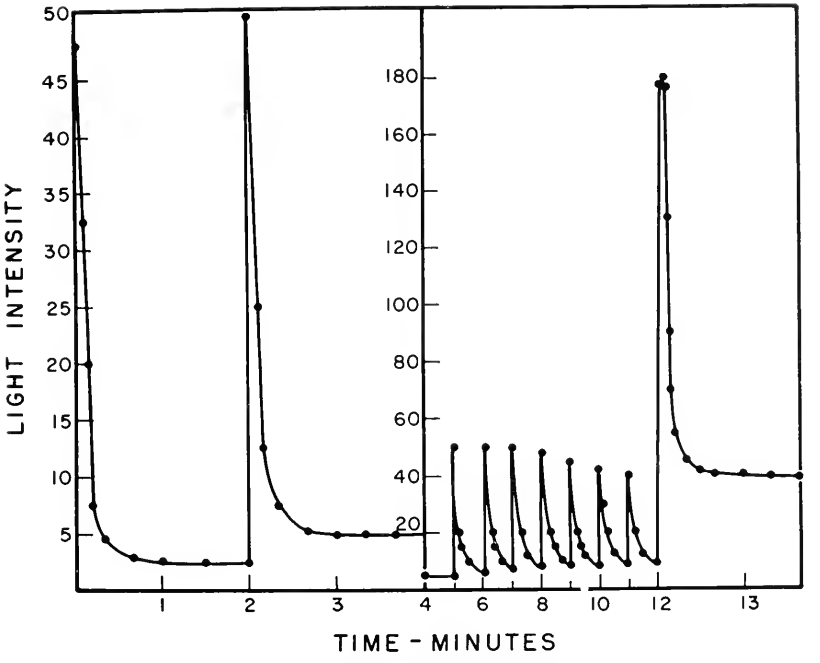

FIG. 4. Luminescent response to the successive addition of luciferase. The reaction mixture contains luciferin and ATP. Additional 0.1 ml of partially purified luciferase was added at the times indicated. At 12 minutes 0.1 ml of inorganic triphosphate was added (final concentration, 0.001 M) (6).

ence of adequate substrates and cofactors. As reported previously, the secondary addition of pyrophosphate to such a reaction mixture will give a flash ($t_{\frac{1}{2}}$ rise time less than 0.1 sec.) whose rate of decay depends upon the concentration of pyrophosphatase.

We believe that pyrophosphate functions in stimulating luminescence by removing an inhibitor from the enzyme surface. Additional details concerning this action of pyrophosphate have been discussed elsewhere (7). Within the photogenic cell we believe that the reactive intermediate is present, but is held

in an inactive state because of enzyme inhibition. The flash presumably occurs when this inhibitory complex is split by pyrophosphate. It is possible that nervous stimulation to the cell could lead to the rapid liberation of inorganic pyrophosphate by means of the typical acetylcholine–coenzyme A–ATP cycle. It is suggestive that the luminous organs contain a very high coenzyme A concentration. Unfortunately more direct evidence concerning this postulated mechanism is not yet available.

ADDENDUM

Since this chapter was prepared there has appeared a paper by Hastings and Buck (*Biol. Bull.* 111: 101-113, 1956) on the effects of different oxygen tensions on the pseudoflash of firefly photogenic tissue, which indirectly supports the theory here presented.

REFERENCES

1. Brown, D. E. S. and C. V. King. *Physiol. Zool.* 4: 287, 1931.
2. Buck, J. *Ann. New York Acad. Sci.* 49: 297, 1948.
3. Buck, John. In: *The Luminescence of Biological Systems.* Washington, D. C.: Am. Assoc. Advance. Sci., 1955, p. 323.
4. Harvey, E. N. *Bioluminescence.* New York: Acad. Press, 1952.
5. Hastings, J. W., W. D. McElroy, and J. Coulombre. *J. Cell & Comp. Physiol.* 42: 137, 1953.
6. McElroy, W. D. and J. W. Hastings. In: *The Luminescence of Biological Systems.* Washington, D. C.: Am. Assoc. Advanc. Sci., 1955, p. 161.
7. McElroy, W. D., J. W. Hastings, J. Coulombre and V. Sonnefeld. *Arch. Biochem.* 46: 399, 1953.

Triggering of Contraction in Skeletal Muscle

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IN NORMAL LIVING MUSCLE the input of a very small amount of energy, giving rise to a wave of excitation, can rapidly precipitate a contraction capable of performing many, many times the work equivalent of the input energy. The complete chain of events leading to this sudden amplification is not known, but many probable links in the chain have been described. Considerable attention has been directed toward the excitation events at the neuromuscular junction, the surface phenomena associated with the resting potential and the action potential, and the properties of various anatomical units ranging from whole muscle down to molecular sub-units of contractile protein. In recent years more emphasis has also been given to the immediate recovery processes taking place in muscle. The discovery of 'relaxing factors,' for example, has opened up a new approach to the study of the contraction-relaxation cycle. However, much is still unknown and the interpretation of much that is known remains controversial. Several excellent papers and reviews have been concerned with the link between excitation and contraction (34, 45, 81, 82, 89). The main purpose of this chapter is to review the issues involved and call attention to some of the more recent work bearing on this problem.

INITIATION OF AN IMPULSE AT THE NEUROMUSCULAR JUNCTION

Although we will be mainly concerned here with the events taking place in the muscle fiber proper, it is pertinent to consider first some of the special features of the nerve-muscle junction and the processes taking place which initiate the wave of excitation in the skeletal muscle fiber. In the generally accepted picture, the incoming nerve impulse induces at the nerve endings a release of acetylcholine (ACh) which is able to bring about an increase in ion-permeability with a depolarization of the end-plate region of the muscle fiber. Sodium ions pour in, accelerating the fall in potential in the end-plate and adjacent regions; and this process ultimately gives rise to the propagated action potential.

Recent work by Fatt and Katz (32, 33) and by del Castillo and Katz (21, 23) suggests that ACh is released in discrete, uniformly-sized spurts from various regions of the nerve endings. The evidence for this is based on the discovery of 'miniature end-plate potentials,' (32). With an internal micro-electrode located in a muscle fiber at the end-plate region, it is found that even during quiescence

small spontaneous potentials of rather uniform size and shape occur. Each rises rapidly (1–2 msec.) to a maximum amplitude of 0.5–1.0 millivolts, and then slowly decays over the course of 20–30 milliseconds. The existence of a minimal amplitude response and a stepwise (rather than a continuous) increase in amplitude suggests some basic functional unit. Extensive, careful work by these investigators has led to the tentative hypothesis that the unit responsible for such a potential is a single active 'carrier' molecule for ACh, each carrier molecule making possible the passage of perhaps several thousand ACh molecules through the nerve ending in the course of a few milliseconds. In this picture the occasional freeing of a carrier molecule from an inactive state gives rise to these randomly occurring, miniature potentials. When, under the effects of a nerve impulse, many of these carrier units are brought simultaneously into action, sufficient ACh can be released at one time to produce a critical depolarization and to bring about a propagated impulse in the muscle fiber. Indirect evidence indicates that the amount of ACh released through the action of a nerve impulse increases with calcium concentration over a range of low concentrations. It is speculated that at least some of the carrier molecules in their normally inactive state are combined with calcium, and that a nerve impulse acts only on this complex to create the active carrier molecule (20). Thus, an increase in calcium concentration could be reflected in an increase in the Ca-carrier concentration and a more effective response to a nerve impulse.

Whether the hypothetical carrier molecule actually performs some sort of rapid shuttle service (being associated momentarily with each ACh molecule released), or whether it acts as a true trigger in simply unlocking the flood-gate for an outpouring of ACh, is not known. But, in any case, the amount of ACh released by a single unit appears to be very nearly constant. The first possibility cannot be dismissed simply on the basis that the required turnover rate would be too high. For example, even with lowered estimates for the molecular weight of cholinesterase, the enzymatic turnover rate for ACh (and hence the rate of complex formation between ACh and the enzyme) might well be of the order required here. Possibily some other molecule (carrier) is equally adept at combining with ACh rapidly and also fleetingly. A single carrier molecule with many binding sites for ACh could also be hypothesized in order to relieve the demands made on a single site without losing the unitary character of the response.

Several years ago it was found that the end-plate regions of a muscle fiber are much more sensitive to ACh than are the non-end-plate portions (12, 60). Recent work has shown that the ACh-sensitive 'receptors' must be localized in the outer surface of the muscle membrane in the end-plate region, since ACh supplied intracellularly to this region fails to cause a response (22). The nature of the receptor is not known. However, kinetic analyses of the initial rate of shortening and the final extent of shortening, for an eserinized nerve-muscle

preparation (frog rectus abdominis muscle) exposed to varying concentrations of ACh, suggest that 'activation' of each receptor molecule is achieved by combination with exactly two ACh molecules (14). (Presumably these measurements reflect the response of only the slow fiber component of this muscle; refs. 14, 62.)

The main effect of ACh at the end-plate is believed to be that of increasing the membrane permeability to sodium ions, although a simultaneous increase in permeability to potassium and possibly other ions also occurs. If sucrose is substituted for NaCl in the external medium, ACh causes only a slight reduction in the end-plate potential, indicating that depolarization due to the influx of the positively charged ACh itself is probably negligible (73).

Since a single carrier molecule or unit makes possible the passage of several thousand ACh molecules, an analogous amplification might be looked for immediately at the end-plate in response to the ACh molecules. It has been estimated that some 10^{-18} moles of ACh are released at an end-plate per nerve impulse (2) and that more than 8×10^{-15} moles of monovalent ions flow across the end-plate in the course of initiation of the propagated impulse in the muscle fiber (22). These figures would correspond to the passage of about 10^4 monovalent ions through the end-plate for each ACh molecule. (Such an estimate, however, embodies several over-simplifications and may be regarded only as a very rough first approximation.)

Energy amplification at the neuromuscular junction is difficult to estimate. But it seems clear that a large amplification occurs in the number of ions involved, since the activation of a single ACh-carrier unit may result in the flow of more than 10^7 ions through the end-plate. It is uncertain whether either of the steps at the neuromuscular junction (ACh release and ACh action at the end-plate) can be considered as triggered or triggering processes. The first of these has been discussed above. Nachmansohn and Wilson (72) have postulated that ACh can combine with a hypothetical receptor substance in nerve membrane and produce a shape change in the receptor which allows a freer passage of ions. If such a mechanism were operative at the end-plate, the action of ACh could readily qualify as a triggering process.

EVENTS AT THE MUSCLE FIBER MEMBRANE

In the resting state of muscle the outside of the fiber membrane is positive relative to the inside, the 'resting potential' being of the order of 85–95 millivolts in frog muscle fibers (65, 74). The ratio of intracellular to extracellular electrolyte concentration is: about 40 or 50 for potassium, 1/6 for sodium, and 1/77 for chloride (summarized, 36, 64). According to Boyle and Conway (7), if the resting membrane is able to keep sodium ions out of the cell and is impermeable to intracellular anions such as negatively charged protein molecules, a Donnan distribution of the more permeable K^+ and Cl^- is such as to account for a resting

potential of about 99 millivolts. Since muscle fibers have been shown to be permeable to sodium, an energy-consuming 'pump' mechanism located in the cell membrane has been postulated (50) to extrude sodium and thereby maintain a low concentration of intracellular sodium. Ling (64) has proposed an alternative 'fixed charge hypothesis' for maintaining electrolyte distributions in muscle. This depends on the existence within the cell of a network of protein chains bearing a large number of negative charges. Since the effective (hydrated) diameter of K^+ is believed to be smaller than that of Na^+, the potassium could, on the average, be more closely associated with the fixed negative charges of the proteins than could the larger sodium ions. By this mechanism, it is postulated that potassium might be selectively accumulated within the cell—and sodium essentially excluded—without appeal to a sodium pump. A similar mechanism is proposed at the cell surface to account for the relatively low permeability to sodium. Although the pump theory has won more general acceptance, the arguments presented by Ling must be carefully considered.

In the normal excitation of muscle twitch fibers, the action potential arising from the end-plate region travels in a wave of depolarization down the surface of the muscle fiber. The effects of this disturbance are transmitted inward, inducing the 'active state' which gives rise to the actual contraction.

According to the theory set forth by Hodgkin (50) and Hodgkin and Huxley (51), the depolarization initiated in the region ahead of the travelling action potential induces a marked increase in the fiber membrane permeability, the action potential reflecting a rapid entry of sodium into the fiber (rising phase of the action potential) followed by an outflow of potassium (declining phase of the action potential). There is considerable circumstantial evidence in support of this theory for the basis of the action potential in muscle as well as nerve (50). With sartorius muscle fibers, the height of the action potential increases linearly with the logarithm of the extracellular sodium ion concentration (74) and decreases correspondingly as the intracellular sodium concentration is raised (24). Similarly, the maximum rate of *rise* of the action potential is proportional to the external sodium concentration and is essentially unaffected by the internal sodium concentration; whereas the maximum rate of *fall* of the action potential is proportional to the internal concentration of potassium and is little affected by the external potassium concentration (24).

It was long ago demonstrated that the propagation of the action potential depends on the local state of the fiber. If the local flow of ions from higher to lower concentrations can be viewed as a down-hill (energy-yielding) process (or, more precisely, if this process results in a decrease in free energy for the system composed of the fiber and the medium in which it is immersed), the cost involved in the propagation of the action potential may be readily taken care of. Here again are the essentials of a triggered process: a presumably small but adequate energy input at the end-plate region rapidly causing an energy amplifica-

tion which depends only on the geometry and ionic environment of the living fiber. Various calculations have been made for nerve and muscle fibers to estimate the energy released during a single impulse (36, 50, 81). If the membrane capacity C (5–6 microfarads cm^{-2}) and the maximum amplitude of the action potential V (.120 v.) are substituted in the equation: energy = CV^2, then for a fiber 1 cm long and 100 μ in diameter the value obtained is about .02 ergs $impulse^{-1}$. Although this value may be considerably larger than the corresponding energy change induced at the end-plate, it is still only about 10^{-3} times the work output available from a single twitch of such a muscle fiber.

The amount of sodium transferred from the outside to the inside of the muscle fiber during one impulse can be similarly calculated (quantity of charge = Q = CV; ref. 50). A value of about 4×10^{-12} moles $impulse^{-1}$ cm^{-2} is found. As Hodgkin (50) has pointed out, calculations based on electrical measurements on the fiber membrane give only a *minimum* estimate of the energy released or the number of moles of electrolyte transferred, since these measurements do not take into account any inflow of sodium which is partially balanced by a simultaneous inflow of chloride or outflow of potassium.

Some recent measurements of the fluxes of Na^{24} and K^{42} have been made on resting muscle (16, 18, 59) and on contracting smooth muscle (6). However, problems of diffusion (42, 58) and the possibility of rapid, undetected reabsorption of potassium and re-extrusion of sodium from incompletely mixed pools in the immediate vicinity of individual fiber boundaries complicate the interpretation of these results. The probable interlinking of the cation fluxes in nerve has been discussed by Hodgkin and Keynes (52). Steinbach (84) has given evidence that in frog sartorius muscle the uptake of potassium is dependent on sodium extrusion, and Keynes has found that the rate of sodium loss from frog sartorius or toe muscle is increased when the muscle is transferred to a potassium-rich medium. Weidman (90) has suggested that the effect of potassium on turtle heart action potentials may be explainable on such a basis. In the turtle heart, which has a protracted action potential lasting several seconds at 10°C, an increase in the extracellular potassium concentration during the action potential (by injection into the coronary artery) brings about an abrupt, early fall in the action potential to about half the resting level (90). The Hodgkin and Huxley scheme (51) would seem to require a *slower* fall in action potential under these conditions. However, if superimposed on this slowed response there is an increased rate of sodium efflux induced by the increased potassium concentration, observations on the turtle heart are not inconsistent with that scheme. It is also noted that increasing the extracellular potassium concentration prior to initiation of the action potential causes a slower rate of rise of the action potential. This suggests that even during the rising phase of this slow-motion action potential, some of the sodium inflow is already being countered-balanced by an increased rate of sodium extrusion.

Calcium also plays a role at the fiber membrane. The effect of increased extracellular potassium, in increasing membrane permeability to ions, can be counteracted by increasing the extracellular Ca^{++} concentration (86), whereas lowering the extracellular Ca^{++} concentration causes a lowering of the resting potential (13) and a general increase in membrane permeability (17).

RELATION OF FIBER SURFACE EVENTS TO CONTRACTION

Fleckenstein (36) expresses the view that the energy available from the inflow of sodium and outflow of potassium across the fiber membrane during excitation is sufficient to account for the work of contraction. In such a picture, the entire amplification of the energy input is accomplished in the events at the fiber surface during excitation. In support of this view, Fleckenstein cites the work of Wilde and O'Brien (92) on the slowly contracting turtle heart. Injection with K^{42} *in vivo* was followed, after death, by perfusion of the heart with non-K^{42} Ringer's solution via the coronary artery. The amount of K^{42} recovered in the venous blood per systole was compared with the amount of work performed by such a heart per systole. The work done was found to be approximately equivalent to the maximum work available from the transfer of the recovered amount of K^{42}—and an equivalent amount of sodium—from higher to lower concentrations across the muscle cell membrane. The difference between this calculation and that based on the membrane electrical properties is certainly striking and illustrates the basis for some of the divergent views on the triggering of muscle contraction.

Sandow (83) has recently reviewed the literature on contracture and has stressed the essential similarity between muscle contracture and contraction. Of considerable interest are the 'slow' muscle fibers (somewhat akin to smooth muscle) present to a greater or lesser degree in several skeletal muscles of the frog. These fibers are characterized, in part, by sluggish response to mild stimulation, by the absence of any propagated action potential, and by the presence of multiple innervation per fiber with graded local contractures in response to stimulation (62). Study of these slow fibers has suggested a simple relationship between the degree of contracture and the extent of depolarization of the fiber membrane. In such fibers contracture can be sustained for long periods of time and can be reversed by repolarization of the membrane (61, 35). On the other hand, Niedergerke (76), studying potassium-induced contracture in strips of frog ventricle, has found that a change in Ringer calcium concentration can greatly increase the contracture tension and rate of tension development without significantly altering the extent and time course of depolarization. Other fibers also give evidence of an indirect connection between surface electrical events and contraction. In a crustacean muscle the mechanical response can be completely abolished by long soaking in the presence of tetrabutylammonium ion, while the action potential remains apparently unimpaired though pro-

longed (31). Other substances may enhance contraction, and this may occur with or without a marked alteration in the action potential. For example, another quarternary ammonium ion has given interesting results when applied to frog sartorius muscle. It is found that when sodium ions are partially replaced by tetraethylammonium ions in the external medium, the height of the action potential is reduced (though not to the extent predicted solely by sodium dilution), the declining phase of the action potential is prolonged, and the isometric twitch tension is enhanced—both in magnitude and duration (39). The membrane conductance during the declining phase of the action potential is somewhat smaller than normal. These results suggest that one of the main effects of this substituted cation is a reduction of the increase in the active membrane permeability to potassium (39).

However, a similar enhancement of twitch tension can be achieved with little or no alteration in the action potential. Extensive work has been done on substances which bring about these tension changes since such investigations offer promise of elucidating connecting links between excitation and contraction. Of special interest are the studies of Kahn and Sandow (56, 57) and of Hill and MacPherson (45) on anions. When bromide, nitrate, or iodide are substituted for chloride in Ringer's solution, again a considerable enhancement and prolongation of the twitch tension in frog sartorius muscle are observed. The tetanus tension in these preparations is unchanged, and until lately no change had been observed in the action potential (56, 57). Recently, however, Etzensperger (29), applying an internal microelectrode to frog sartorius muscle, has reported that although the resting potential and spike potential are unchanged by these abnormal anions, there is a detectable prolongation of the negative after-potential.

The anion effect on tension increases in the order $Cl < Br < NO_3 < I$. Calculations of diffusion times of ions into the inter- and intra-fiber spaces indicate that tension augmentation is essentially complete before the anions have had a chance to penetrate the fibers extensively (45). Also, after long equilibration of a muscle in one of the substituted-anion Ringer solutions, a return to the normal chloride medium causes a rapid loss of the augmented response (correlated with the anion exchange in the extracellular fluid), even though the abnormal anion concentration within the fiber has not yet decreased appreciably (45). It is therefore presumed that the anion effect on contraction is mediated at or near the fiber membrane and is not a direct effect on the contractile mechanism itself. Hill and MacPherson (45) have found that the amount of heat production associated with a twitch in the abnormal anion medium is roughly proportional to the magnitude of the contraction. Fibers contracting repetitively in NO_3-Ringer fatigue more readily than fibers similarly stimulated in Cl-Ringer solution. However, if a fiber in Cl-Ringer is stimulated in such a way (two closely spaced stimuli, delivered repetitively) as to induce a mechanical response

similar to that of the fiber stimulated in NO_3-Ringer, the twitch tension decreases along approximately the same time course as that of the fiber in NO_3^- Ringer (57). Thus, the increased heat production and the early fatiguing of muscle in anion-substituted Ringer solution do not appear to be specific anion effects but rather a reflection of a more rapid depletion of metabolic energy reserves.

Since the *tetanus* tension is unaffected by the abnormal anions, it is presumed that the increase in twitch tension does not reflect an over-all intensification of the 'active state' but rather a prolongation of this state (45). A. V. Hill (44) suggests that elastic components in series with active contractile components in muscle act as 'shock-absorbers' so that some of the force of contraction is expended in stretching these passive elastic elements. In a normal twitch, all the contractile components are presumed to become activated, but this active state does not persist long enough to be manifested as a full tension on the ends of the fiber. However, if the active state can be made to last beyond the period in which the elastic elements are being rapidly extended, an increased external tension appears.

Some years ago it was found that application of pressure (of the order of 100 atmospheres) during the early stages of contraction causes an increase in tension and heat production (10, 11), similar to that observed with the abnormal anions. Podolsky (79) has recently suggested that the effects of hydrostatic pressure and of the anion series may be manifestations of the same phenomenon. It is found, for example, that a twitch of a frog sartorius muscle in chloride Ringer's solution at 80 atmospheres appears to be identical with a twitch in nitrate Ringer's at one atmosphere. The temperature dependence of the tension is also similar in the two cases. It is proposed that an increase in hydrostatic pressure may increase the hydration shell of the chloride ion in such a way that the muscle fiber finds this hydrated anion indistinguishable from, say, a hydrated nitrate ion at one atmosphere pressure. It is known that the rate of sodium efflux in cat erythrocytes decreases when chloride is replaced by Br^-, NO_3^-, or I^-, the greatest reduction occurring with iodide (19). When a hydrostatic pressure of 80 atmospheres was applied to these cells in the presence of chloride ion, the rate of sodium efflux was reduced to that in a corresponding nitrate medium at one atmosphere (79). Thus, in two different systems (muscle fiber and erythrocyte) a nitrate response is mimicked by application of 80 atmospheres of pressure to cells in a chloride medium. With certain other anions (acetate, propionate, or butyrate), pressure was found to have little effect on the rate of sodium efflux in erythrocytes (79). Although these results are not conclusive, they indicate that in the range of pressure used (< 100 atm) there is a specific pressure effect on the chloride ion rather than a general alteration of some property of the fiber in response to pressure.

The studies with abnormal anions and with pressure suggest that normally

some anion, such as chloride, participates in a process taking place at the fiber membrane during the very early phases of contraction, and that this process is closely concerned with activation of the contractile component in muscle. What this process may be is not known, though several possibilities have been discussed (45, 57). Etzensperger (29) suggests that the action of abnormal anions in prolonging the negative after-potential is in accord with the hypothesis that the active state persists as long as the membrane potential remains below some critical value. If one attempts to interpret the negative after-potential effect in terms of ion fluxes, it can be speculated that this after-potential reflects, in part, an influx of chloride. It is known that in muscle, in the presence of excess potassium, the anion penetration rate decreases when abnormal anions are substituted for chloride, the rate effect being in the order $Cl > Br > NO_3$ (15). A prolongation of the negative after-potential might therefore be interpreted in terms of a slower penetration rate of abnormal anions. One might also look for alterations in the recovery processes taking place at the membrane in the wake of the action potential, and speculate that a delay in the initiation of some such process prolongs an inwardly directed activation step. There is indirect evidence, for example, that in nerve sodium can be extruded in company with some unidentified anion (52). Although phosphate, formed as an intracellular breakdown product, would seem a likely candidate, Harris finds only a small loss of phosphate from muscle and essentially no reincorporation (41). It may be that chloride normally participates in a sodium extrusion process already initiated in the early stages of contraction.

Since the immediate sequence of events taking place in the muscle fiber following stimulation is not known, it is difficult to speculate on the role which triggering may play at this level. It is generally agreed that the propagation of the wave of excitation is a triggered process in that the 'output' is independent of the 'input' beyond some critical level of the latter. If one adopts the view that contraction depends *directly* on the depolarization of the fiber membrane—i.e., if there are no intermediate steps between depolarization and contraction—then no further triggered processes can be postulated. Although such a seemingly direct relationship has been described in some muscle fibers, other evidence suggests that the situation is more complex. For example, it can be supposed instead that ion shifts associated with membrane depolarization or repolarization are directly responsible for initiating further changes within the fiber. In this case, the final unleashing of contractile energy can occur at a later stage and the possibility of additional triggered processes can be considered.

Prior to the development of positive tension in stimulated muscle, various manifestations of changes taking place within the fiber have been observed. Sandow has made an extensive study of 'latency relaxation', a term applied to the very small drop in tension immediately preceding the onset of contraction in stimulated muscle fibers. Analysis of the magnitude and time course of this

relaxation indicates that it is sensitive to environmental changes. For example, the abnormal anions discussed above intensify the depth of the latency relaxation (57). At about the time that the latency relaxation is beginning, changes in the optical properties in muscle are also observed (46, 47). D. K. Hill has suggested that these changes may be related to the outflow of potassium ions during the declining phase of the action potential. Abbott and Lowy (1) have found no latency relaxation in mantle muscle of squid, but report an increased transparency during the declining portion of the action potential. These observations suggest the possibility of a sudden change of state in some muscle protein delicately poised near the critical point between its soluble and insoluble states. Such a 'discontinuous' change—in response to, say, a small shift in ion concentrations—is a favorite example of a triggered process and one which may well be operative in muscle.

Following the lead of A. V. Hill in studying the initiation and duration of the active state, Ritchie (80) has recently measured the time at which tension first begins to decline following cessation of multiple-electrode, tetanizing stimulation. He has found a correlation between this time interval and the latent period for contraction, the ratio of these two times being independent of temperature. Although no unique interpretation can be assigned to these results at present, this approach offers one more tool with which to probe the active state.

INITIATION OF CONTRACTION

The largest sub-units within a muscle fiber are the long, more or less cylindrical myofibrils which are parallel to the fiber axis and about 1 micron in diameter. These fibrils display the characteristic A and I bands of striated muscle and, depending on the animal source, may be firmly interconnected at the Z membrane (49). Extensions of the Z membrane substance also appear to be continuous with the fiber sarcolemma (26). Recent reports have again raised the question of whether the Z membrane is a distinct 'disc' or whether it is part of a continuous spiral down the length of the fiber. A. Engelhardt (28), for example, reports a spiral arrangement in the fibers of human eye muscles as well as in frog muscle fibers.

A. F. Huxley and Taylor (53, 54), applying a micropipette to the fiber surface of a frog semitendinosus muscle, observe a localized contraction when a depolarizing current is applied opposite an I band, but no response opposite an A band. (A hyperpolarizing current causes no contraction when applied to either A or I bands.) These investigators support the suggestion of earlier workers that changes initiated by depolarization at the fiber surface are normally conducted to the fiber interior along the structurally continuous Z membrane located in the center of the I band. Exactly how the Z membrane may serve in this capacity is not known. A. V. Hill (43) has calculated that diffusion of some hypothetical activating substance released just inside the

fiber membrane would be much too slow for the transmission of the activating process to the interior of a fiber within the required time span of a few milliseconds. The work of Draper and Hodge (25) suggests a heavy concentration of potassium at the Z membrane; however, since this potassium is presumably in the bound form, there is no assurance that electrical conductivity would be facilitated in this structure. If it is supposed that excitation is somehow rapidly transmitted along the Z membrane, there still remains the problem of 'activating' the contractile protein.

Guba and Biró (38) have reported the existence of a thin myofibrillar membrane. Such a membrane might conceivably support propagated electrical changes (within the length of a sarcomere) analogous to those occurring at the fiber surface. It would also offer, per fiber, a total surface area about 100 times that of the fiber membrane, with the possibility of an energy augmentation due to secondary ion fluxes across a large surface. However, Hodge, Huxley and Spiro (49) have found no evidence for such a structure and it seems doubtful that activation depends on a fibrillar membrane.

Within the myofibril is a regular (hexagonal) array of filaments about 100–200 Å in diameter and 400–500 Å apart (40, 48). Detailed descriptions of the myofibrillar structures and the changes taking place during shortening have recently been given by Hanson and H. E. Huxley (40) and by Hodge (48) based on electron microscope studies. Although speculations on the nature of the contractile process differ in these two studies, it is assumed in both schemes that the filaments are largely composed of actin and myosin, and that thin actin filaments pass into the Z membrane.

Actin, myosin A, myosin B, or some combination of actin and myosin have been variously considered to constitute the 'contractile protein' responsible for contraction and tension in active muscle. The properties of these and other muscle proteins have been discussed at length elsewhere (e.g., 71, 77). Extensive studies have been made of the enzymatic activity of myosin A and myosin B in catalyzing the splitting of the terminal phosphate group from adenosine triphosphate (ATP) and related substances.

Recently a series of substances capable of inducing relaxation in muscle has also been studied. Some of these are naturally occurring enzymes—myokinase (5, 67), ATP-creatine-transphosphorylase + creatine phosphate (37, 66)—and others are much simpler molecules such as pyrophosphate (5, 8) and ethylenediamine tetraacetate (EDTA) (9, 88). The importance of 'relaxing factors' in a discussion of trigger mechanisms for muscle contraction becomes clear when the possibility of identifying 'active state' with a state of 'suspended relaxation' is considered. It has been found that glycerinated muscle fibers, contracted by addition of ATP and Mg, will relax provided relaxing factor is supplied in the presence of ATP and Mg^{++}. Addition of a small amount of Ca^{++} can then induce contraction. It has been speculated that in the relaxed state the contractile

protein forms a four-way complex with relaxing factor, Mg^{++} and ATP. Initiation of the active state can then be pictured as an introduction of Ca^{++} which removes or inactivates the relaxing factor and allows contraction to take place. As long as Ca^{++} is present, relaxation is inhibited. Various other schemes have been proposed for relaxing factor action (4, 5, 9, 88, 89).

Considerable attention was devoted in the earlier part of this chapter to substances which act near the membrane surface and in some way lead to a prolongation of the normal activating process. It has been suggested that one step in this activating process is the release of Ca ions in the fiber interior. If the normal activating route is bypassed and Ca ions are supplied directly through artificial means, contraction might also be expected to take place. It is found that a reversible localized contraction does occur at the site of intrafiber injection of Ca^{++} although similar injections of Mg^{++} and ATP are ineffective (30, 75, 91). A further indication that calcium may be released in the fiber interior during stimulation is the finding that stimulation causes an increased rate of outflow of Ca^{45} from frog sartorius muscle previously soaked in Ca^{45} Ringer's solution (94).

Another means of inducing or prolonging the active state would be to apply to the fiber some other substance which directly inhibits relaxing factor action. Ryanodine appears to be such a substance (55). It is found that muscle (frog rectus abdominis) soaked in Boyle-Conway solution containing a small amount of ryanodine contracts irreversibly. The action potential (27) and the ATPase activity of myosin B (55) are unaffected by ryanodine. In glycerinated rabbit psoas fibers (in which presumably the naturally occurring relaxing factors are no longer present), ryanodine does not appear to affect ATP-induced contraction or EDTA-induced relaxation (55), suggesting that ryanodine does not obscure enzymatic sites or relaxing factor binding sites on the contractile protein. These findings appear to be consistent with the view that ryanodine directly inhibits a naturally occurring relaxing factor.

If it is supposed that the active state is normally precipitated by a release of calcium in the fiber interior, the question arises as to the source of this calcium. The micro-incineration studies of Draper and Hodge (25) indicate that some bound metals, probably calcium and magnesium, occur at 400-Å intervals throughout the A and I bands, except in the immediate vicinity of the Z and M bands. As Weber and Portzehl (89) have pointed out in connection with their theory of the contraction-relaxation cycle, movement of ions through a 100- to 200-Å distance as required here is not an unreasonable demand. Recently Hodge (48) has found a similar spacing of about 400 Å in the protein supporting structure between filaments. He has tentatively suggested that this protein may be tropomyosin. It is of interest that tropomyosin has an exceptionally high negative charge, which would make it a particularly attractive haven for cations, and also has a particle length approximately equal to the estimated

interfilament distance in an undried preparation (3, 87). Philpott and A. G. Szent-Györgyi (78) have found a 400-Å spacing in L-meromyosin crystals (L-meromyosin being the 'light' fraction of myosin A obtained by short tryptic or chymotryptic digestion). L-meromyosin is presumed to contain no heavy metals, and its periodicity has been attributed to a possible overlap of the ends of basic units or to the accumulation of salts between the ends of particles. If L-meromyosin is longitudinally oriented along a filament, its 400-Å periodicity may correspond to the spots where the ends of the interfilament (tropomyosin?) bridges impinge. In this connection it is interesting that, from a comparison of amino acid analyses of actin, myosin A, tropomyosin and the meromyosins, Laki has suggested that L-meromyosin may be largely composed of tropomyosin (63; discussed in 71). However this may be, it seems reasonable to suppose that the interfilament bridges correspond to the sites of the bound metal accumulations observed earlier.

CONCLUDING REMARKS

Numerous theories of contraction are detailed in current literature (e.g., 4, 36, 68, 70, 71, 77, 85, 89, 93). It is recognized that, in order for muscle to do work, chemical energy must ultimately be transformed into mechanical work; but how and at what stage this transformation is accomplished remains controversial.

Various steps leading to the development of the active state have been outlined above. Two possibly triggered processes at the neuromuscular junction are followed by the triggering of the action potential along the fiber membrane. Two related phenomena are associated with the action potential—membrane depolarization and a shift in ion distribution about the fiber membrane. In what way these events may be related to the initiation of contraction is not known. In Fleckenstein's theory, discussed above, it is assumed that the immediate free energy for the work of contraction is derived entirely from the electrochemical processes occurring at the fiber membrane during excitation. Other theories assume further intermediate steps between excitation and contraction and require extensive energy augmentation only at a subsequent stage. In certain of these theories it has been postulated that contractile protein is somehow released from the effect of a 'relaxing factor' and that this release gives rise to the development of the active state.

Within a tenuous framework of fact and fancy, it is possible to construct a picture of the final steps in the initiation of this active state. Through some unknown mechanism (such as invasion by competing cations), inter-filament bridge protein is forced to release some of its bound calcium. It may be speculated that the latency relaxation preceding contraction is due to the relaxation of these bridges under the effects of an ion shift. If the inter-filament bridges normally impose a slight distortion of the filament surface, an increase in the

length of the cross-structure could then be reflected in a small, longitudinally-oriented relaxation. It can be supposed that calcium is released in sufficient quantity to inactivate relaxing factor maximally and does so briefly, establishing the fully active state. During this period the contractile protein is free to develop tension and to function as an enzyme. Calcium can thus be considered here as a triggering device in that the performance of the contractile protein following its release from relaxing factor is independent of the releasing mechanism. A rapid removal of free calcium by readsorption or combination with other anions brings about an early decline in the active state. If more calcium is initially released due to a prolongation of the activating process initiated at the fiber membrane, a greater relaxation of bridge protein, and a more pronounced latency relaxation, would be expected. In addition, the active state would be maintained for a longer period and full development of tension in the fiber could be realized.

REFERENCES

1. Abbott, B. C. and J. Lowy. Transparency changes of unstriated squid muscle during contraction. Abstr. XXth Internat. Physiol. Congr., Brussels, 1956, pp. 8–9.
2. Acheson, G. H. Physiology of neuro-muscular junctions: Chemical aspects. *Federation Proc.* 7: 447–457, 1948.
3. Bailey, K. Tropomyosin: a new asymmetric protein component of the muscle fibril. *Biochem. J.* 43: 271–279, 1948.
4. Bailey, K. Structure Proteins. II. Muscle. In: *The Proteins*, vol. II. Edited by H. Neurath and K. Bailey. New York: Acad. Press, 1954.
5. Bendall, J. R. Relaxing effect of myokinase on muscle fibers: its identity with the Marsh factor. *Proc. Roy. Soc. London, s. B* 142: 409–426, 1954.
6. Born, G. V. R. and E. Bülbring. Movement of potassium between smooth muscle and the surrounding fluid. *J. Physiol.* 131: 690–703, 1956.
7. Boyle, P. J. and E. J. Conway. Potassium accumulation in muscle and associated changes. *J. Physiol.* 100: 1–63, 1941.
8. Bozler, E. Interactions between magnesium, pyrophosphate, and the contractile elements. *J. Gen. Physiol.* 38: 53–58, 1954.
9. Bozler, E. Relaxation in extracted muscle fibers. *J. Gen. Physiol.* 38: 149–159, 1954.
10. Brown, D. E. S. Effect of rapid changes in hydrostatic pressure upon the contraction of skeletal muscle. *J. Cell. & Comp. Physiol.* 4: 257–281, 1934.
11. Brown, D. E. S. Effect of rapid compression upon events in the isometric contraction of skeletal muscle. *J. Cell. & Comp. Physiol.* 8: 141–157, 1936.
12. Buchthal, F. and J. Lindhard. Direct application of acetylcholine to motor end-plates of voluntary muscle fibers. *J. Physiol.* 90: 82–83P, 1937.
13. Bülbring, E., M. E. Holman and H. Lüllman. Membrane potential and spontaneous activity in calcium-deficient striated muscle of the frog. *J. Physiol.* 132: 12P, 1956.
14. Cavanaugh, D. J. and J. Z. Hearon. Kinetics of acetylcholine action on skeletal muscle. *Arch. intern. pharmacodynamie* 100: 68–78, 1954.
15. Conway, E. J. and P. T. Moore. Cation and anion permeability constants for the muscle fiber membrane. *Nature* 156: 170–171, 1945.
16. Creese, R. Measurement of cation fluxes in rat diaphragm. *Proc. Roy. Soc. London, s. B* 142: 497–513, 1954.

17. CREESE, R. AND H. E. ROBERTS. Calcium and muscle sodium. *J. Physiol.* 127: 32P, 1955.
18. CREESE, R., M. W. NEIL, AND G. STEPHENSON. Effect of cell variation on potassium exchange of muscle. *Trans. Faraday Soc.* 52: 1022–32, 1956.
19. DAVSON, H. AND J. F. DANIELLI. *The Permeability of Natural Membranes.* Ch. 12. New York: Macmillan, 1943.
20. DEL CASTILLO, J. AND B. KATZ. Changes in end-plate activity produced by pre-synaptic polarization. *J. Physiol.* 124: 586–604, 1954.
21. DEL CASTILLO, J. AND B. KATZ. Effect of magnesium on the activity of motor nerve endings. *J. Physiol.* 124: 553–559, 1954.
22. DEL CASTILLO, J. AND B. KATZ. On the localization of acetylcholine receptors. *J. Physiol.* 128: 157–181, 1955.
23. DEL CASTILLO, J. AND B. KATZ. Quantal components of the end-plate potential. *J. Physiol.* 124: 560–573, 1954.
24. DESMEDT, J. E. Electrical activity and intracellular sodium concentration in frog muscle. *J. Physiol.* 121: 191–205, 1953.
25. DRAPER, M. H. AND A. J. HODGE. Sub-microscopic localization of minerals in skeletal muscle by internal 'micro-incineration' within the electron microscope. *Nature* 163: 576–577, 1949.
26. DRAPER, M. H. AND A. J. HODGE. Studies on muscle with the electron microscope. I. The ultrastructure of toad muscle. *Australian J. Exp. Biol. & M. Sc.* 27: 465–503, 1949.
27. EDWARDS, G. A., E. A. WEIANT, A. G. SLOCOMBE AND K. D. ROEDER. Action of ryanodine on the contractile process in striated muscle. *Science* 108: 330–332, 1948.
28. ENGELHARDT, A. Über den helikoiden Bau menschlicher Augenmuskelfasern. *Anat. Anz.* 101: 233–236, 1956; *Biol. Abstr.* 30: 1024, 1956.
29. ETZENSPERGER, J. Anions anormaux et activite electrique de la fibre musculaire striee. Abstr., XXth Internat. Physiol. Congr., Brussels, 1956, pp. 277–278.
30. FALK, G. AND R. W. GERARD. Effect of micro-injected salts and adenosinetriphosphate (ATP) on the membrane potential and mechanical response of muscle. *J. Cell. & Comp. Physiol.* 43: 393–403, 1954.
31. FATT, P. AND B. KATZ. Electric responses of single crustacean muscle fibres. *J. Physiol.* 117: 15–16P, 1952.
32. FATT, P. AND B. KATZ. Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* 117: 109–128, 1952.
33. FATT, P. AND B. KATZ. Chemo-receptor activity at the motor end-plate. *Acta Physiol. Scand.* 29: 117–125, 1953.
34. FENN, W. O. Muscles. In: *Physical Chemistry of Cells and Tissues,* Ch. 33. edited by R. HÖBER. Philadelphia: Blakiston, 1945.
35. FLECKENSTEIN, A., H. HILLE AND W. E. ADAM. Aufhebung der Kontraktur-Wirkung depolarisierender Katelektrotonica durch Repolarisation in Anelektrotonus. *Pflügers Arch. ges. Physiol.* 235: 264–282, 1951.
36. FLECKENSTEIN, A. *Der Kalium-Nutrium-Austausch als Energieprinzip in Muskel und Nerv.* Heidelberg: Springer-Verlag, 1955.
37. GOODALL, M. C. AND A. G. SZENT-GYÖRGYI. Relaxing factors in muscle. *Nature,* 172: 84–85, 1953.
38. GUBA, F. AND E. BIRÓ. Certain electronmicroscope observations on cross-striated muscle fibrils. *Agrokémia és Talajtan* 2: 41–43, 1953; *Chem. Abstr.* 243f, 1954.
39. HAGIWARA, S. AND A. WATANABE. Effect of tetraethylammonium chloride on the muscle membrane examined with an intracellular microelectrode. *J. Physiol.* 129: 513–527, 1955.
40. HANSON, J. AND H. E. HUXLEY. Structural basis of contraction in striated muscle. *Symp. Soc. Exp. Biol.* no. IX: 228–264, 1955.

41. Harris, E. J. Phosphate liberation from isolated frog muscle. *J. Physiol.* 122: 366–370, 1953.
42. Harris, E. J. and G. P. Burns. Transfer of sodium and potassium ions between muscle and the surrounding medium. *Trans. Faraday Soc.* 45: 508–528, 1949.
43. Hill, A. V. The abrupt transition from rest to activity. *Proc. Roy. Soc. London, s. B* 136: 399–420, 1949.
44. Hill, A. V. Effect of series compliance on the tension developed in a muscle twitch. *Proc. Roy. Soc. London, s. B* 138: 325–354, 1951.
45. Hill, A. V. and L. MacPherson. Effect of nitrate, iodide, and bromide on the duration of the active state in skeletal muscle. *Proc. Roy. Soc. London, s. B* 143: 81–102, 1954.
46. Hill, D. K. Changes in transparency of muscle during a twitch. *J. Physiol.* 108: 292–302, 1949.
47. Hill, D. K. Effect of stimulation on the diffraction of light by striated muscle. *J. Physiol.* 119: 501–512, 1953.
48. Hodge, A. J. Studies on the structure of muscle. III. Phase contrast and electron microscopy of *Dipteran* flight muscle. *J. Biophys. & Biochem. Cytol.* 1: 361–380, 1955.
49. Hodge, A. J., H. E. Huxley and D. Spiro. Electronmicroscope studies on ultrathin sections of muscle. *J. Exp. Med.* 99: 201–206, 1954.
50. Hodgkin, A. L. The ionic basis of electrical activity in nerve and muscle. *Biol. Rev.* 26: 339–409, 1951.
51. Hodgkin, A. L. and A. F. Huxley. Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* 116: 449–472, 1952.
52. Hodgkin, A. L. and R. D. Keynes. Movements of cations during recovery in nerve. *Symp. Soc. Exp. Biol.* no. VIII: 423–437, 1954.
53. Huxley, A. F. and R. E. Taylor. Activation of a single sarcomere. *J. Physiol.* 130: 40–50P, 1955.
54. Huxley, A. F. and R. E. Taylor. Function of Krause's membrane. *Nature* 176: 1068, 1955.
55. Jenden, D. J., J. J. Blum and C. E. Knight. Action of ryanodine on skeletal muscle. *J. Pharmacol. & Exp. Therap.* 116: 32–33, 1956.
56. Kahn, A. J. and A. Sandow. Effects of anions on the mechanical responses of skeletal muscle. *Federation Proc.* 10: 71, 1951.
57. Kahn, A. J. and A. Sandow. Effects of bromide, nitrate, and iodide on responses of skeletal muscle. *Ann. New York Acad. Sc.* 62: 137–176, 1955.
58. Keynes, R. D. The ionic movements during nervous activity. *J. Physiol.* 114: 119–150, 1951.
59. Keynes, R. D. The ionic fluxes in frog muscle. *Proc. Roy. Soc. London, s. B* 142: 359–382, 1954.
60. Kuffler, S. W. Specific excitability of the end-plate region in normal and denervated muscle. *J. Neurophysiol.* 6: 99–110, 1943.
61. Kuffler, S. W. The relation of electric potential changes to contracture in skeletal muscle. *J. Neurophysiol.* 9: 367–377, 1946.
62. Kuffler, S. W. and E. M. Vaughan. Properties of the 'slow' skeletal muscle fibres of the frog. *J. Physiol.* 121: 318–340, 1953.
63. Laki, K. Unpublished observations.
64. Ling, G. Muscle electrolytes. *Am. J. Phys. Med.* 34: 89–101, 1955.
65. Ling, G. and R. W. Gerard. The normal membrane potential of frog sartorius fibers. *J. Cell. & Comp. Physiol.* 34: 383–395, 1949.
66. Lorand, L. Adenosinetriphosphate-creatine transphosphorylase—a relaxing factor of muscle. *Nature* 172: 1181–3, 1953.

67. MARSH, B. B. Effects of adenosine triphosphate on the fiber volume of a muscle homogenate. *Biochim. et biophys. acta* 9: 247–260, 1952.
68. MOMMAERTS, W. F. H. M. *Muscular Contraction, A Topic in Molecular Physiology*. New York: Interscience, 1950.
69. MORALES, M. F. Is energy transferred from ATP to myosin at the moment that ATP is split? In: *Enzymes: Units of Biological Structure and Function*, edited by O. H. GABLER. New York: Acad. Press, 1956, Ch. 15.
70. MORALES, M. F. AND J. BOTTS. A theory of the primary event in muscle action. In: *Currents in Biochemical Research 1956*, edited by D. E. GREEN. New York: Interscience, 1956, pp. 609–627.
71. MORALES, M. F., J. BOTTS, J. J. BLUM AND T. L. HILL. Elementary processes in muscle action: an examination of current concepts. *Physiol. Rev.* 35: 475–505, 1955.
72. NACHMANSOHN, D. AND I. B. WILSON. Trends in the biochemistry of nerve activity. In: *Currents in Biochemical Research 1956*, edited by D. E. GREEN. New York: Interscience, 1956, pp. 628–652.
73. NASTUK, W. L. Electrical activity of the muscle cell membrane at the neuromuscular junction. *J. Cell. & Comp. Physiol.* 42 (Suppl.): 249–272, 1953.
74. NASTUK, W. L. AND A. L. HODGKIN. Electrical activity of single muscle fibres. *J. Cell. & Comp. Physiol.* 35: 39–73, 1950.
75. NIEDERGERKE, R. Local muscular shortening by intracellularly applied calcium. *J. Physiol.* 128: 12–13P, 1955.
76. NIEDERGERKE, R. KCl-contractures of the frog's heart and their modification by Ca. *J. Physiol.* 132: 45P, 1956.
77. PERRY, S. V. Relation between chemical and contractile function and structure of the skeletal muscle cell. *Physiol. Rev.* 36: 1–76, 1956.
78. PHILPOTT, D. E. AND A. G. SZENT-GYÖRGYI. Structure of light-meromyosin: an electron microscope study. *Biochim. et biophys. acta* 15: 165–173, 1954.
79. PODOLSKY, R. J. A mechanism for the effect of hydrostatic pressure on biological systems. *J. Physiol.* 132: 38–39P, 1956.
80. RITCHIE, J. M.: Duration of the plateau of full activity in frog muscle. *J. Physiol.* 124: 605–612, 1954.
81. SANDOW, A. Excitation-contraction coupling in muscular response. *Yale J. Biol. & Med.* 25: 176–201, 1952.
82. SANDOW, A. Fundamental mechanics of skeletal muscle contraction. *Am. J. Phys. Med.* 31: 103–125, 1952.
83. SANDOW, A. Contracture responses of skeletal muscle. *Am. J. Phys. Med.* 34: 145–160, 1955.
84. STEINBACH, H. B. On the sodium and potassium balance of isolated frog muscles. *Proc. Nat. Acad. Sci.* 38: 451–455, 1952.
85. SZENT-GYÖRGYI, A. *Chemical Physiology of Contraction in Body and Heart Muscle*. New York: Acad. Press, 1953.
86. TAMASHIGE, M. Effect of potassium and calcium ions upon the contractile mechanism of single muscle fiber. *Annot. Zool. Japonenses* 25: 15–20, 1952.
87. TSAO, T. C., K. BAILEY, AND G. S. ADAIR. Size, shape, and aggregation of tropomyosin particles. *Biochem. J.* 49: 27–35, 1951.
88. WATANABE, S. Relaxing effects of EDTA on glycerol-treated muscle fibers. *Arch. Biochem.* 54: 559–562, 1955.
89. WEBER, H. H. AND H. PORTZEHL. Transference of muscle energy in the contractile cycle. In: *Progress Biophysics and Biophysical Chemistry*, edited by J. A. V. BUTLER AND J. T. RANDALL. New York: Acad. Press, 1954, vol. 4, Ch. 2.

90. WEIDMAN, S. Shortening of the cardiac action potential due to a brief injection of KCl following the onset of activity. *J. Physiol.* 132: 157–163, 1956.
91. WIERCINSKI, F. J. Relation of pH to the shortening of muscle protoplasm by cations. *Federation Proc.* 11: 172, 1952.
92. WILDE, W. S. AND J. M. O'BRIEN. Time relation between potassium (K^{42}) outflux, action potential, and the contraction phase of heart muscle as revealed by the efflurogram. Abstr. XIX Intl. Physiol. Congr., Montreal, 1953, pp. 889–890.
93. WILKIE, D. R. Facts and theories about muscle. In: *Progress in Biophysics and Biophysical Chemistry*, edited by J. A. V. BUTLER AND J. T. RANDALL. New York: Acad. Press, 1954, Vol. 4, Ch. 7.
94. WOODWARD, A. A., JR. Release of radioactive Ca^{45} from muscle during stimulation. *Biol. Bull.* 97: 264, 1949.

Triggering of the Contractile Process in Insect Fibrillar Muscle[1]

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IN THE SEQUENCE OF EVENTS that initiates muscle contraction three basic processes are involved: *1*) excitation, the depolarization of the muscle membrane; *2*) coupling, or the transfer of excitation to the contractile parts of the cell; and *3*) activation, the setting up of the active stage in the contractile elements. Once in the active state the contractile elements shorten, if allowed, at a velocity determined by the external force transmitted to them through the series elastic element. In muscle, therefore, several events are triggered in sequence.

Into this general picture of the initiation of contraction must be fitted the anomalous behavior of insect fibrillar muscle. Characterized by its large, easily separated fibrils, fibrillar muscle is specialized to produce vibratory movement. It is found as the flight muscle of the higher insects, as the muscle moving the halteres of flies, and as the tymbal muscle of the song-making apparatus of certain cicadas. Pringle (11), using external electrodes, noted that the action potentials of the flight muscle of flies are of a much lower frequency than the wing movements. This surprising independence of action potentials and muscle cell response was confirmed by experiments in which electrodes were placed within single muscle cells (5). Pringle, observing that the indirect flight muscles of flies did not shorten on stimulation, suggested that the action potentials sensitized the muscles to activation by stretch. Upon being stretched by its antagonist, therefore, each muscle would shorten.

The wing articulation of flies was shown by Boettiger and Furshpan (1) to store elastic energy by straining the thorax at the beginning of each stroke, and to release it at a critical point as an aid in completing the movement. The rapid unloading of the muscle that results from this action should reduce the muscle tension, as in the quick release experiments of Gasser and Hill (6). They had found that when a tetanized, isometrically contracting muscle is allowed to shorten rapidly a small amount, the tension falls to zero and then gradually returns to the level characteristic of the shorter length. The movement cycle

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might, therefore, be accounted for by assuming that the action potentials of the fibrillar muscle were of a frequency to induce tetanus. Upon being quick-released by the click mechanism, the tension in the tetanized shortening muscle would fall. A subsequent quick stretch by the antagonist on the return stroke would bring the muscle to full length before the tension redeveloped (1). To explain the work cycle the muscles must exert higher tensions on shortening than while lengthening.

Experimental work was undertaken in our laboratory to test this theory. McEnroe (8) demonstrated that tension was maintained during tethered flight in the fibrillar muscle of flies. In these experiments, one end of the muscle was attached to a stiff recording lever while the other end, through which the muscle was innervated, remained *in situ*. Typical twitches and tetanus resulted upon electrical stimulation of this preparation (9). The shortening possible in these muscles is extremely small, 0.02–0.04 millimeters, and had escaped previous notice. The processes of excitation, coupling and activation are, therefore, not modified in fibrillar muscle and the physiological trick by which the muscle performs its function is in the contractile mechanism itself. Pringle (12) observed similar behavior in a more favorable preparation, the fibrillar tymbal muscle in the song-making apparatus of certain cicadas. He used the term "deactivation' to describe what happens in the muscle when suddenly unloaded by the click mechanism of the tymbal. Gasser and Hill had explained the momentary loss of tension in frog muscle as due to a more rapid shortening of the elastic element than of the contractile element rather than as a basic change in the contractile mechanism.

The deactivation of the tymbal muscle was considered by Pringle to be of very short duration, the muscle being restretched by the recoil of the tymbal before reactivation could occur. This explanation is not adequate to account for the observed behavior of the flight mechanism. A number of records obtained by us showed the muscle cycle suddenly blocked with one muscle short and the other long for a period equivalent to the duration of several cycles. When movements again began, the longer muscle shortening and stretching the shorter muscle, it was evident that the tension had not returned in the muscle whose restretching had been delayed. One may alter the frequency of movement in the same insect from 40 per second to 330 per second, by changing the wing load. The deactivation-reactivation cycle must have quite a different duration at these extreme frequencies. It is necessary, therefore, to postulate a reactivation by stretch as well as the deactivation by release. That stretching a quick-released fibrillar muscle does increase the rate of rise in tension was shown experimentally by Boettiger and Furshpan, (3, 4).

Recent experiments to be described here have demonstrated oscillatory movements in fibrillar muscle in the absence of a click mechanism. As background for the discussion of these results, it will be necessary to define some of the properties of a vibrating system.

CHARACTERISTICS OF VIBRATING SYSTEMS

The unit of motion in a vibration is a cycle consisting, in the case of a muscle, of a shortening and a lengthening. A vibration always occurs about an equilibrium point, the movements and forces being measured plus or minus the equilibrium values. A perfect spring and a mass set into vibration will maintain continuous sinusoidal motion in the absence of all frictional forces. All real vibrating systems generate heat in frictional damping and so can be maintained in motion either by an external force, a driven oscillation, or by an internally generated force. If the sustaining force is internal and itself non-oscillatory in nature, the movement is called a self-excited vibration. In such a system frequency is determined by the natural oscillating period. The oscillation is maintained by a sustaining force which, in the steady state condition, is just sufficient to overcome all damping during the cycle. The instantaneous value of this force is in some way controlled by the motion, the force being zero when there is no motion. In the simplest case of sinusoidal motion the sustaining force is always equal and opposite to the damping force. Since the damping force increases and decreases with movement velocity, the sustaining force must do so also, its action directed to aid motion throughout the cycle.

If to the simple mass and spring system is added an internal mechanism to generate the sustaining force, we have a model showing many properties of fibrillar muscle. The behavior of this model will be used in discussing the experimental results. The muscle model contributes the elastic and sustaining forces, while the mechanical system with which it operates furnishes the damping and inertia.

Using the principle of D'Alembert and considering inertia as a force, the static method of analysis may be employed. The sum of all forces acting is equal to zero throughout the cycle and the following relation may be written:

$$m\ddot{x} + c_1\dot{x} = c_2\dot{x} - kx \qquad (1)$$

where: $m\ddot{x}$ is the inertia force, mass, m times acceleration, $\ddot{x}$; kx is the apparent elastic force proportional to displacement, x; $c_1\dot{x}$ is the damping force, proportional to velocity, $\dot{x}$; and c_2x is the sustaining force, proportional to velocity, $\dot{x}$. The sustaining force aids the elastic force during the shortening phase when this force is opposed by damping and aids the inertia force when its action in lengthening the spring is also opposed by damping. It aids the inertia force by opposing the elastic force against which the inertia must work.

The relations between the forces throughout the movement cycle are illustrated in figure 1. Figure 1*A* is a vector diagram of the maximum values of the forces; OA, the apparent elastic force; OB, the damping force; OC, the inertia force; and OD, the sustaining force. The damping force OB is in phase with velocity and so leads the elastic force OA in phase with movement, by 90°. The inertia force is in opposite phase to the elastic force, and so leads by 180°. The

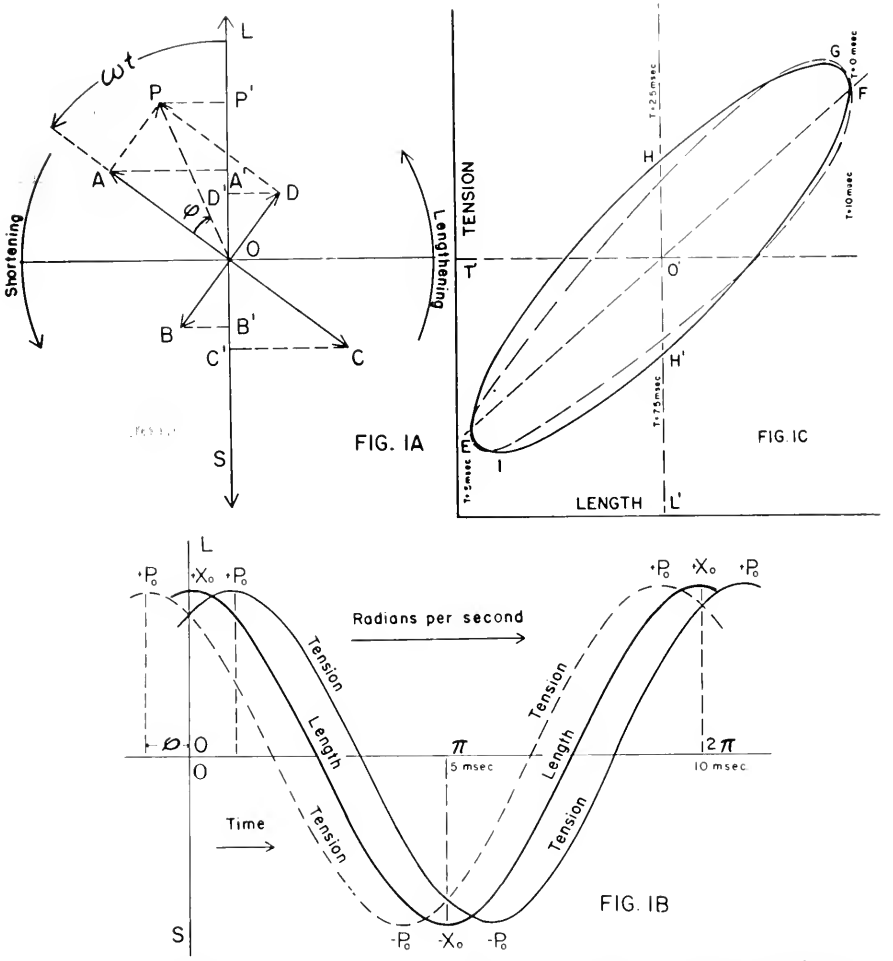

FIG. 1. *A*, force vector diagram of the model vibrating system. OA, the elastic force; OB, the damping force; OC, the inertia force; OD, the sustaining force; OP, the resultant of OD and OA; φ, the phase angle; ω, angular velocity; t, the time. *B*, relative values of tension and length during a 10-msec. cycle of the model system for a positive phase angle, *dotted*, and negative phase angle, *solid*. $\pm P_0$, maximum and minimum tensions; $\pm x_0$ maximum and minimum lengths; φ, phase angle. *C*, dynamic tension-length relation for the model system, *solid line*, and for the muscle, *dashed line*. E, minimum length, F, maximum length, O, equilibrium point. See text.

sustaining force OD opposed the damping force and is opposite to it on the diagram. Applying *equation 1*, these forces must be balanced during the cycle. The maximum force vectors are imagined to rotate counter-clockwise about the zero point, with the same angles maintained between them. The force at any instant is the projection of the vector upon the line LS, the direction of motion; e.g. OA′, OB′, etc. The cycle is considered to begin when length is maximum.

As the elastic force is in phase with motion it is also maximum at this instant so OA lies on line OL.

For a muscle, the apparent elastic and sustaining forces are components of the muscle tension, which in case of the model is represented by OP, the resultant of OD and OA. The resultant OP makes the angle φ with OA, the elastic force, and so also with the length. This angle is the phase angle, the existence of which accounts for the sustaining force. In figure 1*A*, tension OP lags behind length OA. To obtain the fraction of cycle completed at any instant by the tension, the angle φ must be subtracted from the angle ωt completed by the length. If the tension rotates ahead of length, the angle φ must be added. In the first case the phase angle is considered negative, in the second case positive. Only when the phase angle is negative is there a sustaining force component to oppose damping and so a self-excited vibration.

Tension and length are the parameters usually measured. The change in tension during a 10-millisecond cycle of shortening and lengthening is shown in figure 1*B*. For a negative phase angle the tension is given by the full line, for a positive phase angle by the dotted line. As length change is in phase with apparent elastic force (fig. 1*B*), the relative values of this force are also represented by the line showing length. The tension in the muscle is above that of the elastic component during shortening and below during lengthening. The difference between the total tension and that of the elastic component determines the value and direction of the sustaining force.

Vibrations may be conveniently studied by plotting tension as a function of length. This can be done experimentally, using a cathode ray oscilloscope. A tension transducer is connected to give a vertical deflection of the beam, and a length transducer to give horizontal deflection. In figure 1*C* the solid line is a tension-length plot of the model system in sinusoidal motion with a phase angle of 30° and a period of 10 milliseconds. The force of gravity on the mass shifts the equilibrium length and tension to the values L′ and T′. The tension varies from $T' + P_0$ to $T' - P_0$; and the length from $L' + x_0$ to $L' - x_0$. The line EF gives the apparent elastic force at each length, the slope of the line being a measure of the elastic constant. Points on the loop are values of the total tension OP. The direction of movement of the beam in drawing out the loop is clockwise for a positive phase angle, and counterclockwise for a negative phase angle. The difference between the elastic and total forces at any length is a measure of the sustaining force; OH and OH′ represent the maximum values of this force at the two points of the cycle when length equals the equilibrium length L′ and velocity is maximum.

The area of the loop is the external work done by the muscle model against damping each cycle. For sinusoidal motion it can be shown that:

$$\text{work per cycle} = \pi P_0 x_5 \operatorname{Sin} \varphi \qquad (2)$$

$$\text{Power output per second} = \pi f P_0 x_0 \operatorname{Sin} \varphi \qquad (3)$$

where: f is frequency in cycles per second; P_0 is maximum departure of tension plus or minus the equilibrium value, T′; and x_0 is maximum departure of length plus or minus equilibrium value, L′. The work done is therefore a function of the phase angle, the maximum value of the tension above or below the equilibrium value, and the maximum length above or below the equilibrium length. The frequency of the vibration depends chiefly upon the mass and the elastic constant of the spring. A stiffer spring and smaller mass will increase frequency.

In the foregoing discussion the behavior of a model self-excited system is reviewed. The oscillating motion is seen to be maintained by an internal force component that changes sign and magnitude throughout the cycle and so aids the movement on both the shortening and lengthening strokes. This component arises as the result of a shift in the phase relations of tension and length, the length reaching its maximum and minimum values before tension. To what extent does fibrillar muscle resemble this model?

EXPERIMENTAL STUDY OF THE MECHANICAL PROPERTIES OF THE BUMBLE BEE FIBRILLAR MUSCLE PREPARATION

To study fibrillar muscle as a mechanical system, it was necessary to find a muscle that was large, easily attached to the recording system, and readily available. A satisfactory preparation was finally made using the longitudinal flight muscle of the common large bumble bee, Bombus. The head and abdomen are first removed and the posterior phragma to which the muscle is attached, exposed. The thorax is then impaled, posterior end up, on two needles projecting vertically from a small mounting board. With a third needle inserted at right angles to the other two, the thorax is rigidly held. A small wire hook, carefully inserted into each side of the saddle-shaped phragma, is attached through a light chain to an RCA mechano-transducer tube for recording tension. As a last step the arms of the phragma that extend forward to the articulation are cut. Stimulation is accomplished through the two vertical needles.

In the first series of experiments with this preparation, the results obtained with fly muscle (8) and with the tymbal muscle of the cicada (12) were confirmed. The isometric twitch tension was small compared to the tetanus tension. Fusion of the contractions was complete at about 40 stimuli per second. The build-up of tension on stimulation to maximal tetanus, and its decrease at the end of stimulation, were relatively slow; contraction time 0.4 seconds, relaxation time 0.5 second.

The static tension-length curve was determined by allowing the muscle to shorten without a load to the desired length and then to build up tension without shortening. The results are shown in figure 2 and are typical for many types of striated muscle. The active tension curve (OA) is for the muscle

stimulated to maximal tetanus, the passive curve (BC) for the unstimulated muscle. In the insect the muscle is held at the length at which maximum tension is obtained. Due to mechanical limitations in the articulation, the maximum shortening possible in the insect is only about 0.2 millimeter, though greater shortening is shown by the preparations. The maximum shortening of unrestricted muscle, however, is only about 12 per cent of the muscle length, a value much smaller than that usually found in striated muscle but comparing well with the behavior of the tymbal muscle (12).

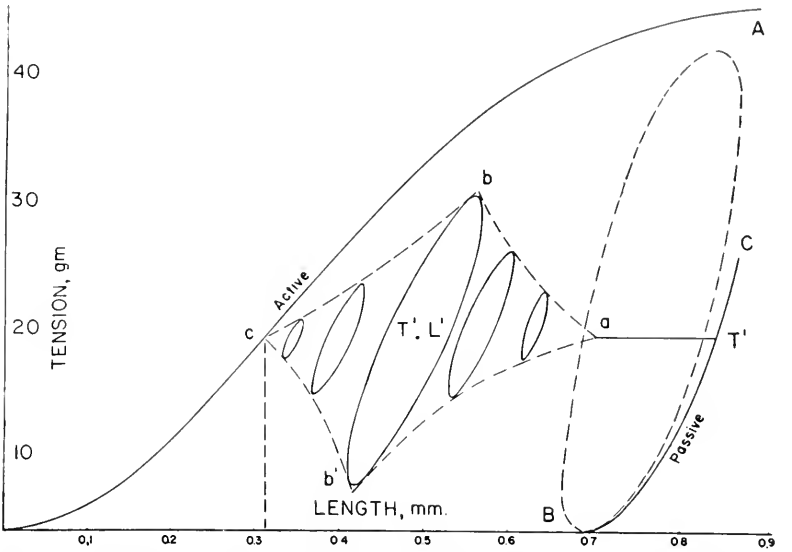

FIG. 2. Tension-length relations of fibrillar muscle. OA, the isometric tension of active muscle as a function of length; BC, elastic behavior of the passive muscle. Length scale in millimeters above minimum muscle length. *Loops* (including bb', the steady state tension length relation of vibrating muscle) are not actual records but show the general behavior of the muscle. *Large dashed loop* is the possible tension-length relation of the muscle in the intact insect.

In all these experiments there was no evidence of oscillatory behavior and the muscle appeared to have no unique properties. However, for a self-excited vibration one would not expect oscillation unless the system had an appreciable period of vibration. The addition of a weight provided the necessary inertia and the muscle, on stimulation, was then set into vibration. The oscillations were recorded as dynamic tension-length diagrams by a slight change in the setup. The mounting board was attached to a short piece of metal hinged at one end to a fixed point. The metal strip provided the load. Additional weight could be added to it as needed. By changing the friction in the hinge, an increase in damping could be achieved. The tension in a small spring attached

to the metal strip was recorded with another mechano-transducer. The output of this transducer was proportional to length, as the tension in a spring is determined by its length. A typical result of stimulating the loaded muscle is diagrammatically shown in figure 2. The load raised the passive tension to T′, equal to the weight. On stimulation the muscle began to shorten to *a* (fig. 2) normally at first, but soon went into vibration, the vibrations increasing in amplitude with further shortening until a steady state (*bb′*) was achieved. The beam moved counterclockwise around the loop, and work, equal to the area of the loop, was done against the damping forces. When damping was increased the area usually became larger.

COMPARISON OF FIBRILLAR MUSCLE WITH THE MODEL SYSTEM

There was considerable variability in the shape of the loops experimentally obtained. Apparently the phase relations between tension and length can vary during a single cycle. Symmetrical loops were, however, found, as illustrated in figure 1*C*, the dotted line. This loop corresponds quite well with the one drawn for the model system, having a constant phase angle of 30° and sinusoidal motion. The characteristics of the mechanical system to which the muscle was attached determined in large part the shape of the loops.

Position of the Loop in the Tension-Length Area. The position of the loop may be discussed in terms of the equilibrium point, T′L′, of the vibration (fig. 1*C*). The tension, T′, is the weight load. If the oscillations are damped out by an external agent, the muscle shortens until it attains the length at which, under static conditions, it can just exert a force equal to the weight. In figure 2 the steady state condition is the loop *bb′*; the tension and length of the completely damped muscle is represented by *c*. When the damping agent is removed, the vibrations reappear and the equilibrium length of the muscle increases to the steady state value, L′ (fig. 2). While in vibration the muscle has an average velocity which determines the average force the muscle can exert, as defined by Hill's characteristic relation. As the amplitude or frequency increases, the average velocity also increases. The tension the muscle is able to exert at higher velocity is less at each length. Consequently, if the tension, T′, is constant, the muscle must elongate as the velocity is increased. By this device the operating loop is forced over into the region of the tension-length area in which the greatest amplitude and tension changes are possible. L′ depends upon amplitude and frequency, T′ on the weight load. As tension drops below the isometric value when the muscle shortens, and as the loop moves to the right in the tension-length area with increasing velocity, the characteristic force-velocity relation of Hill is present in fibrillar muscle.

Work Cycle of Fibrillar Muscle. Any theory explaining the physiology of fibrillar muscle must account for the large energy output. If the muscle behaves as the model in its tension-length relations, the work done can be calculated

from *equations 2* and *3*. In the best cases (fig. 1*C*, dotted line) the experimental loops resemble those of the model when the phase angle is 30°. The following data may therefore by used to calculate the energy output: P_0 = 25 gm, maximum recorded tension was 50 gm and P_0 is one-half the tension change; x_0 = 0.01 cm, one-half the probable movement; f = 100 sec. and the phase angle, 30°. The calculation shows that in the work cycle of one muscle, 375 ergs are generated. In flight the insect uses two muscles in opposition. Assuming they do equal work, the energy output per cycle for the insect is 750 ergs, and the power output per second, 75,000 ergs. As the aerodynamic efficiency of insect wings has been shown to be about 66 per cent (7) the power available to move the insect is 50,000 ergs per second. This value is of the right order of magnitude, though somewhat low considering the large size of the bumble bee. An increase might be achieved if muscle tension and/or the phase angle is greater in the insect than in the preparation.

To get the maximum size loop in the available area (the large loop of fig. 2) the weight load, T′, should be one-half the maximum tension. The click mechanism acts in the same manner as a mass, and so contributes to the value of T′. Wing inertia is also an important factor in determining the position of the loop.

From these calculations it is apparent that the flight muscle must operate with remarkable efficiency and be able to use a very large portion of the tension-length area available to it. This available area is only a part of the total area of figure 2 because of the restrictions to muscle shortening mechanically imposed on the muscle *in situ*. The size of the work area depends upon the position in the tension-length area of the shortening and lengthening curves.

Shortening and Lengthening Phases. During flight the shortening and lengthening strokes of the muscle are phases of a motion cycle. That the motion is nearly sinusoidal has been shown by recording muscle length changes during tethered flight. A tiny mirror was placed on the scutellum of a fly and the time relations of its motion traced out on moving film by a reflected light beam. Movements of the scutellum were shown to follow changes in length of the muscles (2). If the movement is sinusoidal and the work cycle uses most of the available tension-length area, the shortening and lengthening phases during flight must be similar to those of the large loop in figure 2.

Although the data obtained are only semi-quantitative, it appears that the tensions during vibration are below the static tensions (curve OA of fig. 2) at all lengths. In muscle, unless shortening is very slow, the tensions fall below the isometric level by an amount that is a function of the shortening speed. Non-fibrillar muscle driven through a cycle by an external force exhibits at each length lower tensions on shortening than on lengthening. This is opposite to the results reported here, and so in fibrillar muscle the shortening and lengthening phases are reversed. Such a reversal was actually observed in

fibrillar muscle made to shorten and lengthen at various frequencies by an external agent. Unfortunately, the movement was not sinusodal due to mechanical limitations, and so the loops found were quite irregular. At lower frequencies the tension was greater on lengthening and smaller on shortening, the cycle absorbing energy. With increasing frequency the phases gradually reversed so higher tensions occurred during the shortening phase, the muscle

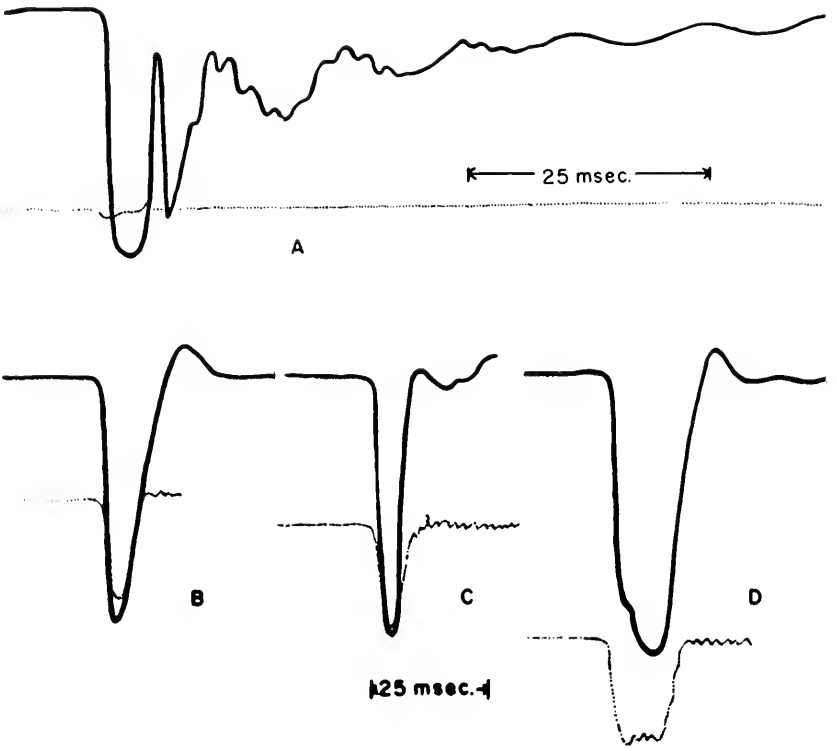

FIG. 3. Effect of quick release and quick stretch on tension of the isometrically tetanized fibrillar muscle preparation. *A*, 1–2 msec. stretch; *B*, a 5-msec. stretch; *C*, control unstimulated muscle stretched to same tension as in *B*; *D*, delayed restretch showing loss of tension at end of release.

now doing work against the driving force. This shift amounts to a change in the phase angle from positive to negative. The crossover point did not occur at the same frequency in all preparations, and with the crude experimental set-up available, quantitative analysis was not possible.

One can further investigate the shortening and lengthening phases by releasing and restretching a tetanized preparation in isometric contraction. The results of such experiments are shown in figure 3. When the muscle is allowed

to shorten rapidly, 0.1 0.2 millimeters, the tension falls to, or nearly to, zero and then slowly returns to a level characteristic of the shorter length. This behavior is apparently a property of all muscles and of the glycerinated psoas preparation as well. In fibrillar muscle, however, as shown in figure 3*D* following the rapid shortening (quick release), the tension continues to fall even though the muscle length is not changing. This fall in tension has not been demonstrated in any other muscle. A still more dramatic difference in behavior is seen when fibrillar muscle is restretched. The full length is attained with a return of only one-third to one-half the initial tension, fig. 3*B*. Following the completion of the stretch, the tension rapidly rises and may even exceed momentarily the level at the time of release. To show that this behavior is not due to some property of the recording system, the changes in length were repeated with the unstimulated muscle stretched passively to the same tension found in the active muscle at the moment of release. The result is shown in figure 3*C*. In this control experiment tension and length are in phase.

There is an optimum speed of stretch for the muscle which is related to the normal operation frequency of the system. In the experiment shown in figure 3*B* there is no break in the curve of tension rise to indicate the end of the stretch. This stretch had a duration of 5 milliseconds. For a full cycle at this rate the period would be 10 milliseconds and the frequency 100 per second, which is the characteristic wing beat frequency in these bumble bees. If the stretch is slower, the rise of tension is slower, a greater portion of the tension rise occurring during the change in length. With more rapid stretches a definite notch appears in the tension record at the end of the stretch. An extreme case is seen in figure 3*A* where the stretch was accomplished in 1–2 milliseconds. The tension rises during the stretch to values near or above the isometric tension, and then falls as quickly almost to the level at which the stretch started. The subsequent rise in tension was erratic, as though some damage had been done to the muscle.

As Pringle (12) has pointed out, there are no adequate experimental results to show changes in tension in a muscle restretched after a rapid shortening. Attempts by us to repeat the above experiments on comparable non-fibrillar muscle have not been fully satisfactory. However, the flight muscle of a moth showed a rapid rise in tension during restretching, the tension overshooting the isometric level and then slowly returning to this level. As this behavior is quite different from that observed in fibrillar muscle, one may conclude that fibrillar muscle is unique in its response to release and restretch. Thus, in any movement the tension changes lag behind the length changes. When the movement is suddenly stopped, the tension change continues until it again comes into phase with length. So after a rapid shortening the tension falls, and after a rapid lengthening the tension rises.

ORIGIN OF THE SELF-EXCITED VIBRATION OF FIBRILLAR MUSCLE

The extensive data obtained by Hill and his co-workers have been interpreted to show that muscle is a two-component system consisting of an undamped series elastic element and a contractile element. The elastic element cannot be studied in the presence of the contractile element unless special precautions are taken. Either the shortening of the contractile element must be calculated and subtracted from the muscle shortening, or the muscle shortening must be so rapid that the contractile element does not have time to shorten. The elastic force component of the vibrating system might be identified with the action of this elastic element. In fibrillar muscle the elastic element is quite stiff, requiring roughly a force of 2.5 kilograms to stretch it 1 centimeter. If the line EF of figure 1*C* is the tension-length relation of the pure elastic element, deviations from these tensions are due to the presence of the contractile element, which must generate the sustaining force by shortening and lengthening. The change in length of the contractile element in the cycle is somehow controlled by the motion or the tension changes during the cycle.

The changes in length of the elastic element EE and the contractile element CE might occur in the following manner. Referring to figure 1*C*, from F to G the CE is shortening while the EE is lengthening to reach its maximum value when the tension is maximum at G. From G to H both EE and CE are shortening, CE reaching its shortest length at H. The CE lengthens from H to E while the EE is shortening, and so the tension falls more rapidly than the pure elastic tension, the slope of the line EF. At E the rate of shortening of the EE equals the rate of lengthening of the CE and so the muscle reaches its shortest length. From E to I the CE lengthens more rapidly than the EE shortens, so the tension falls and the muscle lengthens. At I the EE reaches its shortest length and begins to lengthen and so from I to H′ both the CE and the EE are lengthening. The CE begins to shorten at H′ while the EE is lengthening and at F the rates are equal and the muscle attains its greatest length.

The EE shortens and lengthens in the cycle in phase with muscle tension (the greater the tension the longer the spring) and so lags behind muscle length by the phase angle. The shortening and lengthening cycle of the CE precedes muscle length by 90° and so is ahead of the EE by $90° + \varphi$. The amount of shortening of the CE determines the phase angle between tension and length by setting the value of the sustaining force. If the CE does not go through a symmetrical cycle such as the one discussed above, the loops will be irregular. Because the loading of the muscle is properly controlled during flight, the loops are more symmetrical and larger than those obtained with the isolated preparation.

This behavior of fibrillar muscle cannot be fully explained in the present state of our understanding of muscle physiology. The agent controlling the activity of the contractile element operates continuously throughout the cycle and in relation to the events of the cycle. The agent cannot exert its effect through the diffusion of a substance to and from the active sites, for the complete cycle of deactivation and reactivation can occur in less than 0.5 millisecond. Sotavalta (13) has noted a frequency of 2200 muscle cycles per second, and this high frequency must be accounted for in any theory of mechanism. The controlling mechanism must be held structurally in close association with the active sites of the contractile protein. This could be accomplished by a new fibrous protein system paralleling the actomyosin chains. The tension-sustaining chemical bonds within the contractile protein could be neutralized by bonding laterally with the new protein. Rapid shifts in these bonds result in the cycle. The large size of the fibrils, averaging almost exactly double that of comparable non-fibrillar muscles (10), is at least consistent with this idea.

As the change produced by the agent appears as an alteration in the tension-sustaining ability of the muscle, it can be described as a change in the active state. The change in active state is viewed here, however, not as a reversal of the activation process by which the active state is set up but as a direct independent effect upon the contractile mechanism.

The question posed by the title of this paper may now be answered. The individual changes in length of activated fibrillar muscle are not triggered, but are rather phases of a process that is continuous throughout the cycle. Once the muscle is set into the active state and allowed to shorten with a load, oscillations are initiated. With regard to triggering the active state, as with other properties, fibrillar muscle behaves as a typical striated muscle.

REFERENCES

1. Boettiger, E. G. and E. Furshpan. Observations on the flight motor of Diptera. *Biol. Bull.* 99: 346, 1950.
2. Boettiger, E. G. and E. Furshpan. The mechanics of flight in Diptera. *Biol. Bull.* 102: 200–211, 1952.
3. Boettiger, E. G. and E. Furshpan. The response of fibrillar muscle to rapid release and stretch. *Biol. Bull.* 107: 305, 1954.
4. Boettiger, E. G. and E. Furshpan. Mechanical properties of insect flight muscle. *J. Cell. & Comp. Physiol.* 44: 340, 1954.
5. Boettiger, E. G. and F. McCann. Single fiber action potentials in insect fibrillar muscle. *Federation Proc.* 12: 17, 1953.
6. Gasser, H. S. and A. V. Hill. The dynamics of muscular contraction. *Proc. Roy. Soc. London, s. B* 96: 398–437, 1924.
7. Hocking, B. The intrinsic range and speed of flight of insects. *Tr. Roy. Entomol. Soc. London*, 104: 223–345, 1953.
8. McEnroe, W. Tension-length curves of insect fibrillar muscle. *Federation Proc.* 11: 104 1952.

9. McEnroe, W. Insect fibrillar indirect flight muscle. Master's Thesis. Storrs, Conn.: Univ. of Connecticut, 1954.
10. Pipa, R. L. A comparative histological study of the indirect flight muscles of various insect orders. Master's thesis. Storrs, Conn.: Univ. of Connecticut, 1955.
11. Pringle, J. W. S. The excitation and contraction of the flight muscles of insects. *J. Physiol.* 108: 226–232, 1949.
12. Pringle, J. W. S. The mechanism of the myogenic rhythm of certain insect striated muscles. *J. Physiol.* 124: 269–291, 1954.
13. Sotavalta, O. Recordings of high wing-stroke and thoracic vibration frequency in some midges. *Biol. Bull.* 104: 439–444, 1953.

The Nerve Trigger

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THE NATURE OF NERVE ACTIVITY and the analogy to the operation of a trigger mechanism have long been recognized, and the 'all-or-none law' of excitation and propagation has come to be one of the fundamental principles of physiology. The next questions have been: What is an adequate stimulus or trigger 'pull,' and what does it release?

It has often been assumed, and accumulated evidence strongly suggests that a sufficiently large and rapid decrease of the electrical potential difference across a nerve membrane may be the principal condition for the initiation of nerve activity. Although an increasing emphasis has been placed on the search for an electrochemical system which could be 'exploded' by such a 'trigger,' crucial experiments of the past decade have clearly differentiated the physical and the electrochemical components of the system. The electrochemical problems have been robbed of much accrued drama and glamour, but the questions have been made highly specific and the answers of paramount importance.

At the present stage of development, it cannot be overemphasized that in the control of ionic flows across the nerve membrane there is nothing even remotely resembling a metastable state, and that of itself the ionic system is completely incapable of reacting explosively. On the contrary, these ionic currents are quite docile, rather leisurely, and only slightly greedy in their responses to the controlling electrical potential. It is only with extreme provocation, with an overwhelming and urgent drive, that they allow themselves to be gotten into a position where, as they begin to give way, the pressure upon them becomes even greater. But, at any and all times, upon the removal of this pressure they also revert to the appropriate condition without any snap or jerk and only as a sedate and dignified compliance with a gentle call.

But if the ion movements of themselves are so complacent and entirely cooperative, where lies the basis of the trigger-like sensitivity, the hard-hitting—almost vicious—response that makes a nerve so effectual a channel of information and control? It cannot be in the purely physical electrostatic capacity of the nerve membrane. This seems to be a completely passive, utterly inert, entirely linear and but slightly wasteful structural component under all conditions. Strictly as ion pairs accumulate across it, it becomes stressed and the electrical potential builds up and decreases only as the ion pairs disperse.

The metastability and consequent explosive potentialities lie *only* in the

coupling of these two components in the chemical-physical system that is the functional membrane. The electrical capacity is a reservoir of energy which can be delivered at almost any desired rate and stands in the membrane as inexorable as a taut spring, gravity, thermal energy and a true justice.

As was mentioned in passing, an ion mechanism—that of sodium—is slightly greedy, and as the pressure on it to pass these ions is lessened, it asserts itself and opens the gate wider. But as a little ion current comes from the condenser, the potential provided to the sodium conduction control is diminished and the ion current tends to increase. This combination thus has in it the essential characteristics from which instability can arise. And, indeed, after passing over the hump, the sodium is the victim of its own greed—the more it gets, the more it wants; but it can do so only because the utterly orthodox capacity stands ready, willing and able to gorge it, and at the same time modifying the conditions to further increase the sodium's appetite for more ions until it is satiated.

Other analogies are equally as good. The increase of reaction rate with temperature speeds the increase of temperature in an exothermic reaction and leads to burning or explosion under conditions approaching adiabatic, whereas the isothermal situation, although perhaps fast, is quite without critical properties. The domino-on-end draws upon its gravitational potential energy to restore it after a small tilt, while beyond threshold gravity hastens its fall to the table. But in the absence of gravity, it merely maintains a prosaic initial displacement or rotational velocity. The liar, the embezzler and the murderer may be relatively restrained in their crimes against society so long as that society does not react to force them into ever-increasing jeopardy with more frantic efforts to protect themselves from it.

So we find the electrostatic capacity and the ion control each rather well-behaved and quite lacking in critical characteristics, as has been amply demonstrated and overwhelmingly confirmed for a living nerve membrane. But in combination they can, most fortunately, egg each other on to produce all of the highly effective and spectacular characteristics of nerve performance.

It is not, then, for electrochemistry to look for the basis of a trigger mechanism, a metastable state or an explosive phenomenon in the ions and their reactions at the membrane, if only because the problems are not these. Rather must attention be directed to systems—of themselves entirely stable, completely continuous and fantastically non-linear—which can produce the variations of the ion flows at various membrane potentials, for which the very beautiful experimental facts serve both as a guide and as a stern judge.

Excitation Triggers in Post-Junctional Cells

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PHYSICAL METHODS often permit the electrophysiologist to determine with high precision the onset, magnitude and time course of bioelectric activity of cells. These methods can also provide considerable information regarding some of the phenomena underlying the responses. Thus, although the membrane molecular changes involved in electrogenesis are not yet known, electrophysiological data to some extent provide specifications as to the nature of their trigger mechanisms. Electrogenic responses all have one feature in common. They are all produced by changes in membrane permeability and these change the electrical state of the polarized cell membrane. Specific types of membrane molecular structure, the electrochemical conditions, and probably other factors, determine the nature of the various types of electrical activity of electrogenic cells.

EXCITABLE MEMBRANE AS TRANSDUCER

The bioelectric response is essentially the utilization of potential energy of the cell, and this process may be termed *transducer action*. The overt initial change is an alteration of the ionic permittivity of the membrane in response to a specific stimulus. Emphasis must be placed on the ionic aspects because ion movements are probably the only means for carrying a current across the non-metallic barrier of the membrane.[1] However, as with other types of transducers (e.g. the piezo-electric crystal) the effectiveness of biological transducer action is enhanced by amplification. In the cell the two processes may be closely linked by an interlocking mechanism (129) and are usually considered together (27, 57, 183).

a) Transducer Action. The change in permittivity which results during the response temporarily disturbs the steady state inequalities which exist in the

[1] The possibility of electron transfers playing a role in bioelectrogenesis is an intriguing subject (cf. 47). However, while such systems may be theoretically possible when operating in combination with ionic movements, they must be examined rigorously when considered in the absence of the latter. For example, a scheme in which it is claimed (54, p. 101) that oxidation-reduction potentials "can be measured with non-metallic, i.e., calomel, electrodes" consists of an ordinary redox cell, but with the metal forming the membrane between the two solutions. This transposition does not alter the principle (cf. 108) of action of the cell, although giving the appearance of electron transfer without metals.

ionic distributions between the cell interior and its exterior (109), and which are probably brought about by several types of secretory activity or 'pumping' (23, 114, 171, 181). A flow of ions results and this changes the membrane potential of the cell from its resting value of about 50 to 100 millivolts, inside negative. The electrogenic response may decrease (or even reverse) the resting potential, or increase it, thus producing depolarization or hyperpolarization of the membrane.

Specific characteristics of different electrogenic responses are probably determined by specificities in the type of transducer action with respect to one or another ion, and to the kinetics of these actions. While the response develops on the basis of potential energy stored during the resting state, the electrogenic transducer action probably involves molecular reorientations within the membrane, the nature of which is at present unknown. These changes may be considered chemical reactions. However, in phenomena which occur at surface films, the distinction between chemical and physical processes becomes vague.

Thus, according to the remarkably comprehensive theory of Hodgkin and Huxley (111), electrical excitation of an axon leads to a small depolarization and outward movement of K^+. This causes about a thousand-fold increase of sodium permittivity (or conductance). If this effect is considered as caused by mobilization of 'carrier' molecules, the process appears to be chemical. It may however, be due to a 'valving' caused by the reorientation of molecular pores in the membrane (149). This could be considered essentially a physical process.

b) **Amplification.** The amount of energy which may be required to initiate the transducer action can be small in comparison with the subsequent release of potential energy during the response. The squid giant axon is excited by an electrical stimulus of only 10^{-9} to 10^{-8} coulombs/cm^2 (96, 112), while the ionic transports which occur during the spike involve about 10^{-6} coulombs/cm^2. The amplification between input (stimulus) and output (response) is, therefore, 100- to 1000-fold. Similarly, the end-plate potential (e.p.p.) is associated with a change in charge of about 10^{-9} coulombs (38, 71) which is sufficient to trigger the spike of the muscle fiber. The latter in turn triggers the much larger release of energy of the mechanical response.

c) **Excitation in Conduction and Transmission.** A nerve or muscle can respond to artificially imposed stimuli, of which the electrical are most convenient to produce and to apply in measurable quantity. In their normal functioning in the organism, however, the excitation of electrogenic cells takes a different form. Sensory nerves are excited by various more or less specific stimuli, and conduct messages bearing this information to the central nervous system. Motor and autonomic nerves, on the other hand, carry centrifugally messages which arise at their central terminations. A transfer is then made to the effectors such as muscle or gland cells. In addition, within the nervous system itself,

complex pathways and synaptic connections distribute messages and commands to other nerve cells.

Two general types of excitatory triggers must therefore be distinguished. One is concerned with propagating activity along the extent of the cell in which the bioelectric response is initiated. The other involves transmission, or excitation of one cell (the post-junctional) by the activity of another (the pre-junctional). Propagation or conduction is essentially a point-to-point affair, the electrogenesis in an already active region producing activity in the next neighboring region. This process therefore involves electrical excitation. At each active site the response is a property of local conditions, and when these are uniform, the response is everywhere identical. This type of activity is admirably suited to conduction of messages in a relatively invariant form, or decrementless conduction.

Transmission, on the other hand, is a more complex phenomenon. Efforts to account for it by electrical action (61, 135), such as is responsible for conduction, have proved unsuccessful (63, 98–101). The release of a specific transmitter agent at the terminals of the pre-junctional fiber which then acts to excite the post-junctional cell (56, 61, 141) therefore appears to be the sole mode of transmission across the synaptic junction. Thus, the excitatory phenomena in post-junctional cells generally involve first a triggering of transmission and then of conduction. Furthermore, transmission may also involve processes of negative excitation, or *inhibition*.

VARIETIES OF ELECTROGENIC TRANSDUCERS

Electrogenic cells may be classified according to the nature of the effective stimulus for their transducer response, effective being defined as that stimulus to which the transducer membrane is most sensitive.[2]

Sensory Receptors

a) Mechano-Transducers. A mechano-transducer membrane exists in the terminal sensory dendrites of stretch receptors in lobster and crayfish (68, 69, 188). The electrogenic response of the receptors (68, 69 and fig. 1) is graded in accordance with the degree of stretch, and persists as long as the stimulus is applied. The terminal dendrites in which the mechano-transducer action takes place are apparently incapable of supporting the explosive, all-or-nothing electrical response which the rest of the cell can generate (cf. also 134). The mode of action in the response may be diagrammed as follows:

$$\begin{array}{c} \text{Stimulus} \rightarrow \text{Transducer action} \rightarrow \text{Electrogenic response} \\ \text{Mechanical} \rightarrow \text{Membrane permeability change} \rightarrow \text{Depolarization} \end{array} \qquad (1)$$

[2] Transducer action is not necessarily linked with electrogenesis. Insulin apparently acts at the membrane of some cells to permit entry of glucose (174), but if ionic transports do not occur at the same time this would not involve a changed membrane potential.

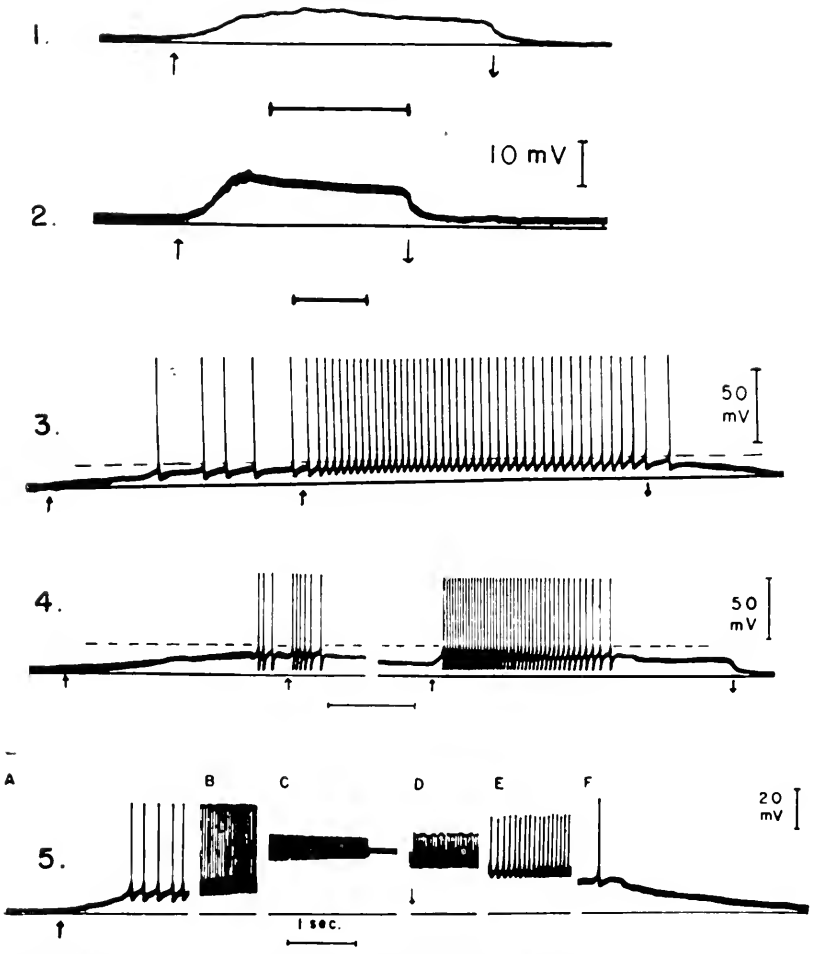

FIG. 1. Mechano-transducer response in crayfish stretch receptors. *1*) Stretch of the terminal dendrites of a slowly adapting receptor causes a maintained depolarization of 6–7 mv at the cell body. *2*) A rapidly adapting cell. The depolarization falls off during a maintained stretch. *3*) At first, slight stretch caused 5 spike discharges in a slowly adapting receptor cell. Increasing the stretch raised the frequency of the discharges to about 12–14/sec., maintained during the stimulus. The depolarization level at which firing of the cell occurred was constant (*broken line*). *4*) The rapidly adapting receptor cell stopped discharging after 3 spikes. A stronger stretch then caused a new burst at higher frequency. The depolarization then decreased and the responses ceased. An additional stretch again called forth a new burst, at high frequency, but again not maintained. The 'adaptation' is associated with decrease of the depolarization level. *5*) Increasing stretch of a slowly adapting cell raised the depolarization level and increased the discharge frequency (*A*, *B*), but the spike decreased and the responsive membrane became inexcitable at a depolarization of about 35 mv (*C*). Recovery shows hysteresis (*D*, *E*, *F*). Time in all records, 1 sec. (composed from ref. 68).

All sensory receptors may be considered as endowed with specialized transducer membrane[3] (27, 57, 183). Auditory and labyrinthine cells probably have deformation-sensitive, mechano-transducer membrane. In some mechano-transducer sense organs, free nerve terminals themselves act as the receptors. In others, the receptor appears to be a specialized cell, and many anatomically distinguished types of these are known. A graded electrical response is recorded in the nerve fiber on mechanical stimulation of some (8, 92, 128). It is not known, however, whether the electrogenic response is generated in these cells themselves and then transmitted to their innervating afferent fibers, or whether the cell is only an adjunct to mechano-transducer membrane in the nerve fiber (cf. 183). Responsiveness of mechano-sensory receptors to, or their sensitization by chemical agents has often been reported (cf. 59). For example, the crayfish stretch receptor dendrites are excited by acetylcholine (188). In this case it is clear that the membrane is certainly a mechano-transducer, and its chemical excitation seems to be an adjunct, often exhibited in other cells and differentiated by the classification of 'adequate' and 'inadequate' stimuli. The responsiveness to chemical as well as mechanical stimulation may be indicative of structural similarities between the transducer molecules. In other cases the specialized receptor cells might be the mechano-transducer, releasing a chemical to activate chemo-receptor membrane of the nerve terminals.

The crayfish stretch receptors also illustrate clearly the differences characteristic among sensory receptors of a given class. The slowly adapting receptor is more sensitive to the mechanical stimulus (cf. 127), to applied acetylcholine, and to Ca^{++} deficiency than is the rapidly adapting receptor cell (188).

b) **Other Types of Sensory Transducers.** Visual and temperature receptors may be considered as possessing specialized membrane for transducing photic or thermal energy. The graded potentials of retinal activity are well known (e.g., 91). The actions of thermal transducers have been measured, as yet, only as spikes generated in their nerve fibers (27, 191). Chemo-transducer membrane probably accounts for the receptors of olfaction, taste, and vascular and respiratory control.

It has been suggested (cf. 45) that the remarkably regular pulsatile discharges of various electric fishes subserve orientating functions. These animals

[3] The behavior of insect flight muscles (16) indicates the possibility that a mechano-transducer with positive feedback is involved, the transducer action being coupled either through electrogenesis or directly to the mechanical response. The self-exciting rhythmic properties of heart muscle, its peculiar electrogenic activity, responses to neural stimuli and to drugs suggest that mechano-transducers may play a role in this tissue under the special conditions of syncytial structures. Mechano-transducer properties, such as are present in muscle spindles, might also account for the increased potential observed (164) in stretched muscle fibers.

may therefore be endowed with extraordinarily sensitive, but gradedly responsive membrane transducing electrical fields into permeability changes with considerable amplification. The highest known amplification appears to be in the thermal receptors of snakes (27).

A general scheme of transducer electrogenic action in sensory receptors is the following:

$$\begin{array}{c} \text{Stimulus} \rightarrow \text{Transducer action} \rightarrow \text{Electrogenic action} \\ \text{Specialized} \rightarrow \text{Permeability change in specialized} \rightarrow \text{Membrane potential change} \\ \text{membrane sites} \end{array} \qquad (2)$$

Electrically Excitable Tissue

a) **Spike Generator.** A very different type of response might be expected if the transducer membrane is electrically actuated and operates according to the scheme:

$$\begin{array}{c} \text{Stimulus} \longrightarrow \text{Transducer action} \longrightarrow \text{Electrogenic response} \\ \text{Membrane depolarization} \longrightarrow \text{Membrane permeability} \longrightarrow \text{Membrane depolarization} \\ \qquad\qquad\qquad\qquad \text{change} \\ \qquad\qquad\qquad\qquad \text{Positive feedback} \end{array} \qquad (3)$$

Once initiated, depolarizing electrogenic activity would tend to cause further change in the membrane permeability and additional membrane electrical change. The process would therefore exhibit regenerative action and an explosive response. An electrical analog of this system is the bistable electronic circuit. A 'trigger' potential change tends to drive this circuit from one level of output voltage to another with no intermediates. While exhibiting this all-or-none property, the response of axons and of many muscle fibers lasts only a characteristic, brief and remarkably constant time, and then returns to the initial condition. This behavior is analogous to a monostable electronic circuit, in which the bistable condition is suppressed by a capacitative leak circuit.

Hodgkin and Huxley (110) have postulated in the specific terms of a biological membrane a sequence of analogous events. Initial increase of sodium conductance by a depolarizing stimulus drives Na^+ inward, depolarizes the membrane still more and this regenerative process rapidly brings the membrane to the reversed 'sodium potential.' The 'leak' is provided by two additional processes, also potential-determined, but proceeding at slower rate. 'Sodium inactivation' decreases the heightened sodium conductance, while potassium conductance increases to drive this ion outward, dissipate the reversed charge and restore the membrane potential to its resting value.

Elegant experimental and mathematical tools enabled Hodgkin and Huxley to deduce the time course of the postulated membrane conductance changes during the squid spike. This sequence also explains satisfactorily a number of

excitation phenomena (110) as well as the time course of increased membrane conductance during the squid spike (9, 44, 101).

Subsequent work (114) indicates that the independence of inward and outward flow of the same ion species, which was assumed in the derivation of the theory, is not experimentally verified. Membrane impedance measurements (9, 172) on the squid giant axon disclosed that the resistance of the membrane rises very considerably above the resting level while the axon is still in the hyperpolarized state, whereas the deduced K^+ conductance (110) is presumed to be still high. It has been suggested (9) that this increased resistance is due to decreased K^+ conductance produced by the hyperpolarization. Thus, while the three processes postulated in the theory appear to exist in squid axon[4] it is likely that some modification of their kinetics would satisfy the new data.

Each of the three processes of the Hodgkin and Huxley theory is a continuous function of membrane potential and is described by an appropriate differential equation. In the totality of their action, momentary excess of inward sodium current over outward potassium current is the immediate trigger for the onset of the spike. The subsequent decrease of sodium current by 'sodium inactivation' below that of potassium current is the trigger for its subsidence. The processes are analogous to those during the triggered pulse of a monostable electronic circuit, in which the events can also be described by differential equations. These processes therefore do not in themselves provide the trigger for the spike, which must be sought in underlying molecular changes of the membrane. The specific differences in rate and the independent course of sodium and potassium conductance, as well as the process of sodium inactivation, indicate that the molecular mechanisms of sodium and potassium conductance are probably different. The species of molecular change can only be a matter of speculation at present and the model chosen depends upon individual preference with respect to the various and conflicting theories of functional membrane structure. In essence the assumed conductance processes represent potential-dependent valving, selective for sodium and potassium, flow of the ions being determined only by their electrochemical gradients. These flows therefore should be independent of metabolic conditions, unlike the secretory transport mechanism of the 'pumps' of the resting membrane. It is customary to postulate that these ion-specific valves are achieved through specific mem-

[4] The frog nerve fiber does not appear to show increased K^+ conductance during the spike (176). The membrane resistance of the eel electroplaque appears to be higher during the falling phase of the spike than at rest (2). The membrane resistance of cardiac fibers rises about three-fold above the resting value during the plateau of the electrical response (185). It is likely, therefore, that sodium conductance and inactivation and potassium conductance have rather different kinetics in different tissues. For example, potassium conductance may be very much delayed in the normal response of heart muscle. This could account for the peculiar form of the response and impedance change. Chemical agents, by altering the 'valving' kinetics, might thus alter the time course of the electrical phenomena.

brane 'carrier' molecules of unknown nature (cf. 113). However, it would also seem possible to construct a model based on membrane pores, electrostatically controlled in size by alteration of their molecular walls (cf. 149). The relative depth of the pores with respect to the ionic diameters would facilitate selective movement of Na^+ inward (114, 173).

b) **Graded Response.** It is not likely that the three processes postulated in the Hodgkin-Huxley theory exhaust the number of membrane molecular events of excitation and electrogenesis. For example (103), depolarization of a 1.2-centimeter region of a squid giant axon by increasing external K^+ rapidly causes loss of responsiveness in this region, which is ascribable to the depolarization of the axon and consequent sodium inactivation. However, after removing the excess K^+, recovery of responsiveness and of propagation develops rapidly while the membrane is still depolarized and, therefore, presumably sodium conductance is still inactivated.[5]

The occurrence of graded responsiveness to electrical stimuli also indicates that additional membrane events are involved in electrogenic activity. When treated with one of a variety of compounds (*d*-tubocurarine, DFP, eserine, procaine, tertiary prostigmine) the eel electroplaque loses all-or-nothing responsiveness (6). The activity is then graded with the strength of the electrical stimulus, and becomes decrementally propagated, but the maximal response resembles the normal spike in form and amplitude. This change must be ascribed to some alterations of membrane constituents by drug action, since the resting potential is not affected and the potential-determined 'sodium inactivation' could, therefore, not play a role.

Graded responsiveness, involving large but localized spike-like activity, and sudden transition to all-or-nothing propagated responses, has also been found during the refractory period of eel electroplaque (fig. 2) and squid, earthworm and crayfish giant axons (7, 98, 102, 122–125), and in dog cardiac muscle (115). Some invertebrate muscle fibers react to electrical stimuli only with graded response (85, 107). Lloyd (140) has described a presynaptic potential in dorsal root afferent terminals, which is similar to the post-synaptic potential of motoneurons (20), and differs radically from the spike of the afferent axon. This response might be graded[6]:

These findings seem to indicate, therefore, that graded responsiveness to electrical stimuli is a general property upon which in certain excitable tissues

[5] The ability of some vertebrate nerve fibers (142) and invertebrate muscle fibers (73) to remain electrogenic, although with a radically altered response, when external Na^+ is substituted by certain organic ions, is still an unexplained phenomenon.

[6] Loss of propagative activity, but persistence of local and therefore presumably graded responsiveness, has been noted in various tissues. Eccles and O'Connor (65) found that at some levels of curarization the neurally excited frog muscle is capable of local 'abortive spikes.' Vertebrate axons are electrically responsive, but do not propagate during the 'functional refractory period' (170). Procaine poisoned muscle is still capable of excitation localized to the cathodal stimulating region (175). An interesting, but as yet inadequately studied phe-

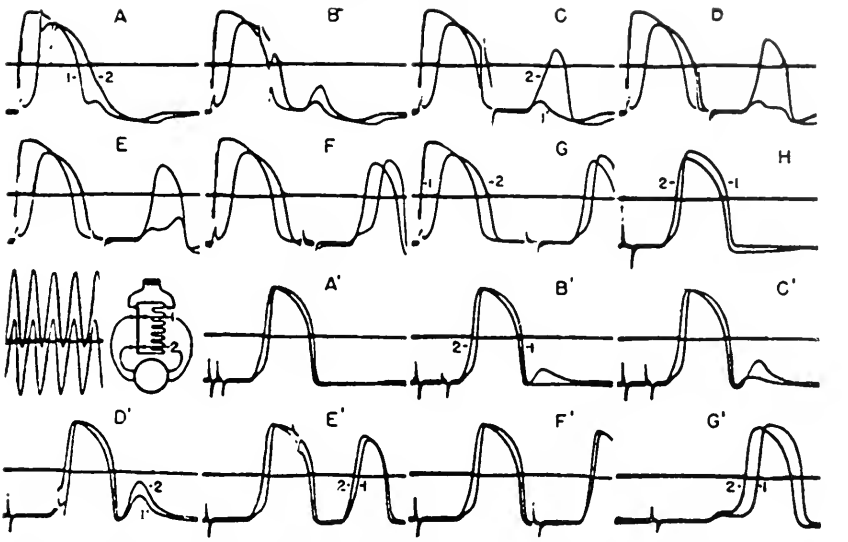

FIG. 2. Responses of eel electroplaque to paired stimuli. *Above:* A direct stimulus was followed by a maximal stimulus to a nerve trunk supplying the cell with a nerve. *Below:* Both stimuli were applied to the same nerve, the first strong, the second somewhat above threshold for a spike. Recording conditions are shown in the diagram. While the cell is still refractory to a second direct stimulus following the conditioning activity, it can develop a p.s.p., seen on the falling phase of the conditioning spikes (*A*). At successively longer intervals between the stimuli (*B–D*) the p.s.p. increases and evokes graded responses which are largest at *site 2*. Still in the relatively refractory period, the neural stimulus then evokes small, delayed spikes (*F–G*). The testing neurally evoked responses are seen in isolation (*H*). *Below:* With the conditioning stimulus also a neural volley (*A'*), the second stimulus does not evoke a p.s.p., because the nerve is in absolute refractoriness. During its recovery (*B'–D'*) a p.s.p. develops and grows, then causes spikes (*E'–F'*). The electroplaque is still relatively refractory, as may be seen from the small spikes in *E'*, but homosynaptic facilitation causes their earlier, almost synchronized development on the p.s.p. in comparison with the effect of the weak testing volley in isolation (*G'*). 100 mv and msec. calibration on each of the recording traces (from ref. 4).

an explosive mechanism is imposed (7, 98). Graded membrane responsiveness to electrical stimuli might result if its transducer elements differed widely in their electrical threshold. A small imposed membrane depolarization then would initiate transducer action only in the most excitable elements, but the resulting

nomenon is the change in electrical excitability of uterine muscle during physiological or induced hormonal changes (17, 55). The anestrous muscle responds to electrical stimuli (17) or to neural (166) only with local activity. In the estrous phase, or after treatment with estrone, the response becomes propagated. Uterine muscle can be converted from propagating responsiveness (estrogen-dominated, ref. 55) to the non-propagating (progesterone-dominated). The relation between the different types of electrical responsiveness and coupling of these to the mechanical response of muscle were considered briefly by Altamirano *et al* (4), but cannot be discussed here.

electrogenic activity would not excite a sufficient number of other elements to cause explosive responsiveness. On the other hand, in tissues normally responding explosively, the electrical thresholds of the membrane transducer elements would be more nearly uniform. However, differences in their recovery rate after absolute refractoriness, or differences increased by chemicals, would convert explosive to graded responsiveness, temporarily in the first case and permanently in the second. Formally, this suggestion is equivalent to the concept of inactivation of sodium 'carrier' in the Hodgkin-Huxley theory It differs from the latter in assuming that the ionic valves of the membrane do not have identical potential dependence and that the latter can be modified by factors such as specific ion effects and drug action. Increasing the spread of

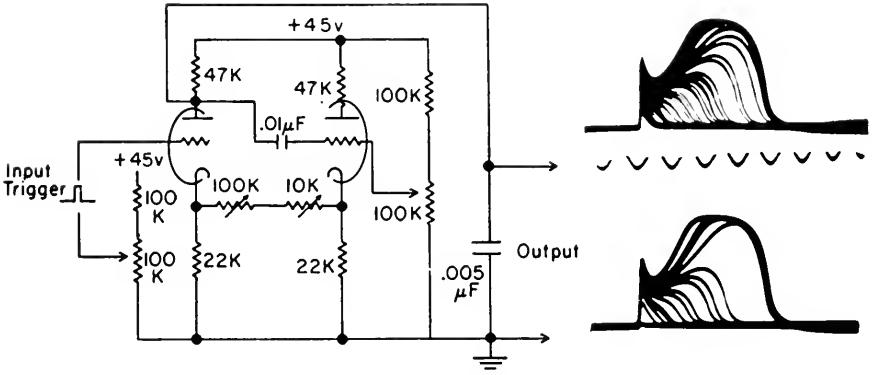

FIG. 3. Graded responsiveness from an electronic circuit model. *Left:* A modified monostable circuit. The resistors between the cathodes of the amplifier tubes (12 AU7) change the amplification in the circuit. *Right:* Oscillographic records (superposed sweeps) of the 'responses' to increasing trigger pulses. The lower set of records shows growth of the 'response' and a sudden transition to maximum output. The amplification was decreased for the upper set, producing continuous gradation in amplitude. Time in msec.

'valve' thresholds is equivalent to decreasing the amplification in a monostable electronic circuit analog of the spike generator. When the loop gain of this circuit is decreased sufficiently, the normal triggered 'all-or-nothing' response becomes fully graded (fig. 3). This behavior of the analog, of course, does not demonstrate the correctness of the hypothesis for the biological system, but is suggestive of further experiments.

As pointed out earlier, (p. 125), a discontinuous event, the spike, may arise from the operation of a series of continuous processes. The concept of a population of 'electrogenic units' involves a fundamentally different mechanism, in which each 'unit' (valve, carrier, etc.) itself behaves as a bistable device. Thus in the immediate region of a 'sodium valve' of the membrane the potential should change abruptly from the resting value ('potassium potential') to that of the reverse 'sodium potential.' The actually recorded potential, however,

would be averaged over the entire membrane by electrotonic spread and would be small if only a few 'units' were active. Thus, step-wise electrogenesis may develop larger or smaller graded responses, or a spike, depending upon this number and upon the temporal relations of the triggering of the population. Recent work (3a) supports these conclusions. Whereas an appropriately poisoned eel electroplaque responds gradedly to punctate electrical stimuli, only a response which is identical with the spike results from simultaneous excitation of the entire membrane. As will be described below, (p. 142) the graded response of postsynaptic membrane is also probably caused by discontinuous electrogenic activity.

Sodium inactivation and potassium conductance probably follow different kinetics in the eel electroplaque than in squid axons (4) and such a difference in crayfish stretch receptor cells could account for the rapidly or slowly adapting types (68, 69, 133, 188). Other data also indicate that sodium inactivation is conditioned by additional factors as well as by membrane depolarization. Ca^{++} deficiency or excess and anesthetics modify sodium inactivation without significantly changing the resting potential (184).

c) **Mediation of Electrical Excitability.** The chemical intermediates of electrical excitability, if any are needed for this process, are unknown. The charges of the local circuit current might themselves cause a breakdown of some complex elements of the membrane, and the resulting change in fixed membrane charges (presumably of long-chain lipoproteins) then might initiate ionic flows. Acetylcholine, which plays an important role in synaptic transmission (56, 60, 141), is also released intracellularly during stimulation of a nerve (13, 33). This fact has been incorporated into a scheme (150) which considers that acetylcholine is released by the action of the local circuit, combines with membrane components ('receptors') to produce the ionic valving of permeability change, and on its destruction by an esterase the response ends. This theory rests on circumstantial evidence (cf. 94) chiefly deriving from correlations of the inactivation of the propagated activity and of cholinesterase by various poisons (28).

The strongest of this evidence (cf. 95, p. 30) was the finding that pretreatment of nerves with eserine prevented their irreversible inactivation by DFP. However, dysfunctions of this enzyme can no longer be considered a primary cause of inactivation of electrically excitable membrane. This is not affected directly (3) by a strong anticholinesterase (Prostigmine), by acetylcholine itself or by its analogs (succinylcholine, carbamylcholine or DMEA), but secondarily by their depolarization of the cell through action on the postsynaptic membrane (fig. 4). Other strong anticholinesterases (eserine, DFP) which inactivate the latter, as do the curares (6, 41), do not destroy the electrogenic capacity of the electrically excitable component, but change it from explosive to graded responsiveness.

The latter finding probably explains the apparent inactivation of axons by

eserine and DFP. The plane surface excitable membrane (4) and the relatively short (*ca* 1 cm) length of the electroplaque, as well as intracellular recording, permitted detection of the decrementally propagated response. The latter was probably missed in the earlier work with axons, in which conduction block was

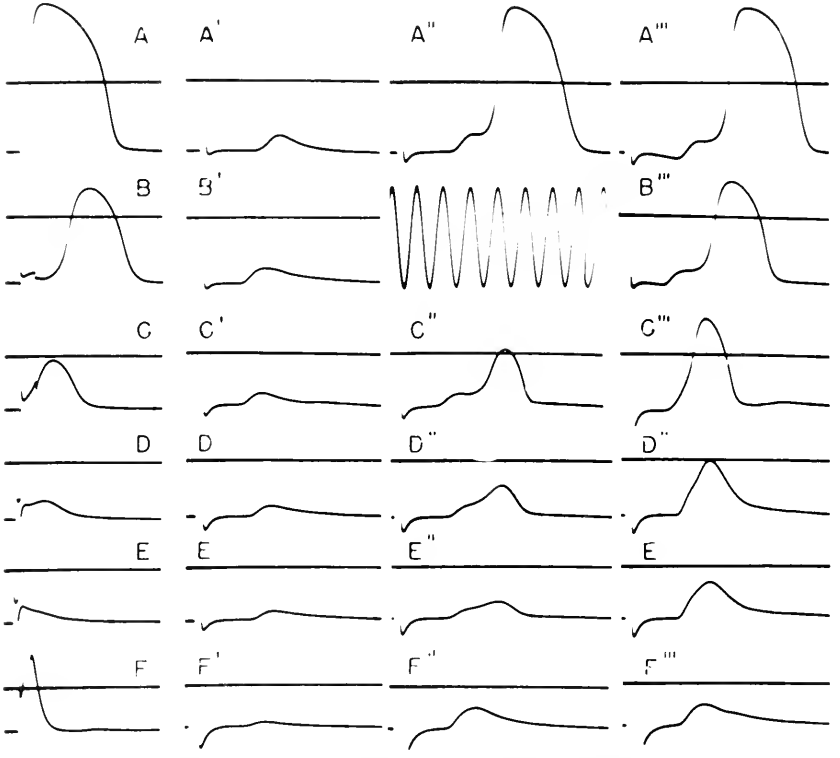

FIG. 4. Differentiation between synaptic and direct excitability in eel electroplaques. *A–F:* direct stimulation of the cell; *A′–F′:* a weak neural volley; *A″–F″:* a neural volley strong enough to elicit a spike; *A‴–F‴:* supramaximal stimulus to the nerve. *Upper row:* Activity in the normal cell. Acetylcholine plus eserine was then applied. *B–B‴:* the spike was depressed after 114 min. Subsequent records at 123, 142, 153 and 156 min. The stronger neural volley evoked a larger, i.e. graded response than did direct stimulation. As depolarization developed, block of the electrically excitable generator occurred, but the p.s.p. was still evoked (*F′–F‴*) while a very strong direct stimulus (*F*) was without effect. Calibration 100 mv and msec. (from ref. 6).

the criterion of 'inactivity.' Graded responses localized to the stimulated region are observed in transmembrane recording from squid giant axons poisoned by external application of eserine or by microinjection of various drugs (102, 126). Therefore, while inactivation of the acetylcholine system probably affects and eventually may block responsiveness, its immediacy in electrogenesis is doubtful.

Lecithinases inactivate and depolarize axons and muscle fibers (179). Some breakdown products of lecithin act likewise. Other enzymes (e.g. collagenase, chymotrypsin, etc.) have no effect, but Tobias (179) is properly cautious with respect to the implications of his findings. As with the chemical events, the significance of various optical and mechanical changes during activity (e.g. 24) is not yet known.

Synaptically Excitable Membrane

a) **Synaptic Electrogenesis.** A specific response appears to be localized to the regions of the post-junctional membrane immediately accessible to the presynaptic stimulus (89). It is designated as the postsynaptic potential (p.s.p.). In most, but not all post-junctional cells, p.s.p. lasts considerably longer than does the spike. It may be in the direction of decreasing the resting potential and therefore excitatory, or oppositely directed and inhibitory. Analysis of several cases has indicated that the mechanism of electrogenesis is different from that causing the spike, and therefore it must involve different types of membrane molecular structures. These differences are also reflected in different pharmacological properties, and particularly in the nature of their initiating stimulus. The preponderance of present evidence supports the view that excitation of synaptic activity is initiated by specialized transmitter agents, released from the presynaptic terminals in the course of their electrical response. The transmitter, and the specialized postsynaptic membrane 'receptor' therefore constitute the triggers of postsynaptic electrogenic activity.

Probably only a few workers still adhere fully to the theory that synaptic transmission is mediated by eddy currents of the impulse in the pre-junctional cell flowing through the post-junctional (150). A number of experimenters have found by direct measurement that the membrane depolarization generated in a cell by activity of its presynaptic innervation or that of neighboring cells is too small to initiate excitation (20, 29, 122–124, 177). Others accept neurohumoral synaptic transmission for some junctions, but consider that in certain synapses, and particularly in those of the vertebrate central nervous system, transmission is electrical (18, 19). Most investigators, whether adhering to strictly neurohumoral (cf. 63, 71, 78) or to the dualistic view (cf. 19, 81) nevertheless appear to accept the possibility that the postsynaptic membrane can be excited by electrical means. Considerable evidence has now accumulated to indicate that this is not the case, and that the postsynaptic membrane probably in all post-junctional cells is one which cannot be excited by electrical stimuli, but is responsive only to specific chemical excitants.

b) **Electroplaques Other Than Those of Eel.** At the end of the last century it was found that the electroplaques of *Malapterurus* (90) and *Torpedo* (86, 87) cannot be excited directly, but only through their nerves (cf. also 80). The denervated electric organ, or the curarized, becomes irresponsive. Their response to neural stimuli is probably like that of the electrically inexcitable *Raia*

clavata (22). The electric organ of *Torpedo* responds electrogenically to acetylcholine (77).

Twitch muscle fibers. Electrical inexcitability of the end-plate of frog skeletal muscle was inferred by Kuffler (131) and by Fatt and Katz (72) and demonstrated by del Castillo and Katz (37). The neurally evoked end-plate potential (e.p.p.) may develop concurrently with a directly excited spike of the fiber, and the two sum in a manner which indicates that they occur independently in different kinds of fiber membrane, are based on different electrogenic mechanisms, and that the e.p.p. is not itself generated by the electrical stimulus of the spike (fig. 5). The exterior of the end-plate membrane responds electrogenically to a jet of less than 10^{-15} M acetylcholine (38, 151, 152), but its interior face is not responsive to injections of large quantities (38).[7] The resistance of the depolarized and reversely polarized, physiologically non-responsive membrane nevertheless decreases upon application of an acetylcholine jet (39). Thus, although the electrogenic action is abolished because the depolarized fiber no longer provides the battery needed for its expression, the trigger response of the postsynaptic membrane, increased conductance, still remains.

Eel electroplaques. The p.s.p. neurally evoked in the eel electroplaque is similarly independent of the spike generator (4). The p.s.p. cannot be evoked by electrical stimuli, nor inhibited by large hyperpolarizing or depolarizing changes in membrane potential It may be elicited while the cell is not responsive to electrical stimuli, either in absolute refractoriness (fig. 2) or when the cell is depolarized (fig. 4). The two responses therefore exhibit different pharmacological and physiological properties.

Axono-axonal synapses. The segmented giant axons of the earthworm and crayfish, in which the occurrence of axono-axonal synapses appears likely (26), also respond with electrogenic activity (122–124) characteristic of synapses. Their p.s.p. is small, graded and repetitive, and may be superimposed upon the spike or elicited during the absolutely refractory period of the fiber. The magnitude of the p.s.p. varies in different parts of the axon, and therefore it is probably localized at synaptic regions. The p.s.p. is absent in the medial giant axons of the crayfish, which are devoid of axono-axonal synapses (187).

Invertebrate and 'slow' vertebrate muscle fibers. Graded, only neurally evoked activity is exhibited in some invertebrate muscle fibers which are not electrically excitable (85, 107, 169, 190). These responses appear to be confined to the multiple myoneural junctions (75). As in the eel electroplaque, very strong hyperpolarization of the muscle fiber does not block the neurally evoked p.s.p. (107). Different nerve fibers innervating the same muscle fiber may initiate different forms of response, and these may vary in different parts of the same

[7] Microinjection of high concentration of acetylcholine is tolerated by the squid axon, although that of dilute concentration is not (97, 126). The injection into muscle fibers of ATP (70) or Mg^{++} (180), without effect, appears to be expected on the basis, at least, of one theory (148).

fiber. In some muscle fibers, specific neural paths produce inhibitory effects which may be manifested as a hyperpolarizing p.s.p. (74).

The 'slow' muscle fiber associated with the crayfish stretch receptor is diffusely innervated, and responds to neural stimulation with a long-lasting, end-

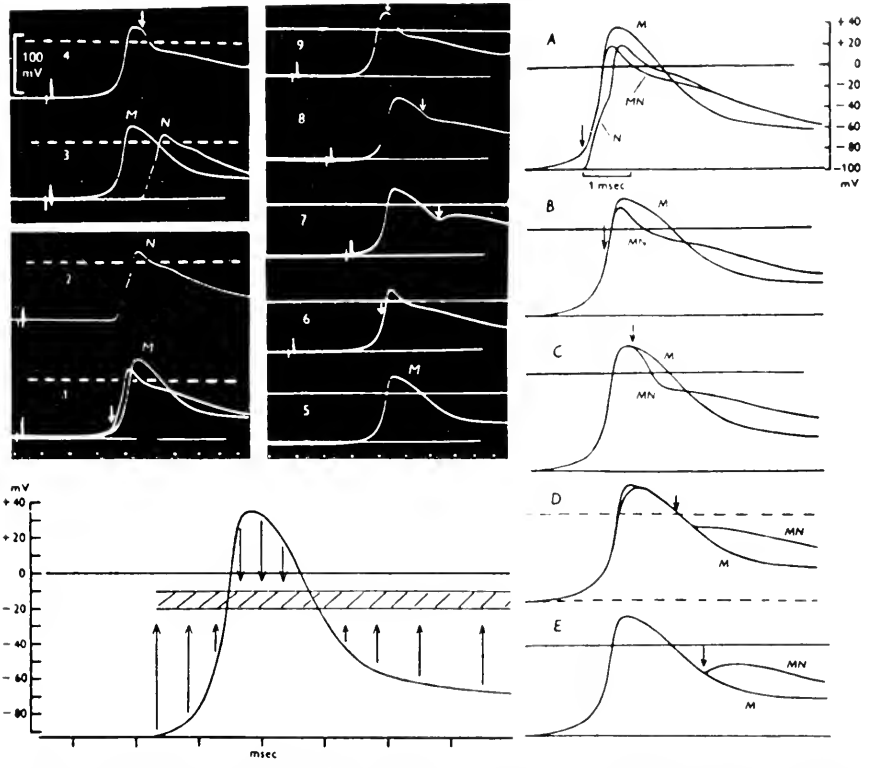

FIG. 5. Interaction of the directly elicited muscle fiber spike and an end-plate potential. *Upper left:* The responses labeled *M* in *1* and *5* show the direct spikes of the muscle fiber recorded at the end-plate region. The response *N* in *record 2* is that obtained by stimulating the nerve. The directly elicited spike is distorted by superposition of the neurally evoked e.p.p. in the other records. Arrows mark the onset of the response to a neural stimulus. *Right:* A reconstruction of the same records, showing how the direct spike is distorted (*M N*) when the neural stimulus is delivered at different phases of *M*. The e.p.p. of the neurally evoked spike (*N*) is seen in the upper tracings (*A*). *Lower left:* A diagrammatic representation of the events The e.p.p. adds to the spike when the latter is more negative than an equilibrium potential of −10 to −20 mv. It subtracts from the spike when the latter is more positive than the equilibrium potential (composed from ref. 37).

plate type of potential. On the other hand, the 'fast' muscle fiber exhibits both an e.p.p. and a spike (133). These muscle fibers also show anatomical differences, and are supplied with different receptors, slowly adapting and rapidly, respectively (133, 188).

The 'slow' muscle fibers of the frog (132) do not respond to electrical stimuli (31), but do so to neural stimulation. The graded, summative depolarization of these 'junctional potentials' (132) is therefore also a p.s.p. As is to be expected for the response of electrically inexcitable membrane, the p.s.p. reverses in sign when strong outward currents are applied across the membrane (32; cf. below, ELECTROGENIC ACTION OF SYNAPTIC TRANSDUCERS).

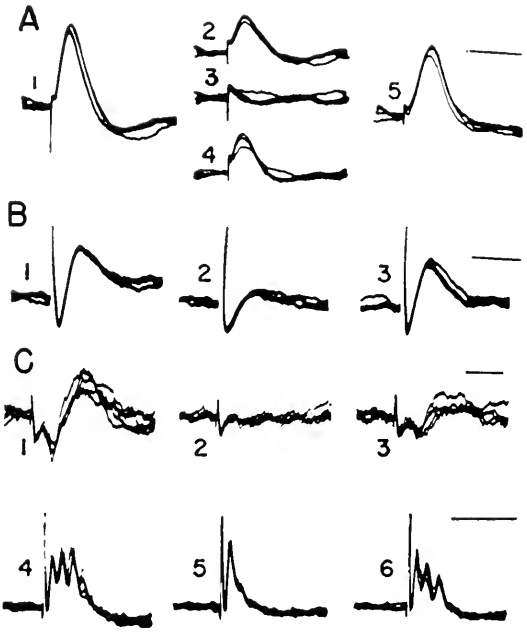

FIG. 6. Electrical inexcitability of the post-synaptic membrane of apical dendrites of the cat cortex. *A:* Stimulating and recording from the cortex. The large normal surface negativity which is the response of cortical dendrites (*1*) is decreased 50 sec. after intravenous injection of 3 mg/kg *d*-tubocurarine chloride (*2*). At 70 sec., it is gone (*3*), to return after 5 min. (*4*) and 20 min. (*5*). Four consecutive records superposed in each set. *B:* Stimulating about 0.8 mm below the cortical surface, recording as in *A*. The dendritic negativity (*1*) disappeared and only the positive response, representing the activity of the directly excited spikes, remained 45 sec. after an injection of drug (*2*). Recovery was rapid, although not complete at 90 sec. (*3*). Three consecutive records superposed in each set. *C:* Response of motor cortex to stimulation of the pyramidal tract (*1*) is composed of two positive deflections preceding the negative dendritic potential. Two minutes after the injection of drug all synaptically induced activity (the second positive component and the negative dendritic response) disappeared, leaving only the first positivity which is ascribed to the antidromic response of pyramidal axons and/or cells in the deep cortical layers (*2*). Recovery after 20 min. (*3*). *4–6:* From the same experiment, stimulating the cortex and recording in the pyramidal tract. The normal response is composed of a directly excited component and several synaptically evoked repetitions (*4*). The latter are absent 5 min. after drug was administered (*5*) and recovered somewhat after 20 min. Five superposed responses in each set. Times 20 msec. for *A* and *B*, 10 msec. for *C* (from ref. 105).

Invertebrate neuronal junctions. Evidence for electrical inexcitability of invertebrate neurons is chiefly indirect. The large cells of the lobster cardiac ganglion develop a graded response when excited synaptically by the smaller, pacemaker neurons of the ganglion, but the soma membrane is not capable of supporting a spike, that response occurring in the axon of the cell (106). The synaptic potential of the squid third order giant axon may be elicited while the

axon is absolutely refractory, after a directly evoked spike (29). Upon neural (orthodromic) excitation, the giant nerve cell of *Aplysia* develops a p.s.p. which is not evoked by electrical stimulation (12a, 178). As in other cases, the p.s.p. can be elicited while the electrically excitable membrane is absolutely refractory (12a).

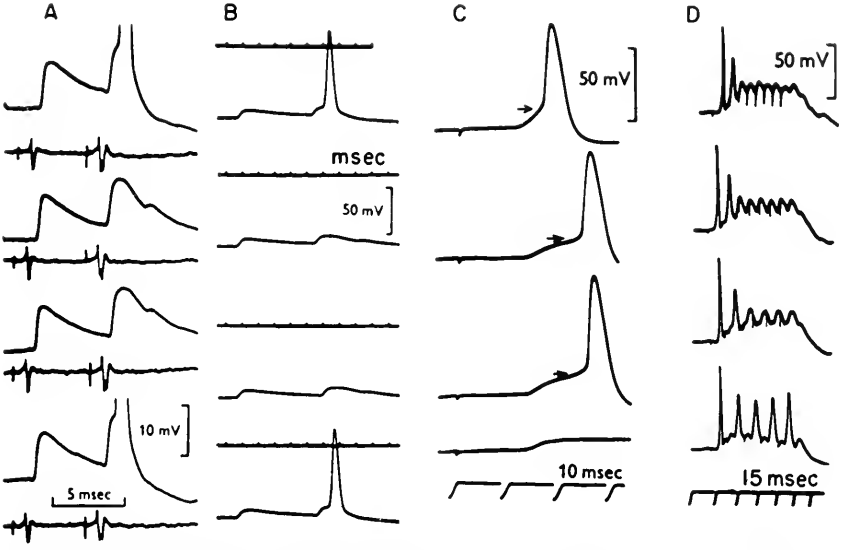

Fig. 7. Excitatory post-synaptic potentials in neurons. *Left:* Cat motoneurons. *A*, p.s.p.'s in response to paired threshold orthodromic stimulation summate, at shorter intervals, causing a spike. The lower trace in each pair shows the arrival of the presynaptic impulses recorded extracellularly at higher gain. Note that this potential is also picked up by the internal electrode, but the change in post-junctional membrane potential is less than 1 mv. *B*, the same experiment at lower amplification. Time is msec. (from ref. 20). *Right:* Responses in the rabbit superior cervical sympathetic ganglion cell, (first column) to a single stimulus of increasing strength (from below up) showing the subthreshold p.s.p., and decreased latency of the spike with larger p.s.p.'s. Second column: The cell cannot generate a train of spikes at frequencies of pre-synaptic stimulation above about 80/sec., but the p.s.p.'s are produced and maintain the depolarization. Presumably sodium inactivation operates to block the depolarized electrically excitable membrane of the cell, but does not affect the post-synaptic (composed from ref. 66).

Vertebrate neuronal synapses. Direct evidence is available (105, 163) that the prolonged, graded response of the apical dendrites of the cat cortex (43, 44) is a postsynaptic potential. Under the action of synaptic blocking agents the dendrite response cannot be evoked by direct stimulation of the cortex. The membrane, at least of the apical dendritic portion, is therefore inexcitable by electric stimuli (fig. 6) although the soma and axon of the same cell are so excitable and respond with characteristic spikes (e.g. 159, 160). Depolarizing

(excitatory) p.s.p.'s evoked by neural stimuli are recorded intracellularly in motoneurons of cat (20, 49, 84) and toad (10); in cat cortical neurons (159, 160); and in rabbit sympathetic ganglion cells (66). The p.s.p. of the latter develops even when the cell does not respond with spikes to repetitive stimuli (fig. 7). Antidromic activation of the motoneuron does not elicit the p.s.p. A hyperpolarizing (inhibitory) p.s.p. also occurs in neurally stimulated motoneurons (10, 20, 48, 50, 64 and fig. 8). The p.s.p.'s of cat and toad motoneurons and turtle autonomic ganglion cells may be elicited during the falling phase of the spike when the cells are absolutely refractory to electrical stimulation (10, 21,

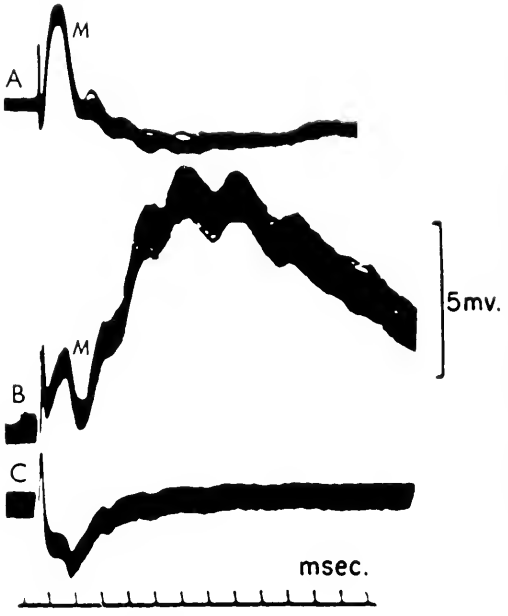

FIG. 8. Reversal of an inhibitory, hyperpolarizing p.s.p. into a depolarizing, cat motoneuron. The initial deflection, *M*, is the antidromic spike which did not reach the soma. Following it (*A*) is a hyperpolarization generated by activation of inhibitory post-synaptic membrane. Passing current through the recording electrode then increased the internal Cl^-. Activation of the inhibitory post-synaptic membrane now led to a depolarizing potential indicating outflow of Cl^-. *C*: Extracellular responses on withdrawing the microelectrode from the cell show the reversed *M* spike, but not the p.s.p. (from ref. 64).

49, 136). The neurally evoked p.s.p. of toad motoneuron develops during strong hyperpolarization of the cell (155).

The neurohumorally, but not electrically excitable transducer action of the synapse may be schematized as follows:

$$\begin{array}{lll} \text{Stimulus} \rightarrow \text{Transducer action} & \rightarrow & \text{Electrogenic action} \\ \text{Specific transmitter agent} \rightarrow \text{Specific change in ionic permeability} & \rightarrow & \text{Depolarization or hyperpolarization} \end{array} \qquad (4)$$

c) **Functional Value of Synaptic Electrical Inexcitability.** It is interesting to speculate about the purposive value of electrical inexcitability of the postsynaptic membrane. The fields of electrical currents generated by active cell and fiber masses of the central nervous system undoubtedly produce electrical

effects in the membrane of adjacent elements which affect their excitability (93, 104, 116, 138, 165). Electrical inexcitability of the postsynaptic membrane therefore would provide a considerable measure of immunity from the perturbing influence of electric field effects to the synaptic pathways upon which the precision of integrated nervous functioning depend (99).

d) Synaptic Transmitters. At least two synaptic transmitters are presently distinguished, acetylcholine and some form of adrenaline-noradrenaline complex (30, 78, 146). However, it is not at all unlikely that others also exist. The synaptic transmitters of the mammalian central nervous system in particular do not seem to fit into the two accepted categories. The agents causing the excitatory and inhibitory p.s.p.'s at the motoneuron probably do not belong to either category (64), although it is believed that the transmitter for excitation of the inhibitory (Renshaw) cells is acetylcholine. Neurohumoral activity has been recently demonstrated in the cortex (cf. 120), particularly by the use of cross-perfused preparations (162), but the transmitter nature is unknown. A possible clue to variously discrepant data is provided by the finding (58) that inactivators particularly effective for pseudocholinesterases are better cortical poisons than are those which specifically attack the 'true' (acetyl-) cholinesterase, while the reverse is the case for the muscle end-plate. Thus, structural analogs of acetylcholine, if not the latter, might act as transmitters at the central nervous synapses. Differential sensitivity to various pharmacological agents (161, 163) also indicates that a number of different synaptic mechanisms probably exist.

The relatively high concentration of cholinesterase present in the eel electric organ, chiefly at the innervated faces of the electroplaques (52), tempts the conclusion that acetylcholine is the synaptic transmitter of this system (cf. 100). The fatigability of synaptic transfer in eel electroplaques with a concommittant exhaustion of acetylcholine (42) supports this. The eel electric organ does not, however, respond to injections of acetylcholine (6) whereas that of *Torpedo*, which is similar in properties to the muscle end-plate (100) does respond electrogenically to the drug (77).

Proportionality between cholinesterase concentration and the voltage produced by blocks of the eel electric organ have been assumed (150) to support the theory that acetylcholine has electrogenic properties in electrically excitable, spike generating membrane. This is probably an unjustified conclusion drawn from two types of parallel data, in which the common factor happens to be the excitable surface of the individual cells. The voltage is proportional to the number of these cells in series (4, 14, 130). Also proportional to the number is the area of the innervated, excitable membrane at which cholinesterase is concentrated (52) probably in relation to synaptic, electrically inexcitable membrane. Recent data from Nachmansohn's laboratory (3) also appear to contradict that theory. The resistance of the electroplaque membrane rises

when it is treated with acetylcholine, whereas were the drug an electrogenic agent, a fall might be expected.

e) **Release of Transmitters.** The process by which a transmitter is released at the synapse is as yet only partially known, and this chiefly from work on the muscle end-plate by Katz and his collaborators (cf. 36, 38, 39). 'Miniature' e.p.p.'s which probably represent spontaneous releases of unit small quantities of transmitter (76) occur more frequently when the pre-junctional nerve fiber is electrotonically polarized (36), suggesting that the neural impulse propagating into these terminals might be an adequate stimulus for release of the transmitter. However, miniature responses still occur when the pre- and post-units have become electrically irresponsive in preparations depolarized by K_2SO_4 and deprived of sodium and chloride (39). Ca^{++} increases the quantity of transmitter released, while Mg^{++} has the opposite effect (34, 35).

f) **Facilitation and Fatigue.** A rather general phenomenon of synaptic transmission is that now termed presynaptic (or posttetanic) facilitation. Autonomic ganglia (121, 137) and eel electroplaques (4–6) for a long time after a single presynaptic stimulus (fig. 2) exhibit enhanced responsiveness to another stimulus, through the same homosynaptic pathway, but not to another (heterosynaptic) pathway or to direct stimulation. There are no persistent changes in membrane potential to accompany the enhancement of responsiveness (4). In other systems (79, 139) the facilitation is manifested best when the presynaptic nerve is stimulated at high rates and for long times. One explanation offered for this phenomenon is that the presynaptic electrical response becomes more effective for releasing the transmitter agent, a greater quantity of the latter becoming available at the synapse perhaps because the presynaptic spikes starts from a baseline of hyperpolarization after the previous activity and is therefore higher in amplitude. The facilitating action is therefore presumed to reside in the presynaptic fiber. Another possibility is that the conditioning stimulation has left behind at the synapse a residue of the transmitter or of the changed state of the transducer molecules which raises the synaptic effectiveness of subsequently emitted transmitters. This view (4) places the facilitatory action at the postsynaptic membrane, but localized to those synapses previously excited and not affecting other, probably even nearby synaptic membrane regions.

The reverse phenomenon of synaptic 'fatigue' is also rather general. Repetitive stimulation of the presynaptic pathway leads to transmission block, although a smaller p.s.p. is usually still elicited (e.g., squid giant axon; ref. 25). The post-junctional cell remains, however, directly excitable (4, 25). The block might result from absolute or relative exhaustion of the store of transmitter at the presynaptic terminals, from secondary, depressant effects elicited by excess of the transmitter, or from secondary changes in the transducer molecules analogous to 'sodium inactivation' of squid giant axons. This phenomenon is

also seen in figure 7 and was first described by Wedensky. The summated depolarization produced by repetitively evoked p.s.p.'s itself probably causes the inactivation of the electrically excitable, spike generating membrane of the ganglion cell. This blockade is therefore similar to that caused by 'depolarizing' or synapse activating (100) drugs.

g) **Synaptic Transducer Membrane.** *Structures observed.* In view of the likelihood that transducer function resides in membrane structures of molecular dimensions, it is not surprising, although disappointing, that electron microscopy has not yet revealed new, functional differentiations between pre- and post-synaptic membrane or between the latter and the electrically excitable spike generator components of the post-junctional cell (156, 167). In the muscle end-plate and at the innervated face of electroplaques, cytological and histochemical differentiations have long been known, but of obscure significance (51–53). In electron micrographs of the eel electroplaque, the differences seen between the responsive, innervated caudal membrane of the cell and the anterior non-responsive membrane are minor (15). Some differentiations are reported in electroplaques of *Torpedo* (53).

Structure deduced from function. Functional considerations provide some specifications for the molecular structure of the postsynaptic membrane. The 'receptor' located at the external face of the membrane (38) probably has specific sites for attachment of, and action by, the transmitter. For the acetylcholine members of the family, two somewhat similar structural schemes have been proposed (46, 189). In some cells of the autonomic system (e.g., cardiac pacemaker cells; refs. 40, 118) the cholinergic transmitter causes inhibition (i.e., tendency toward hyperpolarization) while the adrenergic acts oppositely and is excitant (i.e., tending toward depolarization). Therefore the same cell must be endowed with different and reciprocally-acting synaptic transducer membrane. In other cells, the cholinergic is an excitant and depolarizer, while the adrenergic is a depressant and hyperpolarizer. Thus a double reciprocal relation appears.

Transmitter	Electrogenic response		Transmitter	
	→ *1*) Depolarizing	*2*) Hyperpolarizing ←		
Cholinergic			Adrenergic	(5)
	→ *3*) Hyperpolarizing	*4*) Depolarizing ←		

The cholinergically activated membrane constituent *1* tends to be associated with the adrenergic *type 2*, but when the cholinergic is of *type 3* the adrenergic component is *4*. These relations, therefore, suggest that some common basic molecular structure of the membrane undergoes modification in one direction or another to evolve all four synaptic receptors. A common relation between molecular structures of cholinergic and adrenergic synapses (cf. 180) may also

explain the adjuvant effects of sympathetic stimulation (Orbeli effect) of adrenaline on neuromuscular transmission of the fatigued preparation (117). Suggestive of this are also the effects of adrenergic agents on the cholinergic system of autonomic ganglia (cf. 144).

An instructive and probably a clinically important interrelation is to be seen in the effects of blocking agents on cardiac actions of vagal and sympathetic stimuli (163a). Whereas *d*-tubocurarine has a well-known vagolytic action (i.e., block of vagus-induced depression of cardiac rhythm) a depolarizing synaptic blocker (32), succinylcholine, eliminates the effects of both vagal and sympathetic stimulation in the cat. The mechanism of this action is presumably different from that of the non-depolarizing curare effect. Nevertheless, injection of *d*-turbocurarine eliminates the sympathetic block, but not the vagal, produced by previous administration of succinylcholine. Therefore, the former drug is an adjuvant to adrenergic action and presumably combines with adrenoceptive membrane as well as with cholinoceptive.

The inhibitory transmitter agent for invertebrate muscle fibers exerts its effects not only on the specific synaptic sites tending to hyperpolarizing electrogenesis, but also depresses the sensitivity of the depolarizing, excitatory receptors to their synaptic transmitter (71). This is presumed to be caused by competitive combination of the inhibitory transmitter with the excitatory synaptic receptor, and therefore indicates that both the excitatory and inhibitory receptors of the membrane have a similar molecular structure.

Modifications in a common receptor molecular structure are likely for the chemo-transducers of olfaction, taste and vision. The possibility that mechano-transducer membrane is also chemo-receptive (59, 188) because of a common molecular structure of the transducers and the implications of this for sensitivity to 'adequate' and 'inadequate' stimuli were noted earlier. Elaborations of functional differences based on a common ground structure are also found among the respiratory pigments, enzymes and hormones, and are particularly well known for the biologically active steroids.

Synaptic structure and neurochemistry. Pharmacological and electrophysiological considerations are not, however, sufficient to develop our knowledge of membrane phenomena. Neurochemical investigation has evolved only recently and, despite the growing volume of its work (67, 145, 147, 182), it still has not supplied the important clues to membrane structure in the same sense, for example, as has the much older muscle chemistry (148) to the field of muscle physiology. The difficulties faced by neurochemists are formidable. The immediate structural components of electrogenesis probably reside in the boundary surface of neuroplasm. The manifestations of activity depend upon the integrity of this organized surface, and are evanescent. Even in the largest mass of neural tissue, the brain, neurochemical study is hampered by the intermixture of inactive components and, as has been pointed out in the foregoing, by the

several types of action which are intermingled in electrogenic responses. Thus, even the elementary nature of the excitable membrane, whether it is essentially that of neuroplasm altered by its phase boundary position or whether it is a special structural element, is unknown. Since electron microscopy has not resolved highly differentiated structures comporting with the differentiation of membrane function (156, 167, 168) it seems that the first mentioned possibility is the likelier. Also unknown are the molecular components of this membrane. In consequence, 'models' of electrogenic mechanisms must resort to the vague terms of 'pumps,' 'carriers' and 'receptors.' However, the growing number of analyses of specific chemical processes either in 'resting' brain (e.g. 82) or in 'active' (e.g. 88), and even in single cells (12, 117, 143), gives hope that neurochemistry will in the near future provide needed restrictions on speculation and useful specifications for processes.

h) **Electrogenic Action of Synaptic Transducers.** The permeability changes induced in the excitatory and inhibitory transducers must be different to cause oppositely directed potential changes. Under the normal conditions of ionic distributions, increase only of potassium conductance would drive that ion out of the cell and leave the interior more negative. Increase of chloride permeability would tend to drive this ion inward and thereby also increase the internal negativity. Increase of sodium conductance alone, if it does not involve a regenerative mechanism, could lead to depolarization and even to reversed polarization of any level up to that of the sodium potential. However, there is evidence (37, 72) that the end-plate potential does not exceed an equilibrium level slightly negative to zero membrane potential. Therefore, it is likely that the end-plate and other excitatory p.s.p.'s result from a general increase of membrane conductance to all three ions.[8] In addition to exciting the electrically excitable component of the membrane, the p.s.p. would tend to poise the potential of the latter at its own equilibrium potential, but this effect would generally be small, since in most post-junctional cells the area of synaptic transducer membrane is a small part of the total surface. However, at the muscle end-plate a relatively large part of the fiber membrane is made up of this transducer. The poising (or 'short circuiting,' cf. 72) action then is pronounced. The peak of the spike is driven down while its falling phase is lifted toward the equilibrium value of the p.s.p. (fig. 5).

An interesting example of the electrochemical modification of a synaptically activated system is disclosed by the electrogenic behavior of polarized *Torpedo* electroplaques (100). Depolarizing currents diminish the amplitude of the response, but the latter also reverses with still stronger applied currents (1). This behavior does not comport with that of an electrically excitable membrane

[8] At present the transducer effects on other, chiefly the divalent Ca^{++} and Mg^{++}, ions are neglected, although it appears likely that the membrane conductance for these is also changed during electrogenic responses (83).

generating spikes (110). It is explicable in terms of electrogenic properties of electrically inexcitable, postsynaptic membrane (e.g. fig. 8). While these cells have not yet been studied with intracellular electrodes, it also seems necessary (100) to invoke an early phase of sodium conductance and overshoot in the responses of *Torpedo* electroplaques. This could come about by different kinetics of the several ion conductance changes in the electrogenic reaction of the postsynaptic membrane as in the production of the spike by the electrically excitable membrane.

The membrane component which alters to increase sodium conductance is presumably absent from the transducer of the inhibitory p.s.p., the response normally being in the direction of increasing the internal negativity. As described above, increase in conductance of either potassium or chloride, or both would cause this electrogenic activity. As with the excitatory p.s.p., the maximum of hyper- or repolarization (cf. 134) would depend upon the equilibrium determined by electrochemical conditions. If the interior is already so negative (e.g. by applied hyper-polarization), that increased chloride conductance would drive this ion out, or increased potassium conductance force more of these into the cell, the electrogenic response should be in the direction of decreasing the membrane potential toward its equilibrium value. This has been observed in invertebrate muscle fibers (74), the crayfish stretch receptor (134) and cat motoneurons (fig. 8; refs. 48, 50, 64). On some occasions this depolarizing response may even discharge the cell, presumably when the latter is undergoing some form of anodal or postanodal excitation.

i) **Magnitude and Form of P.S.P.** A specific carrier molecule or a membrane pore probably changes its properties in an all-or-none fashion. Thus, the membrane transducer molecules probably become completely sodium, potassium, or chloride 'electrodes' and, were one able to probe the membrane potential of these molecules in isolation, the swing of the potential would probably be maximal. However, under actual conditions, the effects are averaged over some area, and electrotonic spread of the change in charge at the active loci decreases the amplitude and distorts the form of the electrogenic response. Thus, even the briefest possible electrogenic action would be observed with a rising time and a decay time. Since membrane conductance is higher during the active phase than at rest, the rising phase would be relatively more rapid, while the decaying phase would reflect the time constant of the resting membrane. In short, the response is essentially ballistic. Involvement of a larger number of action units, but still for the same very brief time, would increase the 'throw' of the ballistic system, i.e., the amplitude of the response, but would not alter its form. This is generally found to be the case for many p.s.p.'s, and it is therefore inferred that the action of the synaptic transmitter is brief compared with the time constant of the membrane. The short time of action may be caused by rapid destruction of the transmitter, its inactivation, or diffusion if only a small, threshold

quantity is involved. In other cases, either under normal conditions when the transmitter is stable and present in relatively large quantities or when a rapidly inactivated transmitter is protected against destruction (e.g. as is acetylcholine by anticholinesterases), the transducer response may persist and the p.s.p. duration may then be long (38, 72). In most known cases, the p.s.p. lasts many times longer than the spike of the same cell, but in two known cases their durations are approximately equal (squid, ref. 29; eel electroplaque. ref. 4). However, the significance of this is not known.[9]

j) Role of P.S.P. in Transmission. If depolarizing, the p.s.p. acts as an excitant to adjacent electrically excitable membrane (providing the cell has this) just as an external electrical stimulus or the electrotonic potential of the local circuit does. Small excitatory p.s.p. would tend to elicit the local, graded response and this would sum with the p.s.p. over an area of the membrane. As the p.s.p. is increased, so is the local response until their sum causes sufficient depolarization to trigger the spike (fig. 9).

The inhibitory p.s.p. would of itself cause only conductance changes characteristic of electrotonically anodally polarized membrane (e.g., 62), but these would be opposed by the increased conductance of the activated postsynaptic membrane. In the presence of membrane depolarization it would depress the latter, both by the algebraic summation of opposite membrane charge and also by tending to poise the membrane at its own equilibrium potential, thus preventing development of the depolarizing electrogenic response. In the crayfish stretch receptor, the latter action produced near the sites of the mechano-transducer potential generator decreases the electrotonic spread of the generator e.m.f. to the electrically excitable portions of the cell (134).

k) Synaptic Delay. The response of muscle or nerve to electrical stimulation occurs with vanishingly small latency. Junctional excitation, however, is always associated with a considerable minimum latency, the synaptic delay. This interval, observed in 1882 by Bernstein (100), ranges from about 0.5 milliseconds in cat motoneurons to values of ten or more times longer, as in sympathetic ganglia (66). Slowed conduction in the terminals of the prejunctional nerve fiber may account for some of the synaptic delay (140). However, in the eel electroplaque the stimulus can be applied to the nerve terminals themselves, yet the responses evoked in this way always have a latency of 1 or more milliseconds (4) and must be interpreted otherwise. It appears likely (100, 101) that

[9] The time constant of the eel electroplaque membrane is 0.1–0.2 msec. or less (2, 130), but that of the squid axon membrane is about 1 msec. The p.s.p. of the latter is reported to have a hyperpolarizing phase (29) as does the spike. However, the graded response also has this phase (11, 125) and the apparent hyper-polarization of the p.s.p. may have been caused by addition of local (i.e., graded, electrically excitable) response to the synaptic. The responses of electric organs of *Torpedo* and *Malapterurus*, which are elicited only by neural stimuli are therefore p.s.p.'s. The durations of their discharges are only 2–3 msec., as in *Electrophorus* (*cf.* 100).

at least part of this time is occupied by release and diffusion of transmitter from the nerve terminals to the post-junctional membrane.

1) **Other Interrelations of Pre- and Post-Junctional Units.** The foregoing discussion has focussed on the nature of the electrogenic triggering of junctional transmission. Pre- and post-units cooperate in other ways. Profound chemical and morphological changes occur in many, if not all, post-junctional

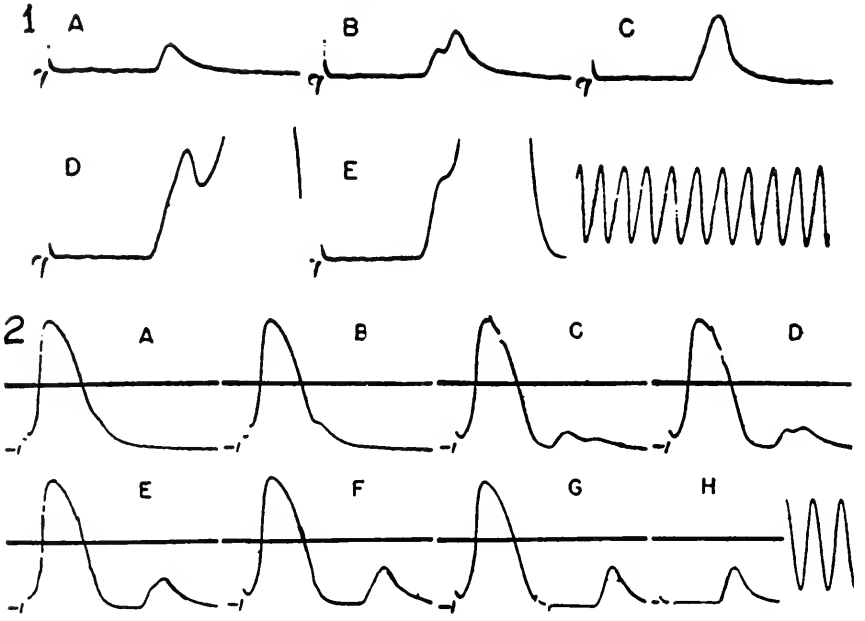

FIG. 9. Mode of transfer of excitation from the synaptic to the electrically excitable membrane. *1*) A graded neural stimulus produces increasing depolarization via increases of p.s.p. (*A*-*C*). This initiates a local response and spike in the eel electroplaque (*D*, *E*). *2*) Another electroplaque in which a maximal neural volley did not discharge the cell (*H*). The depolarization was not purely a p.s.p., however, as seen by applying the neural volley at different times during recovery from a conditioning direct stimulus. (*A*, *B*): The neural stimulus produces a small distortion of the falling phase of the spike, when the electrically excitable membrane is absolutely refractory. Early during relative refractoriness it generates a local response (*C*) which grows and arises earlier (*D*, *E*) to fuse with the p.s.p. (*F*, *G*). Calibration 10 mv and 1 msec. above, and 100 mv, 1 msec, below (from ref. 4).

cells when they are deprived of the pre-junctional supply. The reactions of denervated muscle (cf. 154) and the transneuronal degenerative changes in nerve cells are well established. Complex metabolic and pharmacological changes have been demonstrated in denervated autonomic ganglia (e.g. 157, 158). These phenomena indicate that electrogenic transducer activity triggered by presynaptic action is probably not the only function of the specialized post-

synaptic membrane. Long term functional interrelations might, for example, subserve such phenomena as memory, to which brief, but frequent transducer activity might contribute determinants by its temporary increases of the permeability of the membrane. However, this domain of interactions is still in a purely descriptive state.

m) Synaptic Transmission and Function. The electrogenic responses of the nervous system immediately subserve first, the function of collecting information, and then of effecting actions based on this information. The various sensory transducers convert specific types of stimuli into a single generalized form of response, the electrical, probably graded according to some function of the stimulus intensity. This electrogenic activity can be propagated electrotonically, and thus pass on information, but only for short distances in the cell before it is degraded in form and amplitude to such an extent that it no longer would serve as intelligence. The solution of this problem developed by most animals is similar to that adopted much later by long-lines telephone and telegraph engineers—the insertion of amplifiers to make up for the losses. The specific form used in living organisms is, however, much more elegant in its apparent economy, although, as has been described here, in actuality is dependent on much more complex phenomena. It has inserted, in the form of all-or-nothing, electrically excitable membrane, combined trigger and amplifier mechanisms more or less uniformly distributed along the cell, and thereby capable of propagating information along the full extension of the cell. However, the triggered all-or-nothing response, while making available the precision of that type of information,[10] does not in isolation provide the nuances of which graded responsiveness is capable. In many cells this weakness is overcome by repetitive discharge of all-or-nothing spikes in a latency-frequency-number code. Transmission of this information to the post-junctional cell again initiates in the latter a graded response, more or less proportional to the information coded in the message of the prejunctional unit, and this response in turn may initiate activity suitable for propagating information or commands to another junction. This is a complex mode of action, but it achieves its ends with an economy of means at hand, ionic asymmetries, resting membrane potential, and a membrane of high resistivity, but probably also of extremely high complexity in its molecular structure and in the adaptations of this to the many demands of the organism.

SUMMARY

Trigger mechanisms of electrogenesis operate on the substrate of the resting polarization of the cell. The latter is probably complexly produced because of

[10] Von Neumann (153) has made the illuminating analogy between all-or-nothing responsiveness and the digital computer, and between graded, decrementally propagated responsiveness and the analog computer (cf. also 15a).

the specific properties of the cell membrane, manifested by its high resistivity, 'pump' action, and ion selective behavior. Electrogenic activity develops when the cell membrane is responsive to stimuli, reacting by alteration of its ion permittivity. This transducer action is probably specifically determined by membrane molecular configurations of as yet unknown nature. Certain of these configurations probably respond only to specific stimuli, while others may be excited by several varieties. In some cells the membrane appears to be composed of several transducer types. Junctional systems in general appear to have specialized chemo-transducer membrane at the synaptic sites of the post-junctional cell. These respond to transmitter agents (some of which are now known) released during activity of the pre-junctional cell. They give rise to specific types of transducer action in the postsynaptic membrane and specific electrogenic activity determined by the nature of the transducer response and the electrochemical state of the post-junctional cell. This electrogenic response, the postsynaptic potential, if depolarizing may by its local circuit action in turn serve as a stimulant to electro-transducer membrane neighboring the chemo-transducer type. The resultant electrical response may have an explosive character, derived from the regenerative restimulation of the electro-transducer by the altered membrane polarization. Explosive responsiveness permits all-or-nothing, decrementless propagation of information for long distances in the cell. On the other hand, membrane transducers insensitive to electrical stimuli are capable of electrogenic responsiveness graded in amplitude and duration with the intensity and duration of the stimulus, but rapidly losing intelligibility with distance of propagation in the cell. The combination of graded and all-or-nothing responses developed by the simultaneous presence of different types of transducer membrane may have a functional significance in junctional transmission.

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REFERENCES

1. Albe-Fessard, D. *Arch. sci. physiol.* 5: 45, 1951.
2. Altamirano, M. *J. Cell & Comp. Physiol.* 46: 240, 1955.
3. Altamirano, M. *Biochim. et biophys. acta* 20: 323, 1956.
3a. Altamirano, M. *Federation Proc.* 15: 2, 1956.
4. Altamirano, M., C. W. Coates and H. Grundfest. *J. Gen. Physiol.* 38: 319, 1955.

5. ALTAMIRANO, A., C. W. COATES, H. GRUNDFEST AND D. NACHMANSOHN. *J. Gen. Physiol.* 37: 91, 1953.
6. ALTAMIRANO, M., C. W. COATES, H. GRUNDFEST AND D. NACHMANSOHN. *Biochim. et biophys. acta* 16: 449, 1955.
7. ALTAMIRANO, M. AND H. GRUNDFEST. *Tr. Am. Neurol. A.* 79: 79, 1954.
8. ALVAREZ-BUYLLA, R. AND J. R. DE ARELLANO. *Am. J. Physiol.* 172: 237, 1953.
9. AMATNIEK, E., G. KEIBEL, W. FREYGANG, A. M. SHANES AND H. GRUNDFEST. To be published.
10. ARAKI, T., T. OTANI, AND T. FURUKAWA. *Japan. J. Physiol.* 3: 354, 1953.
11. ARVANITAKI, A. *Arch. internat. physiol.* 49: 209, 1939.
12. ARVANITAKI, A. AND N. CHALAZONITIS. *Arch. sci. physiol.* 3: 45, 1949.
12a. ARVANITAKI, A. AND N. CHALAZONITIS. *Arch. sci. physiol.* 10: 95, 1956.
13. BERGAMI, G. *Boll. soc. ital. biol. sper.* 11: 275, 1936.
14. BERNSTEIN, J. *Elektrobiologie.* Braunschweig: Fr. Vieweg, 1912.
15. BERNSTEIN, P. Unpublished observations.
15a. BISHOP, G. H. *Physiol. Rev.* 36: 370, 1956.
16. BOETTIGER, E. G. *J. Cell. & Comp. Physiol.* 46: 370, 1955.
17. BOZLER, E. *Endocrinology* 29: 224, 1941.
18. BREMER, F., *Editor. Arch. internat. physiol.* 59: 475, 1951.
19. BREMER, F. AND V. BONNET. *Arch. sci. physiol.* 3: 363, 1949.
20. BROCK, L. G., J. S. COOMBS AND J. C. ECCLES. *J. Physiol.* 117: 431, 1952.
21. BROCK, L. G., J. S. COOMBS AND J. C. ECCLES. *J. Physiol.* 122: 429, 1953.
22. BROCK, L. G., R. M. ECCLES AND R. D. KEYNES. *J. Physiol.* 122: 4P, 1953.
23. BROWN, F. A. AND J. F. DANIELLI, *Editors. Active Transport and Secretion* (Biol. Symp. 8). New York: Acad. Press, 1954.
24. BRYANT, S. M. AND J. M. TOBIAS. *J. Cell. & Comp. Physiol.* 46: 71, 1955.
25. BULLOCK, T. H. *J. Neurophysiol.* 11: 343, 1948.
26. BULLOCK, T. H. *Cold Spring Harbor Symp. Quant. Biol.* 17: 267, 1952.
27. BULLOCK, T. H. *Federation Proc.* 12: 666, 1953.
28. BULLOCK, T. H., H. GRUNDFEST, D. NACHMANSOHN AND M. A. ROTHENBERG. *J. Neurophysiol.* 10: 63, 1947.
29. BULLOCK, T. H. AND S. HAGIWARA. *Biol. Bull.* 109: 341, 1955.
30. BURGEN, A. S. V. AND F. C. MACINTOSH. In: *Neurochemistry: The Chemical Dynamics of Brain and Nerve.* Edited by K. A. C. ELLIOTT, I. H. PAGE AND J. H. QUASTEL. Springfield: Thomas, 1955, Ch. 13.
31. BURKE, W. AND B. L. GINSBORG. *J. Physiol.* 129: 9P, 1955.
32. BURNS, R. D. AND W. D. M. PATON. *J. Physiol.* 115: 41, 1951.
33. CALABRO, Q. *Riv. biol.* 15: 299, 1933.
34. DEL CASTILLO, J. AND L. ENGBAEK. *J. Physiol.* 124: 370, 1954.
35. DEL CASTILLO, J. AND B. KATZ. *J. Physiol.* 124: 553, 1954.
36. DEL CASTILLO, J. AND B. KATZ. *J. Physiol.* 124: 586, 1954.
37. DEL CASTILLO, J. AND B. KATZ. *J. Physiol.* 125: 546, 1954.
38. DEL CASTILLO, J. AND B. KATZ. *J. Physiol.* 128: 157, 1955.
39. DEL CASTILLO, J. AND B. KATZ. *J. Physiol.* 128: 396, 1955.
40. DEL CASTILLO, J. AND B. KATZ. *Nature* 175: 1035, 1955.
41. CHAGAS, C. AND D. ALBE-FESSARD. *Acta physiol. latinoam.* 4: 49, 1954.
42. CHAGAS, C., L. SOLLERO, H. MARTINS-FERREIRA AND H. C. PARREIRA. *Anais acad. brasil. cienc.* 25: 327, 1953.
43. CHANG, H. T. *J. Neurophysiol.* 14: 1, 1951.
44. CLARE, M. H. AND G. H. BISHOP. *Electroencephalog. & Clin. Neurophysiol.* 7: 85, 1955.
45. COATES, C. W., A. ALTAMIRANO AND H. GRUNDFEST. *Science* 120: 845, 1954.

46. Cohen, J. A. and C. H. Posthumus. *Acta physiol. et pharmacol. neerl.* 4: 17, 1955.
47. Conway, E. J. In: *International Review of Cytology*, edited by G. H. Bourne and J. F. Danielli. New York: Acad. Press, 1955, vol. IV.
48. Coombs, J. S., J. C. Eccles and P. Fatt. *J. Physiol.* 130: 326, 1955.
49. Coombs, J. S., J. C. Eccles and P. Fatt. *J. Physiol.* 130: 374, 1955.
50. Coombs, J. S., J. C. Eccles and P. Fatt. *J. Physiol.* 130: 396, 1955.
51. Couceiro, A. and M. Akerman. *Anais acad. bras. cienc.* 20: 383, 1948.
52. Couceiro, A., D. F. de Almeida and M. Miranda. *Anais acad. bras. cienc.* 27: 49, 1955.
53. Couteaux, R. In: *International Review of Cytology*, edited by G. H. Bourne and J. F. Danielli. New York: Acad. Press, 1955, vol. IV.
54. Crane, E. E. In: *Progress in Biophysics and Biophysical Chemistry*. New York: Acad. Press, 1950, vol. 1.
55. Csapo, A. *Nature* 173: 1019, 1954.
56. Dale, H. H. *J. Pharmacol.* 6: 147, 1914.
57. Davis, H. *Federation Proc.* 12: 661, 1953.
58. Desmedt, J. E. and G. La Grotta. *J. Physiol.* 129: 46P, 1955.
59. Diamond, J. *J. Physiol.* 130: 513, 1955.
60. Dixon, W. E. *Med. Mag.* 16: 454, 1907.
61. Du Bois-Reymond, E. In: *Ges. Abh. allgemein. Muskel- und Nervenphysik*. Leipzig: Veit, 1877, cf. Vol. 2, pp. 698–736.
62. Eccles, J. C. *Ann. New York Acad. Sci.* 47: 429, 1946.
63. Eccles, J. C. *The Neurophysiological Basis of Mind*. Oxford: Clarendon, 1953.
64. Eccles, J. C., P. Fatt and K. Koketsu. *J. Physiol.* 126: 524, 1954.
65. Eccles, J. C. and W. J. O'Connor. *J. Physiol.* 100: 318, 1941.
66. Eccles, R. M. *J. Physiol.* 130: 572, 1955.
67. Elliott, K. A. C., I. H. Page and J. H. Quastel, *Editors*. *Neurochemistry: The Chemical Dynamics of Brain and Nerve*. Springfield: Thomas, 1955.
68. Eyzaguirre, C. and S. W. Kuffler. *J. Gen. Physiol.* 39: 87, 1955.
69. Eyzaguirre, C. and S. W. Kuffler. *J. Gen. Physiol.* 39: 121, 1955.
70. Falk, G. and R. W. Gerard. *J. Cell & Comp. Physiol.* 43, 393, 1954.
71. Fatt, P. *Physiol. Rev.* 34: 674, 1954.
72. Fatt, P. and B. Katz. *J. Physiol.* 115: 320, 1951
73. Fatt, P. and B. Katz. *J. Physiol.* 120: 171, 1953.
74. Fatt, P. and B. Katz. *J. Physiol.* 121: 374, 1953.
75. Fatt, P. and B. Katz. *J. Exper. Biol.* 30: 433, 1953.
76. Feldberg, W. *Physiol. Rev.* 25: 596, 1945.
77. Feldberg, W. and A. Fessard. *J. Physiol.* 101: 200, 1942.
78. Feldberg, W. and D. Whitteridge. In: *Human Physiology*, edited by F. R. Winton and L. E. Bayliss. Boston: Little, 1955.
79. Feng, T. P. *Chinese J. Physiol.* 16, 341, 1941.
80. Fessard, A. *Proc. Roy. Soc. London, s.B.* 140: 186, 1952.
81. Fessard, A. and J. Posternak. *J. physiol., Paris* 42: 319, 1950.
82. Florey, E. and H. McLennan. *J. Physiol.* 130: 446, 1955.
83. Fluckiger, E. and R. D. Keynes. *J. Physiol.* 128: 41P, 1955.
84. Frank, K. and M. G. F. Fuortes. *J. Physiol.* 130: 625, 1955.
85. Furshpan, E. J. Studies on certain sensory and motor systems of decapod crustaceans. Ph.D. Thesis, California Inst. of Technology, 1955.
86. Garten, S. *Abh. math-wiss Kl. K. Sachs-Ges. Wiss.* 25: 253, 1899.
87. Garten, S. In: *Handbuch der Vergleichen der Physiologie*, 3 pt. 2, edited by H. Winterstein, 1910–1914, pp. 105–224.

88. GEIGER, A., S. YAMASAKI AND R. LYONS. *Federation Proc.* 14: 54, 1955.
89. GÖPFERT, H. AND H. SCHAEFFER. *Arch. ges. Physiol.* 239: 597, 1938.
90. GOTCH, F. AND G. J. BURCH. *Phil. Trans. Roy. Soc. London* 187: 347, 1896.
91. GRANIT, R. *Receptors and Sensory Perception.* New Haven: Yale, 1955.
92. GRAY, J. A. B. AND M. SATO. *J. Physiol.* 122: 610, 1953.
93. GRUNDFEST, H. *Ann. Rev. Physiol.* 2: 213, 1940.
94. GRUNDFEST, H. *Ann. Rev. Physiol.* 9: 477, 1947.
95. GRUNDFEST, H. In: *Progress in Neurology and Psychiatry,* edited by E. A. SPIEGEL. New York: Grune, 1950, vol. V.
96. GRUNDFEST, H. In: *Modern Trends in Physiology and Biochemistry,* edited by E. S. G. BARRON. New York; Acad. Press, 1952.
97. GRUNDFEST, H. *Arch. exper. Path. u. Pharmakol.* 220: 136, 1953.
98. GRUNDFEST, H. In: *Fifth Conference on the Nerve Impulse.* New York: Macy, 1956.
99. GRUNDFEST, H. Electric field effects and synaptic potentials in the functioning of the nervous system. *Volume in Honor of Prof. I. Beritashvili (Beritoff).* Acad. Sci. Georgian S.S.R. In press.
100. GRUNDFEST, H. In: *Progress in Biophysics, Vol. 7,* edited by J. A. V. BUTLER AND B. KATZ. London: Pergamon Press. 1957.
101. GRUNDFEST, H. *Ann. New York Acad. Sci.* 66: Art. 3, 1956.
102. GRUNDFEST, H. AND C. Y. KAO. *Federation Proc.* 14: 65, 1955.
103. GRUNDFEST, H. AND C. Y. KAO. To be published.
104. GRUNDFEST, H. AND J. MAGNES. *Am. J. Physiol.*, 164: 502, 1951.
105. GRUNDFEST, H. AND D. P. PURPURA. *Nature,* 178: 416, 1956.
106. HAGIWARA, S. AND T. H. BULLOCK. *Biol. Bull.* 109: 341, 1955.
107. HAGIWARA, S. AND A. WATANABE. *Japan. J. Physiol.* 4: 65, 1954.
108. HÖBER, R. *Physical Chemistry of Cells and Tissues.* Philadelphia: Blakiston, 1945.
109. HODGKIN, A. L. *Biol. Revs.* 26: 339, 1951.
110. HODGKIN, A. L. AND A. F. HUXLEY. *J. Physiol.* 117: 500, 1952.
111. HODGKIN, A. L., A. F. HUXLEY AND B. KATZ. *J. Physiol.* 116: 424, 1952.
112. HODGKIN, A. L., AND B. KATZ. *J. Physiol.* 108: 37, 1949.
113. HODGKIN, A. L. AND R. D. KEYNES. *J. Physiol.* 128: 61, 1955.
114. HODGKIN, A. L. AND R. D. KEYNES. *J. Physiol.* 128: 28, 1955.
115. HOFFMAN, B. F. AND C. Y. KAO. *Federation Proc.* 15: 302, 1956.
116. HOWLAND, B., J. Y. LETTVIN, W. S. MCCULLOCH, W. PITTS AND P. D. WALL. *J. Neurophysiol.* 18: 1, 1955.
117. HUTTER, O. F. AND W. R. LOEWENSTEIN. *J. Physiol.* 130: 559, 1955.
118. HUTTER, O. F. AND W. TRAUTWEIN. *Nature* 176: 512, 1955.
119. HYDEN, H. In: *Biochemistry of the Developing Nervous System,* edited by H. WAELSCH. New York: Acad. Press, 1955.
120. INGVAR, D. H. *Acta physiol. scandinav.* 33: 169, 1955.
121. JOB, C. AND A. LUNDBERG. *Acta physiol. scandinav.* 28: 14, 1953.
122. KAO, C. Y. AND H. GRUNDFEST. *Federation Proc.* 15: 104, 1956.
123. KAO, C. Y. AND H. GRUNDFEST. To be published.
124. KAO, C. Y. AND H. GRUNDFEST. To be published.
125. KAO, C. Y. AND H. GRUNDFEST. To be published.
126. KAO, C. Y. AND H. GRUNDFEST. To be published.
127. KATSUKI, Y., YOSHINO, S. AND J. CHENG. *Jap. J. Physiol.* 1: 264, 1951.
128. KATZ, B. *J. Physiol.* 11: 261, 1950.
129. KATZ, B. *Symp. Soc. Exptl. Biol.* 4: 16, 1952.
130. KEYNES, R. D. AND H. MARTINS-FERREIRA. *J. Physiol.* 119: 315, 1954.
131. KUFFLER, S. W. *Arch. Sci. Physiol.* 3: 459, 1949.

132. Kuffler, S. W. and E. M. Vaughan-Williams. *J. Physiol.* 121: 318, 1953.
133. Kuffler, S. W. *J. Neurophysiol.* 17: 558, 1954.
134. Kuffler, S. W. and C. Eyzaguirre. *J. Gen. Physiol.* 39: 155, 1955.
135. Kuhne, W. *Proc. Roy. Soc. London, s.B.* 44: 427, 1888.
136. Laporte, Y. and R. Lorente de Nó. *J. Cell. & Comp. Physiol.* 35: Suppl. 2, 107, 1950
137. Larrabee, M. G. and D. W. Bronk. *J. Neurophysiol.* 10: 139, 1947.
138. Lloyd, D. P. C. *J. Neurophysiol.* 6: 143, 1943.
139. Lloyd, D. P. C. *J. Gen. Physiol.* 33: 147, 1949.
140. Lloyd, D. P. C. In: *Textbook of Physiology* 17th ed., edited by J. F. Fulton. Philadelphia: Saunders, 1955, pp. 1–122, figs. 61, 71.
141. Loewi, O. *Pfluger's Arch. ges. Physiol.* 189: 239, 1921.
142. Lorente de Nó, R. *J. Cell. & Comp. Physiol.* 33: Suppl. 1, 231, 1949.
143. Lowrey, O. H. In: *Biochemistry of the Developing Nervous System* edited by H. Waelsch. New York: Acad. Press, 1955.
144. Marmejac, J. *J. Physiol.* 130: 497, 1955.
145. McIlwain, H. *Biochemistry and the Central Nervous System.* Boston: Little, 1955.
146. Minz, B. *The Role of Humoral Agents in Nervous Activity.* Springfield: Thomas, 1955.
147. Modern Trends in Neuropathology. (Symposium). *J. Neuropath. & Exper. Neurol.* 14: 1–83, 1955.
148. Morales, M. F., J. Botts, J. J. Blum and T. L. Hill. *Physiol. Rev.* 35: 475, 1955.
149. Mullins, L. J. In: *Molecular Structure and Function of Neurons.* Washington, D. C.: Am. Inst. Biol. Sci., 1956.
150. Nachmansohn, D. In: *Textbook of Physiology* (17th ed.) edited by J. Fulton. Philadelphia: Saunders, 1955.
151. Nastuk, W. L. *Federation Proc.* 10: 96, 1951.
152. Nastuk, W. L. *Federation Proc.* 12: 102, 1953.
153. von Neumann, J. In: *Cerebral Mechanisms in Behavior* edited by L. A. Jeffress. New York: Wiley, 1951.
154. Nicholls, J. G. *J. Physiol.* 131: 1, 1956.
155. Otani, T. Personal communication.
156. Palay, S. L. and G. E. Palade. *J. Biophys. Biochem. Cytol.* 1: 69, 1955.
157. Perry, W. L. M. and H. Reinert. *J. Physiol.* 126: 101, 1954.
158. Perry, W. L. M. and H. Reinert. *J. Physiol.* 130: 156, 1955.
159. Phillips, C. G. *Quart. J. Exper. Physiol.* 41: 58, 1955.
160. Phillips, C. G. *Quart. J. Exper. Physiol.* 41: 70, 1955.
161. Purpura, D. P. *Tr. Am. Neurol. A.* In press.
162. Purpura, D. P. *Am. J. Physiol.* 186: 250, 1956.
163. Purpura, D. P. and H. Grundfest. *Federation Proc.* 15: 476, 1956.
163a. Purpura, D. P. and H. Grundfest. *Science* 124: 319, 1056.
164. Ralston, H. J. and B. Libet. *Am. J. Physiol.* 173: 449, 1953.
165. Renshaw, B. *J. Neurophysiol.* 4: 167, 1941.
166. Reynolds, S. R. M. and S. Kaminester. *Am. J. Physiol.* 112: 640, 1935.
167. DeRobertis, E. D. P. and H. S. Bennett. *J. Biophys. Biochem. Cytol.* 1: 47, 1955.
168. Robertson, J. D. *J. Biophys. Biochem. Cytol.* 1: 271, 1955.
169. Roeder, K. D. and E. A. Weiant. *J. Exper. Biol.* 27: 1, 1950.
170. Rosenblueth, A., J. Alanis and J. Mandoki. *J. Cell. & Comp. Physiol.* 33: 405, 1949.
171. Shanes, A. M. and M. D. Berman. *Federation Proc.* 15: 168, 1956.
172. Shanes, A. M., H. Grundfest and W. Freygang. *J. Gen. Physiol.* 37: 39, 1953.
173. Sollner, K. In: *Electrochemistry in Biology and Medicine*, edited by T. Shedlovsky. New York: Wiley, 1955.

174. Soskin, S. and R. Levine. *Carbohydrate Metabolism.* Chicago: Univ. Chicago Press, 1952.
175. Sten-Knudsen, O. *J. Physiol.* 125: 396, 1954.
176. Tasaki, I. and W. Freygang. *J. Gen. Physiol.* 39: 211, 1955.
177. Tasaki, I., E. H. Polley and F. Orrego. *J. Neurophysiol.* 17: 454, 1954.
178. Tauc, L. *J. physiol., Paris* 47: 769, 1955.
179. Tobias, J. M. *J. Cell. & Comp. Physiol.* 46: 183, 1955.
180. Toman, J. E. P. Neurotropic drugs. In: *Neurochemistry*, edited by K. A. C. Elliott, I. H. Page and J. H. Quastel. Springfield: Thomas, 1955.
181. Ussing, H. H. In: *Ion Transport Across Membranes*, edited by H. T. Clarke and D. Nachmansohn. New York: Acad. Press, 1954.
182. Waelsch, H., *Editor. Biochemistry of the Developing Nervous System.* New York: Acad. Press, 1955.
183. Weddell, G., E. Palmer and W. Pallie. *Biol. Revs.* 30: 159, 1955.
184. Weidmann, S. *J. Physiol.* 129: 568, 1955.
185. Weidmann, S. *Elektrophysiologie der Herzmuskelfaser.* Bern: Hans Huber, 1956.
186. Wiercinski, F. J. *Federation Proc.* 11: 172, 1952.
187. Wiersma, C. A. G. *Cold Spring Harbor Symp. Quant. Biol.* 17: 155, 1952.
188. Wiersma, C. A. G., E. J. Furshpan and E. Florey. *J. Exper. Biol.* 30: 136, 1953.
189. Wilson, I. B. and D. Nachmansohn. In: *Ion Transport Across Membranes*, edited by H. T. Clarke and D. Nachmansohn. New York: Acad. Press, 1954.
190. Wilson, V. J. *J. Exper. Biol.* 31: 280, 1954.
191. Zotterman, Y. In: *Fourth Conference on Nerve Impulse.* New York: Macy, 1954.

Primary Mechanisms of Hormonal Action on Target Cells[1,2]

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IT SEEMS SCARCELY NECESSARY to point out to this sophisticated group that participation of the endocrinologist in the present symposium is fitting and proper. The time is long past for the view, based largely on the early descriptive phase of the metamorphosis of this field, that endocrine regulation is characterized by relatively gross and imprecise responses which defy quantitative evaluation.

Administration of a hormone to an appropriate test object gives impetus to a series of biochemical events which may culminate in functional and morphological changes in the target tissues. In the present discussion, attention will be directed primarily to the action of certain steroid hormones upon a typical target organ, the uterus, and emphasis will be placed upon analysis of *a*) the metabolic pathways upon which the hormone exerts its primary or 'triggering' influence; *b*) the relationship between this primary effect and the succeeding, relatively nonspecific, consequences of hormonal stimulation; and *c*) the mode of interaction between the hormone and the receptor which, in effect, *together* constitute the 'trigger.'

Such an analysis requires gross oversimplification because of our lack of precise knowledge in these areas. It would appear, however, that the time is ripe for advancing a working hypothesis of the nature of hormone action (cf. 24, 36) which lends itself to experimental testing. In essence, this may be stated as follows *for the special set of circumstances indicated above*. The 'trigger' of hormone action may be considered to be the resultant new biochemical entity formed by the interaction of the hormone and an active receptor site, most likely protein, of the target cell. The primary reaction 'triggered' by the presence of this complex would appear to be the enhanced rate of penetration into the cell of one or more key metabolites. The nonspecific biochemical responses secondary to this fundamental transfer reaction may simply be a function of the increased availability of the critical substance to the enzymatic machinery of the cell.

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[2] Several aspects of this topic have been discussed in previous reviews with the collaboration of Dr. Sidney Roberts, Department of Physiological Chemistry, School of Medicine, University of California Medical Center, Los Angeles (cf. 24, 26, 36).

Gross alterations in structure and function of the target cell would result from enhanced and directed metabolism.

The extension of this mechanism with certain modifications to hormones other than selected steroids can also be demonstrated. Only a few of the more striking examples of the latter will be mentioned.

It is scarcely necessary to emphasize the provisional nature of these propositions. This undoubtedly premature attempt to extend physicochemical principles to the overwhelmingly complex target cell entails an inordinate number of assumptions.

INFLUENCE OF STEROIDS ON UTERINE WEIGHT AND WATER CONTENT

In attempts to dissect the primary effect from the surrounding shell of nonspecific consequences of estrogen action upon the uterus, reliance must be placed upon the time-course of the metabolic events which follow hormonal stimulation. Thus, while the cumulative effects of the changes which occur in response to estrogen are hypertrophy and hyperplasia, it is generally recognized that true growth of this organ is preceded by, and, in fact, predicated upon, earlier modifications of cellular composition and enzymatic activity.

The early period is characterized by an acute shift in water and electrolyte distribution, associated with enhancement in blood supply. Profound changes in energy metabolism and respiratory activity may also be detected. The second, or true growth phase is distinguished by the generally accepted evidences of increase in protoplasmic substance: increment in dry weight, deposition of organic and nitrogenous constituents, and full-blown morphological signs of cellular proliferative activity.

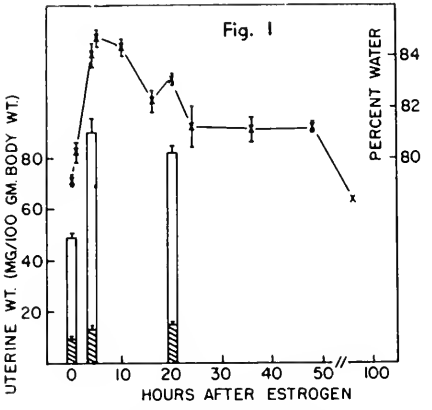
Figure 1 depicts the time-course of water imbibition by the uterus of the adult ovariectomized rat after a single intravenous dose of 0.5 microgram of estradiol-17 β in saline (23) per 100 grams body weight.

It will be noted that a striking augmentation in the water content of the uterus occurred within 4–6 hours; a secondary peak may have taken place at 20 hours. The total uterine weights at these two periods were quite similar, but the 4-hour increase was attributable almost entirely to water imbibition, whereas the 20-hour increase was associated with significantly elevated uterine solids and histological evidences of cellular proliferation. Accordingly, these two time-intervals of duration of hormonal stimulation, as likely to be representative of the primary and secondary phases of the response complex, were chosen for most of the studies reported herein.

The results shown in figure 1 are quite similar to those noted by Astwood (1) after the subcutaneous administration of estradiol or estrone to the immature rat. This investigator and co-workers first directed attention to the biphasic character of the uterine growth response to endogenous or exogenous estrogenic stimulation, and also observed that the rapid early change in intra- and extra-

cellular uterine water may have been preceded by an acute redistribution of its electrolyte pattern (41).

In view of the implication of secretions of the adrenal cortex in the regulation of osmotic phenomena, it seemed advisable to determine whether these early indices of estrogen activity might be subject to modification by the activity of the pituitary-adrenal system. It was found (35) that the increase in uterine weight produced 4 hours after the intravenous administration of estradiol could



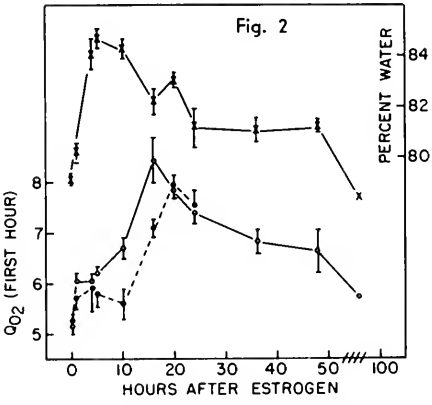


FIG. 1. Influence of time after estrogen administration on weight and water content of the uterus of the castrated rat. *Total height* of bars represents the average uterine wet weight; *cross-hatched portion*, uterine dry weight; *crosses*, % uterine water. Each point represents the mean ± the standard error of values obtained from at least 7 animals, except at 96 hours where only 4 determinations were made of uterine water content. In the case of the bars, only the positive segment of the standard error is shown. All animals except the untreated control group at 0 hours, were injected intravenously with 0.5 μg estradiol-17 β per 100 gm body weight. Estrogens were administered as sodium salts in saline, prepared as previously described (23). Autopsy was performed at the times indicated. (Reprinted from ref. 36 by permission of the editor and publishers.)

FIG. 2. Influence of time after estrogen injection on oxygen consumption *in vitro* and water content of the uterus of the castrated rat. Each point represents the mean ± the standard error of values obtained in 6–66 experiments. *Open circles*, first hour oxygen consumption in medium without added glucose; *solid circles*, oxygen consumption in medium with added glucose; *crosses*, % uterine water. Dosage of estradiol, 0.5 μg/100 gm body weight, injected intravenously. (Reprinted from ref. 25 by permission of the editors.)

be completely prevented by the concurrent injection of ACTH (adrenocorticotrophic hormone). The adrenocortical steroids found (32) to be the most active in reproducing the ACTH effect were cortisol and cortisone (Kendall's Compounds F and E, respectively). Cortisol appeared to be the most effective steroid in this regard, and in other respects as well, and was chosen for most of the subsequent studies (cf. 36). Thus, the 17-hydroxy and 11-oxy functions appeared to be necessary for the highest activity. As will be discussed below, the

occurrence of this antagonistic relationship between estradiol and certain structurally related compounds furnishes a valuable clue to the mechanism of steroid hormone action and interaction, particularly since it has permitted a certain degree of dissociation of the so-called primary from the secondary phenomena (cf. 24, 36).

INFLUENCE OF STEROIDS ON UTERINE RESPIRATION AND GLYCOLYSIS

It may be anticipated that the metabolic transformations which accompany growth in a hormonally stimulated sexual target structure would be associated with enhancement in energy metabolism. In fact, respiratory and glycolytic mechanisms in this organ have previously been shown to be stimulated about 24 hours after the subcutaneous administration of estrogenic hormone (6, 16). In our studies (25), significant changes in the respiration of surviving uterine tissue obtained from ovariectomized rats could be demonstrated within a few hours after the intravenous administration of 0.5 microgram of estradiol per 100 grams body weight (fig. 2). Actually, in the absence of added glucose in the incubation medium (*open circles*), significant increases were noted in the first hour after estrogen injection. The presence of glucose (*solid circles*) in almost every instance depressed the respiration of surviving uterine tissue from estrogen-treated castrated rats and tended to mask these early evidences of stimulation. The total oxidative metabolism of this tissue reached a maximum about 16–20 hours after estrogen administration. A second peak in water imbibition also occurred during this period (*uppermost curve*). Both processes were presumably associated with the stimulation of true growth of the uterus which occurs about this time.

In the presence of added glucose, aerobic incubation of the uterus of the untreated castrated rat resulted in the disappearance of large amounts of this carbohydrate, as depicted by the *open circle* at zero time in figure 3. About two-thirds of this could be accounted for by the accumulation of lactate (*solid circle*).

The early electrolyte and water changes in the uterus, occurring a few hours after the intravenous administration of estrogen, were accompanied by a striking enhancement in carbohydrate utilization by this organ. Much of the extra glucose which disappeared at this time (4 hr.) was apparently converted to lactate. Since the increase in uterine respiration in the glucose medium at this time was of only limited significance (fig. 3, *crosses*), it must be assumed that metabolic shifts occurred within the uterus as a result of estrogenic stimulation, which facilitated the increased permeation and utilization of glucose and the sparing of endogenous substrate.

It will also be noted in figure 3 that a slight decrease in glucose utilization occurred after the 4-hour peak. A renewed disappearance of carbohydrate became evident during the secondary growth phase and reached a maximum

rate about 20 hours after the intravenous administration of estradiol. In contrast to the metabolic events occurring at 4 hours, stimulation of glucose disappearance 20 hours after estrogen was accompanied by a marked depression in lactate accumulation. Only about one-fourth of the glucose disappearing at the 20-hour peak appeared as lactate. Presumably, a large portion of the remainder was completely oxidized and served as the source of the added energy represented by the large increase in oxygen utilization at this time. Under anaerobic conditions the glycolytic rate was somewhat higher than that observed under aerobic conditions, and most of the glucose disappearing could be accounted for on the basis of formed lactate.

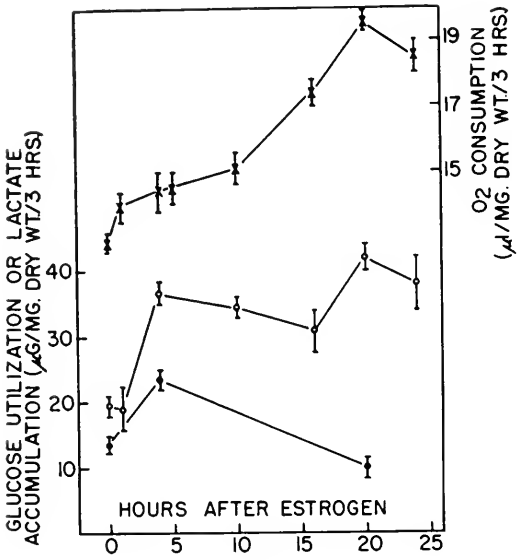


FIG. 3. Influence of time after estrogen injection on aerobic glycolysis by the uterus of the castrated rat. Each point represents the mean ± standard error of values obtained in 6–41 experiments. *Open circles*, glucose disappearance; *solid circles*, lactate accumulation; *crosses*, oxygen consumption. All figures are the total values for the 3-hour incubation period. Dosage of estradiol, 0.5 μg/100 gm body weight. (Reprinted from ref. 25 by permission of the editors.)

Quite analogous data have been obtained by Levey and Szego (18) in studying the glycolytic and oxidative metabolism of the seminal vesicle of the castrated guinea pig with and without androgen treatment.

It will be recalled that the respiration of the uterus of the castrated rat, incubated in a glucose medium, was essentially unchanged 4 hours after the intravenous administration of estradiol alone (cf. fig. 2). This is shown again in figure 4, from which it is also evident that cortisol or desoxycorticosterone, injected simultaneously with estradiol 4 hours earlier, were likewise without effect on the respiration of the uterus. On the other hand, the marked stimulation of uterine respiration 20 hours after estrogen injection (*solid circles*) was almost completely prevented by the simultaneous administration of cortisol (*squares*). Desoxycorticosterone (*triangles*), given together with estradiol, exhibited no antagonistic effect. Moreover, the increase in aerobic glycolysis,

seen both 4 and 20 hours after estrogen administration, was completely inhibited by cortisol and unchanged by desoxycorticosterone.

A number of conclusions may be drawn from these studies. It is apparent that the early changes in permeability and in electrolyte and water metabolism in the estrogen-stimulated uterus were accompanied by evidences of shifts in energy-yielding processes. It does *not* seem, however, that there was a marked increase in energy requirements at this time, since total oxidative metabolism was relatively unchanged 4 hours after the intravenous administration of estradiol. The increase in glucose utilization at this time must, therefore, be associated with a diminished utilization of endogenous substrate, and may be a result of the increased permeability of the uterine cell to glucose. In like manner, the antagonistic effect of cortisol on glucose disappearance at the 4-hour period may be accomplished by preventing these permeability changes

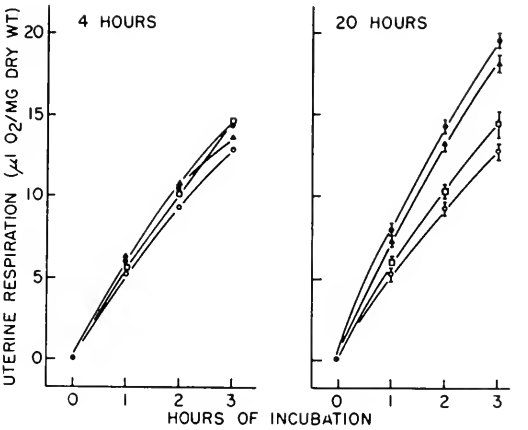


FIG. 4. Influence of steroids injected 4–20 hours earlier on uterine respiration *in vitro*. Each point is the mean ± the standard error of 6–55 experiments. *Open circles*, uninjected castrate; *solid circles*, estradiol-injected; *squares*, estradiol plus cortisol acetate (FA); *triangles*, estradiol plus desoxycorticosterone acetate (DCA). Dosage of steroids: estradiol, 0.5 μg/100 gm body weight; FA and DCA, 2.5 mg/100 gm body weight. (Reprinted from ref. 25 by permission of the editors.)

after estrogen administration. The possibility that a significant portion of estrogen-adrenocortical antagonism may be ascribed to counteractive influences on permeability is certainly consistent with the experimental evidence as earlier reviewed from this laboratory (cf. 24). However, a number of other explanations is possible. These have been discussed at length elsewhere (24, 36). What portion of the later (20-hr.) inhibitory influence of cortisol on the metabolism and growth of the estrogen-stimulated uterus may be a reflection of earlier (4-hr.) antagonistic effects cannot at present be decided. This dissociability of the primary and secondary effects of the estrogenic hormones on the uterus, under the influence of the adrenal steroids, appears to provide a valuable tool for the study of steroid action and interaction.

From the data thus far presented it would appear that reopening of the issue of modification of selective permeability by certain biocatalytic substances, advanced many years ago on the basis of classical pharmacological studies as a

mechanism of action for certain drugs, may be indicated (cf. 24). Indeed, some striking parallelisms present themselves.

The absence of well-documented instances of characteristic hormonal action in homogenates is but one facet of the accumulating evidence in support of this concept, although alternative mechanisms are not excluded on this basis alone. Rapid examination of the array of hormones whose action has been studied in some detail reveals further data consistent with the general thesis. In each instance, the rather specific transfer of a key metabolite appears to underlie the subsequent, relatively nonspecific results of hormone action. For example, the recent studies of Levine and Goldstein (20), and of Drury and Wick (9), now widely confirmed (cf. 31), appear to establish as one mode of action of insulin, its participation in enhancing the permeability of the muscle cell to glucose (and to other carbohydrates of rigidly specific structure), a response which is abolished by freezing and thawing of the tissue (19).

Similarly, the requirement of cellular integrity (12, 14, 26) for the characteristic *in vitro* action of ACTH in promoting steroid biosynthesis in adrenal preparations, coupled with the unrestrained rates of synthesis in homogenates (21), would appear to suggest (cf. 11) that ACTH may act on its target organ by removing cytostructural barriers to substrate-enzyme interaction. The facilitation by ACTH of the rate of penetration of inorganic P^{32} into adrenocortical cells *in vivo* (cf. 26), the augmentation of the effect of ACTH on corticoid biosynthesis in the perfused adrenal by high levels of potassium in the perfusion fluid (43), the requirement of calcium (4) and of glucose (28) for ACTH action on adrenal slices *in vitro* notwithstanding the failure of calcium (4) or glucose (28) deficiency to affect the rate of steroid biosynthesis in the absence of ACTH, and a number of other considerations (cf. 14), suggest that the postulated role of ACTH in regulation of permeability relationships of the intact target cell is consistent with the data thus far available (cf. 13, 14).

As is well known, shifts in ionic environment of the cell may influence in a striking manner its metabolic activities. Altered ion permeability of the cell membrane through the influence of a given biocatalytic substance may thus have far-reaching consequences to the metabolic processes within the target cell (cf. 24). This mechanism has been invoked by Szent-Györgyi in connection with the mode of action of epinephrine in affecting cardiac muscle tension, secondary to changes in intracellular cationic environment (cf. 12). Hajdu and Szent-Györgyi have described antagonistic effects of individual steroids upon heart muscular tension, and have ascribed these effects to counteractive influences upon intracellular ionic environment, achieved through modification of permeability of the cell membrane (cf. 40). Analogous studies conducted by Csapo and colleagues (cf. 8, 15) on estrogen- and progesterone-dominated rabbit uterine muscle give strong support to a similar mechanism.

One additional example of the potential importance of membrane permeability alterations as a common denominator for ubiquitous actions of the hormones

is the extension of various modifications of this postulate to the plant auxins by a number of workers (cf. 2, 5, 17, 22, 42). Bonner and associates have indeed suggested (cf. 5) that the multiplicity of growth and metabolic responses of plant cells to auxin is predicated upon a fundamental, primary one—namely, the facilitation of a water transport system which leads to active water accumulation in the auxin-stimulated plant cell. The state of this subject is extremely controversial in terms of whether the profound water shift is a primary reaction, or secondary to other phenomena (cf. 2, 17, 22). The observations presented at these meetings by Skoog (30), on the potentiating effect of certain amino acids on auxin- and kinetin-induced growth may, similarly, reflect enhancement of a translocation mechanism for these substrates which participate in secondary anabolic reactions in the target cell.

While it is most unlikely that the multifarious effects of the several plant and animal hormones of grossly dissimilar configuration, elicited on widely divergent target cells, may be explained by a single, unified hypothesis, it is tempting to see how far the analogies can be carried in the light of existing data.

HORMONAL COMPLEXES WITH PROTEIN

It would seem self-evident that interactions of the hormones with appropriate, relatively specific, protoplasmic receptors must lie at the root of their influence on the metabolic activities of their several and specific target structures. This consideration would appear axiomatic in view of the extremely low order of magnitude of the dosage requirements, and is in line with the general concept advanced time and again for the mode of action of many highly potent drugs (cf. 7, 10). Such combinations could occur at the target cell or elsewhere, and result in the new biochemical entity which 'triggers' the response complex, not dissociated from the consequences to specific permeability phenomena discussed above (cf. also 24, 36).

Here again, there are a number of signposts which indicate that a broad generalized mechanism may exist among hormonally active compounds of grossly dissimilar configuration. The affinity of insulin for proteins of the muscle cell to which it appears to be attached before exerting its characteristic effects on glucose transfer (cf. 31), the occurrence of circulating thyroid hormone in association with a specific α-globulin (cf. 3), the demonstration of auxin-protein complexes in the plant (cf. 17), some of which require high-energy phosphate for their formation (29), all appear to point in this direction. Attention may also be directed to a preliminary observation by Saffran and Bayliss (27), who noted persistence of the *in vitro* effectiveness of ACTH on adrenocortical steroid biosynthesis, after initial exposure followed by rinsing of the tissue in buffer. This finding, by analogy to that of Stadie and his group with insulin (cf. 31), may likewise represent combination of the hormone with target cell protein.

A considerable literature is available on the *in vivo* and *in vitro* interaction

of certain steroid hormones with specific proteins (cf. 24, 26, 36). A portion of the studies from our own laboratories will be presented, with the rationale that such combinations have potentially great significance in elucidation of transport and triggering mechanisms *in vivo*.

The observations from these laboratories that the liver was essential for estrogenic activity (cf. 36), coupled with the well-established role of this organ in the elaboration of certain of the plasma proteins, suggested that hepatic tissue may be the site of formation or combination of the estroprotein complex present in the circulating plasma of several species. Accordingly, *in vitro* studies were undertaken of the capacity of the liver and other tissues to influence the formation of this complex. A portion of these studies has been presented in detail elsewhere (33, 34). They were made possible by the availability of estrone

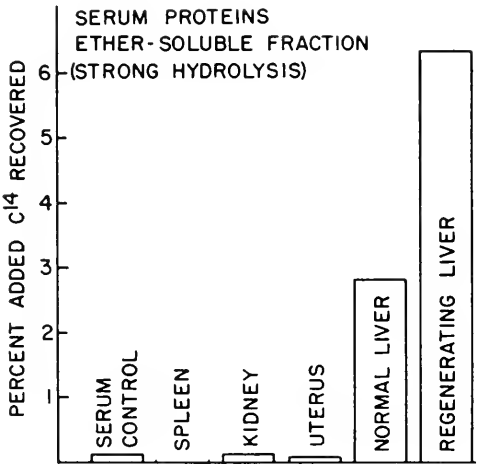


FIG. 5. Incorporation of C^{14} into serum proteins after incubation of estrone-16-C^{14} with different tissues. The ether-soluble fraction of the serum proteins following strong acid hydrolysis is shown. Incubations were carried out for 2 hours. Height of the bars for serum control, normal and regenerating liver samples represents an average of 3–5 samples; values for the other tissues are the result of single determinations. (Reprinted from ref. 33 by permission of the editor and publishers.)

and estradiol labeled at the 16-position with C^{14}. The use of these materials allowed experiments to be designed in which a few micrograms of the steroid, approaching physiological orders of magnitude, could be incubated with rat tissues in an homologous serum medium. The distribution of the estrogen or its metabolic products in various fractions of the incubation mixture could later be determined by radioactivity measurements. Figure 5 demonstrates that of the tissues studied, only surviving liver influenced the incorporation of radioactivity derived from estrone-16-C^{14} into the proteins of the serum medium. The extent of the binding was correlated with the functional state of the tissue and with time of incubation. These, and related observations reported elsewhere, suggest that the binding of estrogen or its metabolic products to the serum proteins is catalyzed by a specific enzyme system found in liver, but not in spleen, uterus or kidney. This *in vitro* model system may be representa-

tive of a mechanism by which 'activated' transport forms of the steroid as specific lipoprotein complexes are synthesized *in vivo*.

These investigations have been extended to the resolution of the above incubation media by paper electrophoretic and cold ethanol fractionation techniques (37, 39). The results demonstrate that the *in vitro* association of the steroidal moiety with the serum proteins appears to occur selectively with albumin in the presence of rat liver tissue. The data substantiate the conclusion that this association is a reflection of estrogen-protein binding, rather than simple coprecipitation. Whether or not endogenous estrogen is present in rat serum bound to albumin is unknown. It should be noted, however, that in animals other than man, albumin may assume a lipide-transporting role in the absence of those β-lipoproteins peculiar to human plasma (cf. 39).

These methods have been useful in the analysis of the antagonistic influence exhibited by the 11-oxy, 17-hydroxy adrenocortical steroids upon the metabolic responses of the uterus to estrogenic stimulation. At the time these observations were reported (cf. 24, 26) the suggestion was advanced that the phenomenon might be due to competition by the two classes of steroid for active sites on protein molecules which have functions essential for steroid activity—either in terms of transport complexes apparently formed in the liver, or at the locus of the target organ itself. This suggestion was predicated upon the following lines of evidence: *a*) the specific structural requirements for antagonistic effects, *b*) the 'titrability' of these effects with increasing dosage of the appropriate antagonist, *c*) the observation that the antagonists were found to have no effect per se on the metabolic processes which were stimulated by the estrogens, even though they were capable of inhibiting estrogenic stimulation of these processes, and *d*) the demonstrated affinity of certain of the steroid hormones for specific proteins.

This hypothesis appears to gain support from recent studies of the influence of increasing levels of unlabeled cortisol upon the liver-catalyzed association of isotopic estrone with rat serum albumin (38, 39). It was observed that 625 micrograms of cortisol (compound F) significantly inhibited the degree of estroprotein formation seen in the control containing isotopic estrone alone, and that doubling the cortisol level virtually abolished the process. The large divergence in order of magnitude of the adrenocortical vs. estrogenic steroids is in line with the *in vivo* observations, and suggests that the relative affinities of the two groups of hormones for the protein is vastly in favor of the acidic estrogens, which appear by all criteria to be the most firmly bound of all the steroids thus far investigated (cf. 26). This is in harmony with the extremely low order of magnitude of their physiological dosage requirements, and if protein binding, as has been postulated, *is* a necessary preliminary to steroid hormone action, a rational explanation for the greater activity of estrogenic substances would appear at hand.

SUMMARY AND CONCLUSIONS

The principles illustrated by this rapid analysis of our admittedly meager knowledge of hormonal action on the target cell may be restated briefly:

It is apparent that while a host of biochemical reactions are influenced (cf. 24, 36) during the complex processes of growth and differentiation which constitute the over-all response of the uterus to estrogenic stimulation, the primary loci of hormonal action in the target organ are few. Similarly, from available data on non-steroidal hormones, it is likely that direct and indirect responses are distinguishable.

The manner in which the hormone registers its influence in triggering, at least quantitatively, the chain of metabolic events in the target organ, appears related to the formation of essential links with protoplasmic receptors of a protein nature. These combinations may occur at the cell surface, and lead to modification of the semipermeability of the cell membrane, leading in turn to indirect alterations in enzymatic activity as a result of shifts in ionic constituents and other diffusible regulating substances or substrates. Hormonal alteration of the highly specific semipermeability of the cell membrane could be accomplished not only by orientation of the active molecules on the cell surface, but also by interaction with the metabolic systems responsible for maintaining the state of the cell membrane. Evidence is available for the affinity of the steroid hormones for active sites on protein molecules with potential biocatalytic functions. These observations have not been stressed in the present discussion because of the pharmacological dosage requirements, and because of difficulties in reconciling conflicting *in vivo* and *in vitro* data which are frequently diametrically opposed (cf. 24).

The demonstration of antagonistic interactions among the steroids may furnish clues to the specific loci of their combination with protoplasmic receptors (cf. 38, 39), and thus, ultimately, permit analysis of primary vs. secondary effects.

The gross and metabolic evidences of growth stimulation in response to the appropriate sex steroid, and growth depression as a result of steroid antagonism, are secondary to parallel variations in permeability and carbohydrate metabolism of the reproductive target organ. At the present stage of development of these problems, it is impossible to distinguish these interrelated phenomena in point of time. A more complete understanding of the mode of action and interaction of the steroid hormones must await elucidation of the precise relationship between selective permeability and metabolic phenomena in the cell. It is not unlikely that such information may have wide-spread applicability to the general phenomenon of biocatalytic regulation.

REFERENCES

1. Astwood, E. B. *Endocrinology* 23: 25, 1938.
2. Audus, L. J. *Biol. Rev. Cambridge Phil. Soc.* 24: 51, 1949.

3. Barker, S. B. In: *The Thyroid.* Brookhaven National Laboratories Symposium, June 9–11, 1954, p. 74.
4. Birmingham, M. K., F. H. Elliott and P. H.-L. Valère. *Endocrinology* 53: 687, 1953.
5. Bonner, J. and R. S. Bandurski. *Ann. Rev. Plant Physiol.* 3: 59, 1952.
6. Carroll, W. R. *Proc. Soc. Exper. Biol. & Med.* 49: 50, 1942.
7. Clark, A. J. *Mode of Action of Drugs on Cells.* London: Arnold, 1933.
8. Csapo, A. *Recent Progr. Hormone Research* 12, 405, 1956.
9. Drury, D. R. and A. N. Wick. *Diabetes* 4: 203, 1955.
10. Goldstein, A. *Pharmacol. Rev.* 1: 102, 1949.
11. Hayano, M., N. Saba, R. I. Dorfman and O. Hechter. *Recent Progr. Hormone Research* 12: 79, 1956.
12. Hechter, O. *Vitamins and Hormones* 13: 203, 1955.
13. Hechter, O. *Tr. Third Conf. Adrenal Cortex,* edited by E. Ralli. New York: Macy, 1952, p. 115.
14. Hechter, O. and G. Pincus, *Physiol. Rev.* 34: 450, 1954.
15. Horvath, B. *Proc. Nat. Acad. Sc.* 40: 515, 1954.
16. Kerly, M. *Biochem. J.* 34: 814, 1940.
17. Leopold, A. C. *Auxins and Plant Growth.* Berkeley and Los Angeles: Univ. Calif. Press, 1955.
18. Levey, H. A. and C. M. Szego. *Am. J. Physiol.* 183: 371, 1955.
19. Levine, R. Unpublished observations cited in (12).
20. Levine, R. and M. S. Goldstein. *Recent Progr. Hormone Research* 11: 343, 1955.
21. Macchi, I., and O. Hechter, *Arch. Biochem.* 53: 305, 1954.
22. Olson, R. A. *Physiol. Rev.* 35: 57, 1955.
23. Roberts, S. and C. M. Szego, *Endocrinology* 40: 73, 1947.
24. Roberts, S. and C. M. Szego. *Physiol. Rev.* 33: 593, 1953.
25. Roberts, S. and C. M. Szego. *J. Biol. Chem.* 201: 21, 1953.
26. Roberts, S. and C. M. Szego. *Ann. Rev. Biochem.* 24: 543, 1955.
27. Saffran, M. and M. J. Bayliss. *Endocrinology* 52: 140, 1953.
28. Schonbaum and Birmingham, cited by M. Saffran. *Recent Progr. Hormone Research* 11: 379, 1955.
29. Siegel, S. M. and A. W. Galston. *Proc. Nat. Acad. Sc.* 39: 1111, 1953.
30. Skoog, F. Unpublished observations.
31. Stadie, W. C. *Physiol. Rev.* 34: 52, 1954.
32. Szego, C. M. *Endocrinology* 50: 429, 1952.
33. Szego, C. M. *Endocrinology* 52: 669, 1953.
34. Szego, C. M. *Endocrinology* 57: 541, 1955.
35. Szego, C. M. and S. Roberts, *Am. J. Physiol.* 152: 131, 1948.
36. Szego, C. M. and S. Roberts. *Recent Progr. Hormone Research* 8: 419, 1953.
37. Szego, C. M. and S. Roberts. *Federation Proc.* 14: 150, 1955.
38. Szego, C. M. and S. Roberts. Proc. Third Intl. Congr. Biochem., Brussels, 1955, p. 65.
39. Szego, C. M. and S. Roberts. *J. Biol. Chem.* 221: 619, 1956.
40. Szent-Györgyi, A. *Chemical Physiology of Contraction in Body and Heart Muscle.* New York: Acad. Press, 1953.
41. Talbot, N. B., O. H. Lowry and E. B. Astwood. *J. Biol. Chem.* 132: 1, 1940.
42. Veldstra, H. *Biochim. et biophys. acta* 1: 364, 1947.
43. Vogt, M. *J. Physiol.* 113: 129, 1951.

Triggering of the Pituitary by the Central Nervous System[1]

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FOR SEVERAL YEARS it has been realized that the anterior lobe of the pituitary gland, though all but lacking in nerve fibers, is nevertheless partially controlled by the nervous system. The secretions of adrenocorticotrophin, thyrotrophin and gonadotrophin from the adenohypophysis are influenced by extrinsic stimuli and psychic factors via nervous pathways. There is strong evidence (16), generally though not universally (44) accepted, that the final pathway to the hypophysis involves humoral mediation via the hypophyseal portal system (42, 13). Although we have been interested in certain aspects of humoral mediation (22–24, 28, 36–39), the present discussion is concerned, not with this final pathway, but with recent work in our laboratory on more central mechanisms triggering the release of gonadotrophin. Topographically, the area under consideration is the hypothalamus and its afferent nervous pathways.

In studies on nervous control of the release of gonadotrophin the rabbit and the cat are excellent experimental subjects, since neither ovulates spontaneously but only after the nervous system triggers the release of pituitary ovulating hormone at copulation. These forms have been used to test the effects of artificial stimulation and nervous lesions and to record changes in the electrical activity of the brain accompanying various types of artificial stimulation, with ovulation as the index of effectiveness of stimulation and intactness of nervous pathways.

The estrous cat appears to have a lower threshold to afferent stimulation of gonadotrophin release than does the rabbit. It was discovered some 20 years ago by Greulich (14) that the estrous cat would ovulate in response to stimulating the vagina with a glass rod about as readily as to natural coitus. With Everett (33), we found that the anestrous cat could be primed with estrogen and equine (PMS) gonadotrophin to a state at which it would likewise ovulate after vaginal stimulation. Behaviorally, such a cat undergoes the typical 'after-reaction' which characteristically follows mating and perhaps signifies orgasm:

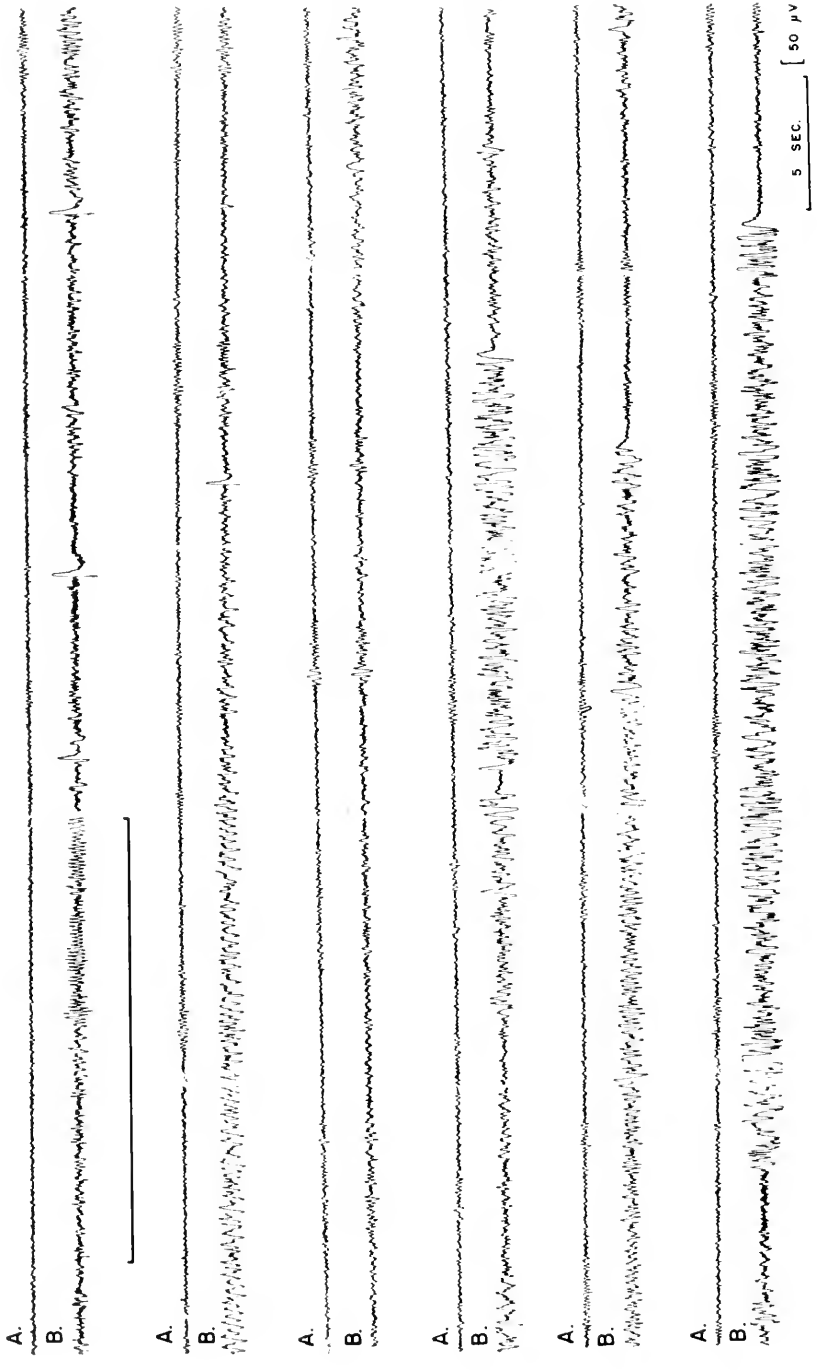
[1] Several of the investigations herein reviewed were supported in part by a grant B-334, from the National Institutes of Health.

violent rolling, neck-rubbing, etc. (43), which continues intermittently for several minutes.

Use has been made of the estrous or primed cat to study, with Dr. Porter and Mr. Cavanaugh (25), the effects of vaginal stimulation on the electrical activity of the hypothalamus. Six electrodes were stereotaxically placed within the hypothalamus and multiple recording was carried out under cyclopropane, chloralosane or curare. Two consistent patterns of altered electrical activity were revealed in the lateral hypothalamic area in and around the medial forebrain bundle. The first was characterized by an increased frequency up to 18 cycles per second, and heightened amplitude of the waves, beginning during stimulation, persisting some 15–45 seconds and returning as spindle-like bursts during the next 2–5 minutes. In unanesthetized cats under curare an additional type of activity was noted: recurrent bursts of synchronous activity with a two-fold increase in amplitude and a slow frequency of 4–6 cycles per second, continuing up to about 5 minutes after cessation of stimulation (fig. 1). Although curare prevented movement during the recording, the bursts appear to be temporally related to the active periods of the after-reaction in the unrestrained non-curarized cat. While present in 10 of 13 estrous or primed cats, the altered electrical patterns were absent in 9 anestrous animals which failed to give evidence of either behavioral after-reactions or pituitary stimulation following vaginal stimulation.

Also employing the stereotaxic technic in cats with Mr. Robison (27), we have been stimulating electrically various regions of the hypothalamus and, at the end of stimulation, making electrolytic lesions with the stimulating electrodes. The areas responding positively to electrical stimulation under chloralose anesthesia by inducing ovulation also react to lesions by blocking subsequent copulation-induced ovulation. These regions are considerably more extensive than the area from which the changes in electrical activity were recorded. They appear to extend from the ventromedial nuclear region back to the mammillary bodies, but not forward into the anterior hypothalamus. Because some of the cats in this investigation are still living, precise localization must await autopsy and a histological study of the brains.

In the rabbit brain, widespread areas of the hypothalamus from the preoptic region to the mammillary bodies have responded to direct electrical stimulation by activating release of ovulating hormone (15, 17, 20, 23). Negative results have been reported from stimulating too near the midline or too far laterally; these parasympathetic zones actually inhibit ovulation (20). Koikegami *et al.* (19) recently reported ovulation in rabbits following strong electrical stimulation of the amygdaloid nuclei. With Dr. Tokizane (40), we have more recently stimulated various amygdaloid nuclei, while simultaneously recording electrical activity in various cortical and subcortical regions approached stereotaxically (34). It appears that stimulating lateral amygdaloid nuclei at a voltage


A.
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level just above the threshold for inducing subcortical seizures is ineffective in activating the pituitary (0/5 ovulated) while similarly gauged stimuli applied to medial amygdaloid nuclei are at least partially effective in triggering the release of ovulating hormone (3/5 ovulated). The medial nuclei project via the stria terminalis to the ventromedian nucleus of the hypothalamus (12). These experiments are continuing, and again the precise positioning of stimulating electrodes awaits histological confirmation.

Relatively immense lesions in the rabbit central and peripheral nervous systems (5) have been ineffective in blocking copulation-induced ovulation. These include anesthetizing or removing the proximal half of the vagina, all

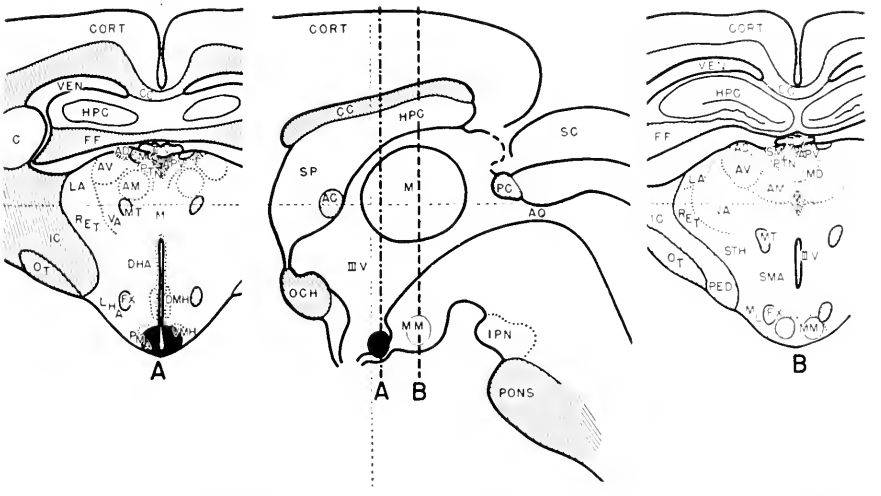

FIG. 2. Sites of hypothalamic lesions in rabbit brain. Lesions at base of ventromedial nucleus in cross-section *A* blocked copulation-induced ovulation. Lesions in medial mammillary nuclei at cross-section *B* (stippled) blocked mating activity in spite of added extrinsic estrogen and maintenance of well-developed ovaries. Tracings from the rabbit atlas (34).

of the uterus, cutting or removing the lumbosacral cord, the abdominal sympathetics, the eyes, olfactory bulbs, middle ear or neocortex. On the efferent side, removing the cervical sympathetics (18) or the greater superficial petrosal nerve (41) failed to block, but cutting the pituitary stalk (6) did interrupt the copulation-ovulation sequence.

In our laboratory (31) recent surgical or electrolytic lesions have bilaterally severed or destroyed in each of five rabbits the olfactory bulbs, orbitofrontal

FIG. 1. Five minutes of continuous EEG record from cat medial hypothalamus (*A*) and lateral hypothalamic area (*B*) in the region of the medial forebrain bundle. Period of artificial stimulation of the vagina is underscored in the first line. Recurrent periods of high amplitude slow waves are apparent in *B*.

cortex and fornix, without blocking the neuroendocrine reflex. Similarly ineffective have been relatively large lesions in the septum, lateral amygdaloid

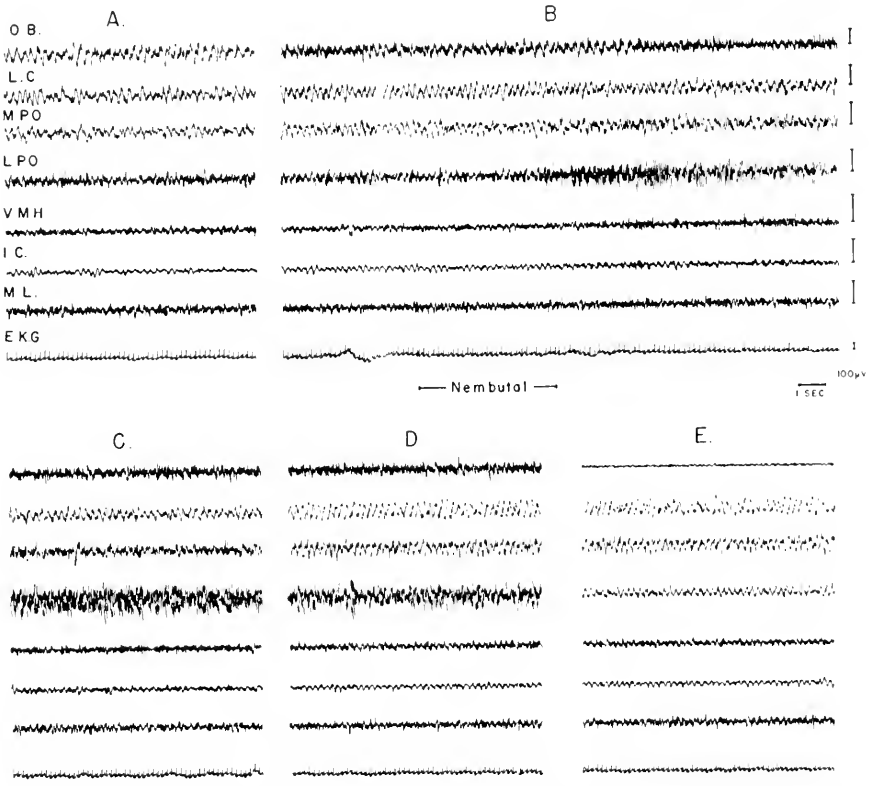

FIG. 3. Effect of histamine-Nembutal on EEG of rabbit brain. *A*, control run under ether. *B*, 10 minutes after 0.5 mg histamine intraventricularly and more ether. Nembutal dosage, 6 mg/kg, i.v. *C*, 10 minutes after *B*, 20 minutes after histamine. *D*, 10 minutes after *C*, 2 minutes after $AgNO_3$ on orbitofrontal cortex, $^1/_2$ minute before cutting ofactory bulb, *E*, $^1/_2$ minute after transecting left ofactory bulb. (From *Am. J. Physiol.* 180: 37, 1955.)

Abbreviations used in this and subsequent records and diagrams: AC, Anterior commissure; AMYG, amygdaloid nuclei; C, caudate nucleus; CC, corpus callosum; EKG, electrocardiogram; FC, frontal cortex; IC, internal capsule; LC, limbic cortex; LHA, lateral hypothalamic area; LPO, lateral preoptic area; MFB, medial forebrain bundle; ML, lateral mammillary nucleus; MM, medial mammillary nucleus; MP, mammillary peduncle; MPO, medial preoptic area; OB, olfactory bulb; OCH, optic chiasma; PO, preoptic area; PUT, putamen; SO, supraoptic nucleus; SP, septum; VHPC, ventral hippocampus; VMH, ventromedial hypothalamic nucleus.

nuclei, thalamus and dorsal hypothalamus. Large anterior hypothalamic and preoptic lesions were usually fatal, probably due to an upset thermoregulatory mechanism, before they could be tested by mating. Five hypothalamic lesions

which effectively blocked copulation-induced ovulation were found, on superimposing graph-paper reconstructions, to have a common site in the ventromedial tuberal region (fig. 2*A*). The hypophyseal portal veins in each of these animals were seen on semi-microdissection to have at least several intact channels. Another interesting common-site lesion, seen in figure 2*B*, centered in the mammillary bodies and was characterized as blocking mating behavior. These rabbits remained anestrous for months. In the case of four individuals of this group, the anestrous state was maintained in spite of treatment with

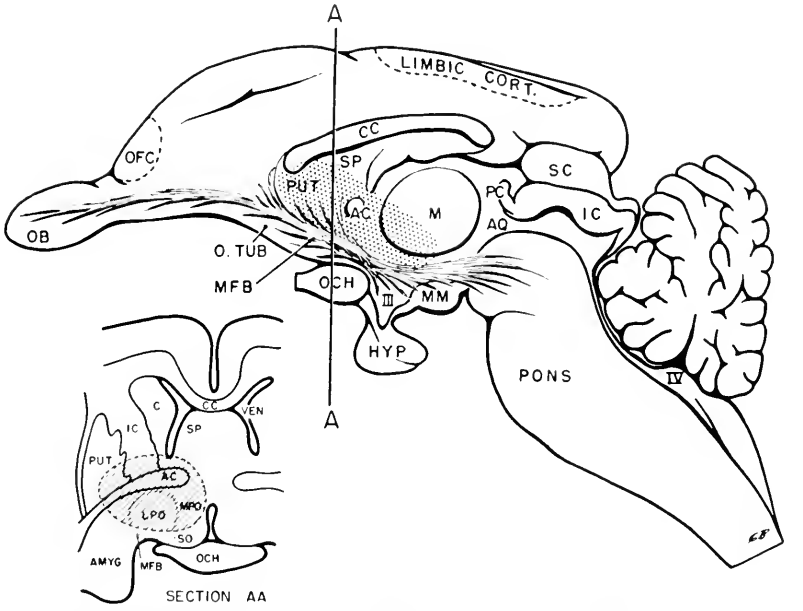

FIG. 4. Diagram of sagittal section of rabbit brain (from *Am. J. Physiol.* 180: 37, 1955). Location of lenticular nucleus indicated in dotted lines. 'Limbic cortex' refers to area from which LC recordings were taken rather than outlining the whole limbic area. Insert shows area from which 'intrinsic olfactory' activity was recorded (*diagonal lines*). Medial forebrain bundle circled in dotted lines. For key abbreviations, see fig. 3.

extrinsic estrogen. Nevertheless, their ovaries remained in good condition and they were ovulated by triggering the pituitary with intravenous copper acetate (35). Similar behavioral upsets have been reported for the cat (1) and for the guinea pig (8), resulting from lesions at the rostral mesencephalic posterior hypothalamic level.

An artificial means of triggering the rabbit hypophysis via the nervous system was revealed on injecting histamine into the third ventricle of the brain under weak Nembutal anesthesia (30). This treatment induced ovulation in 8 of 9 animals. The changes in electrical activity of the brain under these conditions

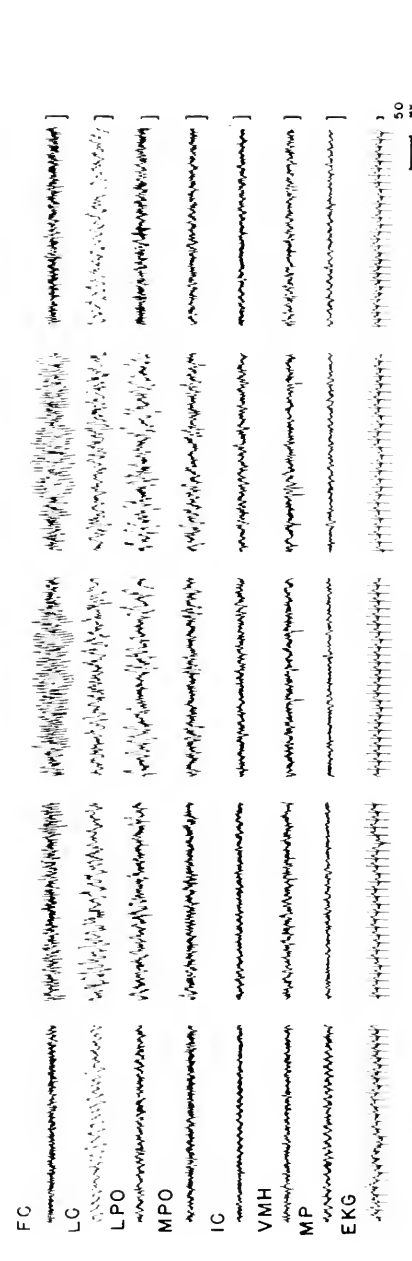

FIG. 5. Effects on EEG of estrogen-treated female rabbit of artificial stimulation of the vagina. For commentary, see text; for abbreviations, see figure 3.

were characterized by the appearance of high amplitude fast (30/sec) activity (fig. 3) centering in the lateral preoptic region. Both the altered electrical activity and the capacity of the treatment to activate the pituitary were eliminated by transecting the olfactory tracts just behind the olfactory bulbs. The areas from which the 'intrinsic olfactory activity' was recorded are seen in figure 4. This activity is most likely transmitted to hypothalamic nuclei via the medial forebrain bundle. An alternative route is via the amygdala and the stria terminalis. Perhaps both channels transmit the artificially induced rhinencephalic activity to its hypothalamic target. The role of histamine in these experiments cannot be that of a final neurohumoral mediator to the hypophysis for, if so, it should have been equally effective in the absence of the olfactory bulbs.

The naturally estrous female rabbit, at least in the New Zealand strain most commonly employed by us, does not ovulate in response to artificial stimulation of the vagina with a glass rod. Primed with extrinsic estrogen, however, these rabbits will respond positively to this treatment in about 50 per cent of the cases (29).

Multiple recordings (31) have been made of the electrical activity of various regions of the stereotaxically approached brains in several such rabbits. An example of the changes evoked by vaginal stimulation is seen in figure 5. Usually between a half minute and a minute after stimulation high amplitude slow waves appeared in the limbic cortex and lateral preoptic region, spindles in the frontal cortex and some spikes in the ventromedial region. The changes could not be correlated with a behavioral after-reaction, which is lacking in the rabbit, but they lasted from 5–7 minutes and then disappeared abruptly. No such changes in the electrical record were observed on similar stimulation of the anestrous rabbit.

Various nerve-blocking agents, including atropine sulfate, have blocked the release of pituitary gonadotrophin not only following artificial stimulation of the vagina but also after the natural copulation-ovulation reflex, if injected within a half minute post-coitum (37). In pituitary-blocking dosages atropine sulfate induces high amplitude slow waves in the rabbit electroencephalogram

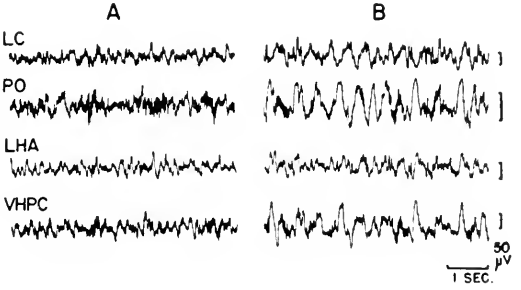

FIG. 6. Effects of a pituitary-blocking dose of intravenous atropine on rabbit EEG. High amplitude slow waves were evident in *B* in less than a minute after atropine sulfate (15 mg/kg) had been injected intravenously, and the threshold of arousal (not illustrated) was simultaneously elevated. For abbreviations, see figure 3.

(EEG) within seconds after intravenous injection (fig. 6). It also raises the threshold of EEG arousal on afferent or direct stimulation of the midbrain reticular formation. Similar results with atropine by Rinaldi and Himwich (26) have led them to propose the existence of a cholinergic mechanism within the reticular activating center. Irrespective of this interpretation, the present results suggest that afferent impulses concerned with pituitary activation may have to traverse the multisynaptic extralemniscal reticular formation en route to higher centers, or that a certain degree of arousal of the reticular formation is required to permit or facilitate activation of higher centers. It is perhaps significant that atropine blocks copulation-induced ovulation if an effective dose reaches the brain prior to the onset of the aforementioned post-coital EEG changes, roughly half a minute after stimulation.

In pituitary-ovarian control mechanisms, the rat differs from the cat and rabbit and more closely resembles the human in that it ovulates in a so-called "spontaneous' manner. Working on the rat cycle with Everett and Markee (9–11), we were able to show, with the use of such nerve-blocking agents as

atropine, dibenamine and Nembutal, that the hypophysis was ordinarily stimulated via the nervous system on the day of proestrus. Under controlled lighting conditions the stimulation occurred between 2–4 P.M. on that day, and the process took from 20–35 minutes. More recently with Barraclough (2–4) we have found that morphine, reserpine and chlorpromazine all block the process,

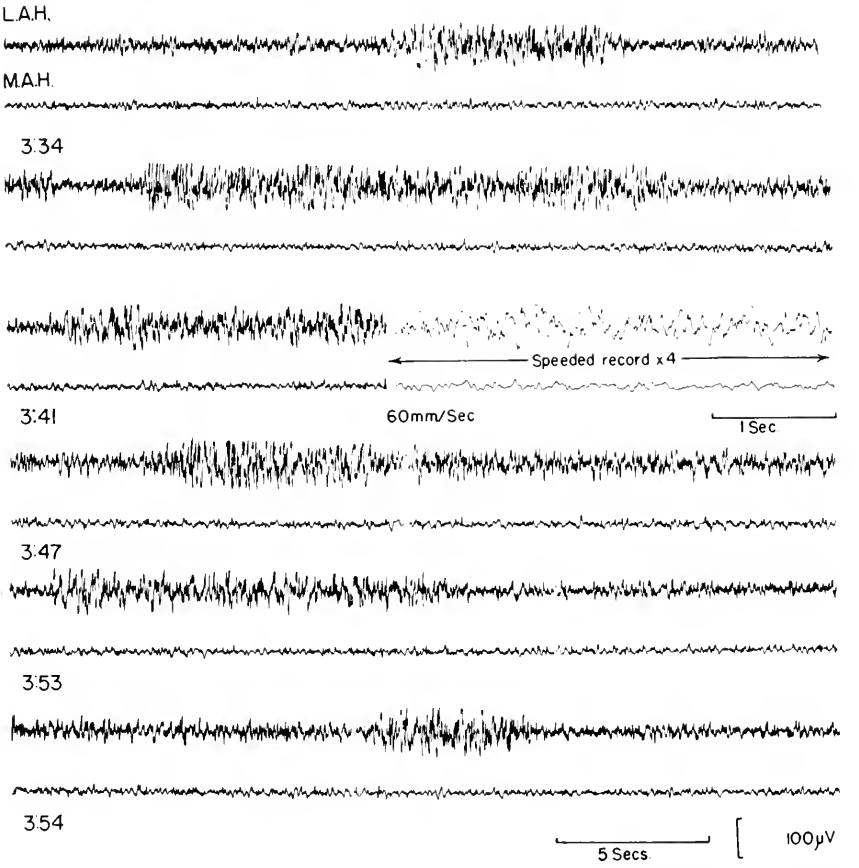

FIG. 7. EEG changes observed in rat lateral anterior hypothalamus (LAH) in the critical period of the day of proestrus during which the adenohypophysis is stimulated to release pituitary ovulating hormone. MAH, medial anterior hypothalamus.

in most cases apparently by raising the threshold of the reticular activating center (32). Morphine is of special interest since this drug has been known for years to induce amenorrhea and infertility in the human female.

In our laboratory Mr. Critchlow (7) has been recording from various regions of the cerebral cortex and brainstem of the rat during various hours and days of

the estrus cycle. He has observed in a few animals, during the critical 2–4 P.M. period on the day of proestrus, marked localized electrical changes characterized by an amplitude of 100–200 μV, a frequency of approximately 15 cycles per second and a duration of about 20 minutes (fig. 7). A limited area in the lateral preoptic region and lateral anterior hypothalamus is the only site thus far observed to display these electrical changes. The work is still in progress, but the results suggest that this rather discrete 'spontaneous' electrical activity may represent part of the central nervous process responsible for activation of the adenohypophysis and the resultant release of ovulating hormone.

SUMMARY

In three different species with varied sexual behavior patterns and two fundamentally different afferent mechanisms controlling release of pituitary gonadotrophin, similar EEG changes have been recorded during periods in which the adenohypophysis is triggered by the central nervous system. Artificial stimulation and blocking experiments suggest that the rhinencephalon and the reticular activating system may contribute directly to the triggering process. The ventromedial nucleus of the hypothalamus and its afferent projections, the medial forebrain bundle (21) and the stria terminalis from rhinencephalic centers, have all been implicated in one or more species to play important roles in the pituitary activation sequence. However, in the process of unearthing the whole chain of events from periphery to pituitary, the missing links probably far outnumber those so far described; their discovery and clarification offer challenging problems for future research.

The present approach to the trigger problem in these examples of higher nervous initiation of behavioral and endocrine activities consists of localizing the most directly involved structures and placing them in functional sequence. Recent work may be said to represent significant progress in this area, and it is perhaps a fair sample of the type of analytical advance now occurring in the central neurophysiology of behavioral and neuroendocrine triggers.

REFERENCES

1. Bard, P. *Res. Publ., A. Nerv. & Ment. Dis.* 19: 190, 1939.
2. Barraclough, C. A. *Federation Proc.* 14: 9, 1955.
3. Barraclough, C. A. Unpublished observations.
4. Barraclough, C. A. and C. H. Sawyer. *Endocrinology* 57: 329, 1955.
5. Brooks, C. M. *Am. J. Physiol.* 120: 544, 1937.
6. Brooks, C. M. *Am. J. Physiol.* 121: 157, 1938.
7. Critchlow, B. V. and C. H. Sawyer. *Federation Proc.* 14: 32, 1955.
8. Dempsey, E. W. and D. M. Rioch. *J. Neurophysiol.* 2: 9, 1939.
9. Everett, J. W. and C. H. Sawyer. *Endocrinology* 47: 198, 1950.
10. Everett, J. W. and C. H. Sawyer. *Endocrinology* 52: 83, 1953.
11. Everett, J. W., C. H. Sawyer and J. E. Markee. *Endocrinology* 44: 234, 1949.
12. Gloor, P. *Electroencephalog. & Clin. Neurophysiol.* 7: 223, 1955.

13. Green, J. D. *Am. J. Anat.* 88: 225, 1951.
14. Greulich, W. W. *Anat. Rec.* 58: 217, 1934.
15. Harris, G. W. *Proc. Roy. Soc., London, s. B.* 122: 374, 1937.
16. Harris, G. W. *Ciba Foundation Colloquia on Endocrinology* 8: 531, 1955.
17. Haterius, H. O. and A. J. Derbyshire. *Am. J. Physiol.* 119: 329, 1937.
18. Hinsey, J. C. and J. E. Markee. *Proc. Soc. Exper. Biol. & Med.* 31: 270, 1933.
19. Koikegami, H., T. Yamada and U. Kan'ichi. *Folia Psychiat. et Neurol. Japonica* 8: 7, 1953.
20. Kurotsu, T., K. Kurachi and T. Ban. *Med. J. Osaka Univ.* 2: 1, 1950.
21. LeGros Clark, W. E. and M. Meyer. *Brit. M. Bull.* 6: 341, 1950.
22. Markee, J. E., J. W. Everett and C. H. Sawyer. *Recent Progr. Hormone Research* 7: 139, 1952.
23. Markee, J. E., C. H. Sawyer and W. H. Hollinshead. *Endocrinology* 38: 345, 1946.
24. Markee, J. E., C. H. Sawyer and W. H. Hollinshead. *Recent Progr. Hormone Research* 2: 117, 1948.
25. Porter, R. W., E. B. Cavanaugh and C. H. Sawyer. *Anat. Rec.* 120: 775, 1954.
26. Rinaldi, F. and H. E. Himwich. *Arch. Neurol. & Psychiat.* 73: 387, 1955.
27. Robison, B. and C. H. Sawyer. Unpublished observations.
28. Sawyer, C. H. *J. Exper. Zool.* 106: 145, 1947.
29. Sawyer, C. H. *Anat. Rec.* 103: 502, 1949.
30. Sawyer, C. H. *Am. J. Physiol.* 180: 37, 1955.
31. Sawyer, C. H. Unpublished observations.
32. Sawyer, C. H., B. V. Critchlow and C. A. Barraclough. *Endocrinology* 57: 345, 1955.
33. Sawyer, C. H. and J. W. Everett. *Proc. Soc. Exper. Biol. & Med.* 83: 820, 1953.
34. Sawyer, C. H., J. W. Everett and J. D. Green. *J. Comp. Neurol.* 101: 801, 1954.
35. Sawyer, C. H. and J. E. Markee. *Endocrinology* 46: 177, 1950.
36. Sawyer, C. H., J. E. Markee and J. W. Everett. *J. Exper. Zool.* 113: 659, 1950.
37. Sawyer, C. H., J. E. Markee and B. F. Townsend. *Endocrinology* 44: 18, 1949.
38. Sawyer, C. H. and G. R. Parkerson. *Endocrinology* 52: 346, 1953.
39. Semonsen, C. P. and C. H. Sawyer. *Am. J. Physiol.* 177: 405, 1954.
40. Tokizane, T. and C. H. Sawyer. Unpublished observations.
41. Vogt, M. *J. Physiol.* 100: 410, 1942.
42. Wislocki, G. B. and L. S. King. *Am. J. Anat.* 58: 421, 1936.
43. Young, W. C. *Quart. Rev. Biol.* 16: 135, 311, 1941.
44. Zuckerman, S. *Ciba Foundation Colloquia on Endocrinology* 8: 551, 1955.

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[1]Certain abbreviated forms have been used. ACTH—corticotropin; ATP—adenosinetriphosphate; DFP—diisopropyl fluorophosphate.

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