Recent Advances in Invertebrate Physiology

A Symposium

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Bradley T. Scheer, Editor
Theodore H. Bullock
Lewis H. Kleinholz
Arthur W. Martin, Associate Editors

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FOREWORD

The idea of a meeting on the Pacific Coast of physiologists interested in the invertebrates was first conceived by Professor A. W. Martin of the University of Washington. In December 1954, he asked the members of the editorial committee for this volume to work with him in organizing a symposium on recent advances in the physiology of the invertebrates. When plans were well along, in the early spring of 1955, certain actions of the administration of the University of Washington were interpreted by many of the invited speakers and members of the committee as prejudicial to accepted principles of academic freedom. As a result, the present writer was asked to accept responsibility for completing the organization so well begun by Professor Martin, and the meeting was held on the campus of the University of Oregon in Eugene in September 1955. We are especially indebted to the National Science Foundation, which provided the bulk of the funds for travel expenses, board, and lodging for the participants in the meeting, and underwrote the publication of this volume. The Tektronix Foundation of Portland, Oregon also contributed generously to the support of the meeting, and the University of Oregon provided facilities and secretarial and administrative assistance.

The primary aim of the symposium was to afford an opportunity for physiologists interested in the invertebrates to become better acquainted personally, and to exchange information and ideas. In this aim, the meeting was eminently successful. Limitations of time and funds made it impossible to bring together more than a small group; the present volume is designed to bring to a wider audience some of the material presented at the symposium.

The committee wished to place as few restrictions as possible on the free interchange of views. Consequently, no attempt was made to obtain verbatim accounts of the formal presentations or of the subsequent discussion. The papers in this volume were prepared by the authors to cover the same material as their oral presentations, but are not necessarily identical with the papers as they were read. It will be obvious to the reader that the papers are of various types. Some are reviews of a large amount of material from an entire field; others are accounts of personal research in a more limited field. Two papers presented at the meeting, by C. L. Prosser and T. H. Waterman, are not included here. B. J. Krijgsman, whose paper is included, was unable to attend the meeting. It was impossible, within our limitations of space, time, and funds, to cover the whole vast subject of invertebrate physiology; the selection of subjects included

here, though to some extent arbitrary, may be said to give a fair representation of the most active areas of research at the present time.

I should like to take this opportunity to express my personal gratitude to the other members of the committee for their helpful cooperation in planning the meeting, and in the preparation of this volume; to Miss Marjorie Foxworthy (now Mrs. Charles Turbyfill) for her many services before, during, and after the meeting; to Mr. Robert P. Bronson for his help with travel arrangements; and to my students, A. S. Hu, R. M. Myers, J. H. McAlear, and R. V. Crisera, for their help during the meeting. Mr. George N. Belknap, University editor, and Mr. Donald Shepardson, superintendent of the University Press, have been very helpful in seeing the book through the press.

Bradley T. Scheer
Eugene, Oregon

CONTENTS

NEURONAL INTEGRATIVE MECHANISMS*

THEODORE HOLMES BULLOCK
University of California at Los Angeles

Integration is to put parts together into a whole. Such a process occurs in organisms at many levels, from the subcellular to that of the community. The levels of interest for the present purpose begin with a whole neuron, therefore do not embrace an analysis of the mechanisms in the cell or its membrane, and extend only one or two steps up the hierarchy, through closely knit groups of neurons to relations between such groups, but not so far as the level of the whole nervous system of any animal. This limitation is not one of appropriateness but is imposed by our methods and present understanding, as physiologists.

Indeed our understanding of the actual mechanism of nervous integration, our insight into the unit behavior which might account for this subtle and complex result, is so meager that it may be asked, what can we say? This paper makes no pretense of accounting for very much normal behavior, and will conclude by emphatically invoking as yet unknown levels of interaction; but it makes an effort to say what can be said today about the properties of neurons which must be involved in, and in certain cases appear even to account for, the observed integration. It goes little beyond a list of the properties—each of which provides a degree of freedom or an available mechanism for altering the input-output function. These properties generally are additive, so that with only a few it is possible to obtain rather complex permutations. Still our knowledge permits a very limited foray into the vast field of higher nervous integration, and I am emboldened for it only because so few have undertaken to bring together the several mechanisms that are now known (see Fessard, 1954, 1956), while one often still hears instances of thinking on these problems in which the neurons are treated as purely digital or are otherwise oversimplified.

One further disclaimer is necessary. We must deal with observed properties of neural units even though they cannot be explained by current theories of cellular mechanism. So we are not accounting for the properties; rather in enunciating the phenomena which may explain higher levels, we are formulating the problems awaiting attack at molecular levels.

Let us return to the definition of our problem. Integration, I said, paraphrasing the dictionary, is to put parts together into a whole.

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Now, what is the whole which is referred to? At the levels below behavior, neurophysiologists today regard it as a pattern in time and space of quantal events, each event brief compared to the events of behavior; these are the impulses in the efferent nerve fibers. At the level of the single neuron we may perhaps best express the whole as the probability of firing within the next given interval of time, or we may revise "firing" to read "change of state influencing another neuron," since it seems to me important to recognize the possibility of subthreshold events as adequate stimuli even though clear cases are not yet known. We can formulate our definition in simple terms like these if we but recognize, and then put aside for the present, those variables which the neuron integrates into its probability of firing which are not immediately determined by other neurons, e.g., general chemical milieu, physical deformation, and temperature. Expressed in terms of impulses or changes of state effective upon other neurons, integration at the unit level then becomes in the general case a relation between input and output which is either more or less than one. Usually this means the algebraic summing of separate neuronal channels one or more of which produces more or less than one output pulse for each input pulse; so long as this is true, the channels may have equal or different weights in their effect upon output and the same or opposite sign, i.e., excitation or inhibition. But we have to admit also the case where only one input channel reaches the neuron under consideration, for in our type of system a qualitative difference between inputs is not an essential condition; the integrating cell does not know whether the signals come in the same or different channels. The essential feature is that the neuron place some value, other than one, upon at least some of its incoming signals, according to their intensity, time course, time of arrival, and the locus upon the neuron where they impinge. This definition of integration at the neuron level will then include all junctions except those that are purely 1:1 relays. It will certainly include many neuroeffector junctions in which therefore the nonnervous cell is the integrating cell. Sensory neurons certainly integrate in the broad meaning given first, putting together different quantities in the milieu into a probability of firing. And they may do this in part by means or with properties which will help us to illuminate junctions. But if any should object to the notion that receptors already integrate, they may wish to exclude receptors on the ground that there is no input from other neurons —it is not nervous integration. But sometimes there is! The same cell, the same terminal ramifications may be transducers of mechanical simuli and postsynaptic elements under nervous control (e.g., Kuffler and Eyzaguirre, 1955; Lowenstein, 1956).

You have patiently listened to my definition of integration. We are supposed to talk about recent advances in invertebrates, and I must accord-

ingly confide in you my definition of this category. For present purposes invertebrates shall mean any animals which an invertebrate zoologist finds interesting.

Some Properties of Units Permitting or Influencing Integration

At the level of the single neuron we may first list a number of the properties or conditions which classical or recent experimental facts indicate as the probable bases of the ability of the neuron to integrate incoming signals. Obviously all the static and dynamic characteristics of the cell more or less directly permit or determine the activity, but we shall enumerate only some of them, at the same time expressing the hope that extension of intracellular analysis like those of Hodgkin, Huxley, Katz, Cole, Grundfest, Eccles, and others will isolate further factors and show their degree of lability and variation in junctional membranes.

The *resting potential* is often thought of as a fixed character which has only one value which is "normal," its maximum value. The evidence, however, can be construed to suggest that some synaptic regions normally have a membrane potential which is less than its maximum, and can be pushed either way and maintained at new levels. The level of this potential affects not only the spike height but the excitability and the magnitude and sign of after-potentials and of subthreshold responses.

Spike threshold and its time course after activity, the excitability cycle, require no development here beyond the reminder that we have little information on these crucial properties in synaptic regions of various preparations and animals. *Accommodation*, in particular, has not been examined comparatively or in junctional regions; and examination is the more necessary since the recent discovery that the classical rise in threshold with slowly rising stimuli does not in fact obtain in the frog axon free of connective tissue. (Tasaki and Sakaguchi, 1950; Diecke, 1954). There are, however, other effects of maintained or slowly changing subthreshold depolarization, as on the form and size of the spike. This is important for us because of the possibility that the action of terminals, dendrites, and somata may be similarly influenced (see below under subthreshold lability). There may be great differences in the minimal slopes, below which threshold is never reached, in different types of neurons.

Based on the distribution of thresholds or of synaptic endings in a group of neurons, there can be curves of various shapes relating input (number of synchronized fibers) to output (number of postunits firing). The variable relation of input to output becomes of greater potential value as an integrative mechanism when the output of one group of neurons is in

turn the input for another order of neurons and both have nonlinear curves. Addition of output-input curves can in the extreme case readily produce a step function, representing a kind of labile multiunit threshold providing stability and discrimination (Fessard, 1954).

Besides the spike threshold we must recognize a separately variable *subthreshold excitability*. This is manifested as a nonlinear increase in the membrane potential with increase in stimulating current below spike threshold (Fig. 1). The active, graded response which does not propagate does not have a threshold, but it has a very labile excitability.

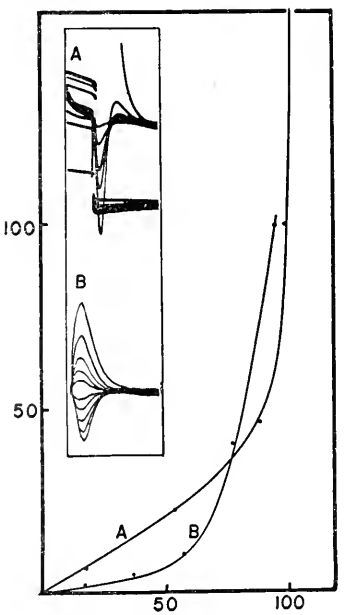

Fig. 1. Stimulus-response relation of the subthreshold local potential in the third-order giant fiber of the squid. Stimulus applied to the stellate ganglion directly; recording from the same ganglion. Two experiments are shown: A. Cathodal stimuli, whose voltages are shown by the upward deflected square tops, elicit local responses, whose amplitudes are shown by the downwards deflected triangular waves, plus one spike which goes off scale and is seen as a descending phase above the base line. Plotted as per cent of threshold on abscissa, per cent of maximum recorded local potential on ordinate (spike off scale). B. Cathodal stimuli (not shown) give responses above base line, anodal stimuli of same intensity below. Plotted as above but ordinates are cathodal response minus anodal to show the development of nonlinear, nonelectrotonic, "active" local response. (From Bullock, 1948; reprinted with permission of the *Journal of Neurophysiology*.)

Subthreshold activity exhibits lability also in other ways (Bullock, 1948; Bullock and Hagiwara, 1955). Its rate of rise and especially its rate of fall vary even from moment to moment under repeated low-frequency stimulation. It may hesitate for many milliseconds before growing up into a spike or starting its fall. Its spatial decrement may vary, possibly as a consequence of change in time course or possibly as a consequence of a labile decremental propagation. In some conditions, there is a heightened excitability after a subthreshold response, beginning without any refractory period (e.g., fresh axon of squid). But in other conditions there occurs a depression after such a response, with a recovery which requires many milliseconds (e.g., fatigued axon or synapse of giant fibers of squid). This depression may be followed by a supernormal period. These considerations

gain in significance if we believe that integrative neurons are typically under tonic subthreshold influence.

The excitability we have spoken of so far is excitability to artificial stimulation of limited kinds. But the outstanding characteristic of synapses is their sensitivity to some consequence of activity in other neurons, and we need not be concerned here whether that is one or more specific chemicals or nonspecific ions. We do need to note: (1) that the response may be *excitation or inhibition;* (2) that one and the *same input channel* (presynaptic fiber) can cause one or the other, depending on the level of the membrane potential of the postsynaptic neuron; (3) that different presynaptic fibers can cause response of the same sign but *different rates of rise, facilitation, maximum height, etc.*, as in crustacean muscle; (4) that these differences may be *discontinuous and unequal* in the proportional rôle of the several characters measured; (5) that summation of the different kinds of input in the same postjunctional cell may be complex (crustacean muscle, crayfish central giant-to-motoneuron synapse); and (6) that inhibition is not just the reciprocal of excitation as measured by its effect on various aspects of activity.

A highly variable property of the utmost importance for integration is the *response of the postsynaptic unit to repeated presynaptic impulses.* In some cases (e.g., *inhibitory escape* in the cardiac ganglion of lobsters) the initial effect of a sustained barrage gradually diminishes as measured by the output of the postunit, reaching a plateau at a new level (Fig. 2). In the example referred to this happens in some seconds at a frequency of presynaptic activity of 20-40 per sec. In other cases (e.g., the synapse of pacemaker upon follower cell in the same ganglion) there is actually what may be called *defacilitation*—the postsynaptic potential to the second presynaptic impulse is less than to the first, the difference being proportional to the frequency. This may be regarded as a consequence of relative refractoriness. Its importance lies in the fact that it happens in a frequency range within the normal firing range of these ganglion cells. The more familiar cases are those of *facilitation*—which should be distinguished from temporal summation by the criterion that each response or increment is greater than the last. The magnitude of facilitation and its rate of growth and decay vary widely. As an example, their consequences can be clearly seen in Wiersma's comparison (1952b) of the responses to the same average frequency delivered with alternately long and short intervals and delivered with uniform intervals. Some junctions give the same response (small, slowly decaying facilitation), others respond enormously more to the paired train (large, rapidly decaying facilitation).

Another group of properties with profound influence upon output, especially in the formation of patterned bursts, is in the domain of *after-*

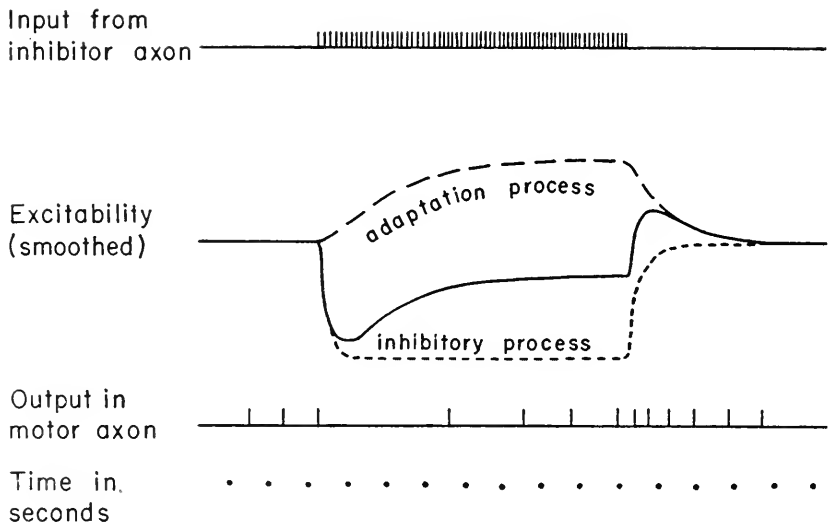

Fig. 2. Diagram of the response of a single unit of the cardiac ganglion of a lobster to stimulation of the inhibitory axon. Each vertical line represents an impulse. Maintained activity in the inhibitor produces, first, deep depression, followed by partial escape or adaptation. Termination of inhibitor activity produces a postinhibitory excitation after a brief latency or inhibitory after-effect. The observed input and output are related by an algebraically summing series of processes, including many of those listed in the text. Just two of these, which are moreover not directly measured but inferred, are shown here. (After Maynard.)

effects. These may be positive or negative or both in sequence, of various relative durations and magnitudes. To say the same thing in more familiar terms, there may be an after-discharge following cessation of presynaptic excitatory bombardment or an after-inhibition following the end of inhibitory influx, and there may be, with or without this positive after-effect, a rebound effect—postexcitatory depression or postinhibitory excitation. When phases of opposite sign succeed each other, they may be affected independently by various factors, as though manifesting separate underlying processes.

Recently we have distinguished another property which is of importance in permitting repetitive firing in response to a single presynaptic impulse in the cardiac ganglion of lobsters (Hagiwara and Bullock, 1955). This may be described as a *safety factor* of much less than one so that the postsynaptic impulse, once initiated in the axon, cannot invade the cell body (antidromically). This protects the latter from the possibility of loss of any partial depolarization it may have built up. Its significance depends on the asymmetrical relation between cell body and axon—the slow synaptic potential of the soma can spread electronically into the axon with less

attenuation for any given time constant of the membrane than the brief spike potential of the axon can spread into the soma; thus the soma can excite repetitive discharge of the axon with a single, long-lasting synaptic potential, while the resulting spikes are seen in the soma as tiny, five millivolt electrotonic deflections (Fig. 3). This case also illustrates the interaction of several factors in determining when a neuron fires. It is obvious that a fixed threshold voltage across the membrane of the soma does not exist; something else interacts with voltage so that successive spikes occur at smaller depolarizations.

A somewhat similar situation may account for the activity of dendrites as analyzed by Clare and Bishop (1955a,b). Dendrites in the vertebrate cortex appear not to conduct all-or-none impulses toward the cell body, as has been classically supposed from the law of dynamic polarity of Cajal (1909). Instead slow potentials generated in dendrites and spread electrotonically may influence the spike-initiating region of the soma. One can think of two interacting regions of integration, the dendrites and the soma; in this way the vertebrate cortex differs from most invertebrate nervous tissue where the soma probably plays little rôle and cell-free neuropiles are responsible for integration (Bullock, 1952).

The last property to which we will allude here is *spontaneity*. This varies not only in the degree to which it is developed but also in the form by which it is manifest, and the question is still unanswered whether these several forms are different in underlying mechanism. Spontaneous subthreshold activity seems to be of two kinds but these are possibly basically the same. In some cases it is quasi-sinusoidal, in others it rises (depolarization) more or less linearly to a point where it reaches a threshold and initiates a spike the repolarization of which carries the membrane back to the high level from which the so-called generator potential can begin again. These two forms of subthreshold change differ at least superficially as a sine wave oscillation from a relaxation oscillation; that is, one form can continue to go through successive cycles without an all-or-none discharge, the other requires such a discharge to restart the cycle.[1] The generator potential is best known in certain sense organs and in the specialized muscle cells pacing the vertebrate heart, but it is also present in integrative neurons which control other neurons, i.e., pacemaker interneurons in the lobster cardiac ganglion.

Spontaneous activity may be relatively rhythmic or nonrhythmic. Even in the same unit a continuous spectrum may be shown between very small and very large standard deviations of the intervals between spikes—the

[1] We have recently found, in spontaneous ganglion cells of the lobster cardiac ganglion, that a propagated spike is not necessary to repolarize the soma to a high level and restart the cycle. An active but graded form of soma potential suffices.

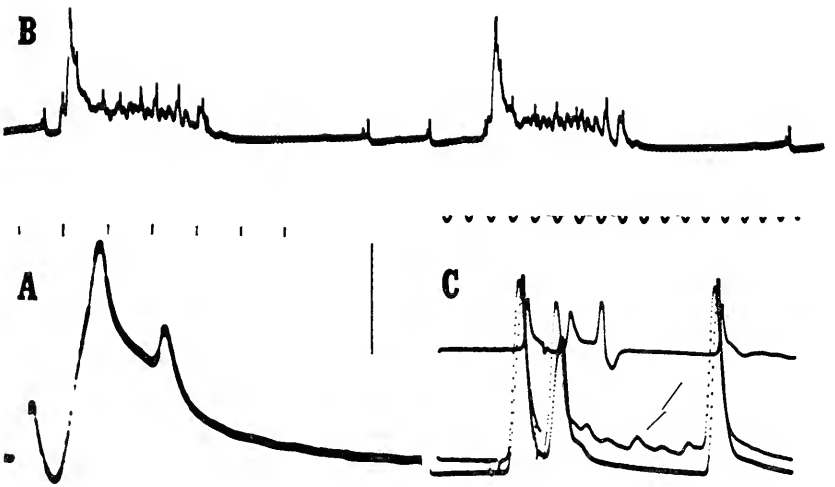

Fig. 3. A. Intracellular potential of the soma of a large neuron of the cardiac ganglion of a lobster. The activity results from stimulation applied to the ganglion a few mm. away. The first deflection is an antidromically conducted spike. The large deflection is interpreted as a synaptic potential resulting from arrival of an impulse in a presynaptic fiber; it is all or none by this form of presynaptic stimulation. The record shows two spikes arising from the synaptic potential, one at its peak and one on the falling phase; other records show from one to five, the number apparently determined by the condition of the postsynaptic element, not by a difference in the number of presynaptic elements. Time marks, 10 msec.; vertical calibration, 10 mv.

B. Intracellular potential of the soma of a large neuron of the cardiac ganglion of a lobster. The activity is spontaneous in the ganglion and in the form of bursts, one for each heart beat. The large deflection is regarded as a synaptic potential resulting from arrival of presynaptic impulses, and the smaller slower deflections are interpreted similarly. The sharp spikes (7-9 per burst) are the impulses in the axon leaving this soma; they arise from or near the crest of a synaptic potential. Note that, as in A and C, they do not have a fixed voltage threshold but a threshold which depends on other factors, e.g., time, even in naturally occurring repetitive firing. The spikes between bursts are preceded by a slow depolarization or generator potential; these are regarded as spontaneous activity of the cell we are in. The record illustrates the complexity of the interaction of integrative properties in a simple ganglion. The record is 3.6 seconds long.

C. Intracellular potentials of a median giant fiber of an earthworm. Two microelectrodes are inside the fiber, 11.3 mm. apart, the one farther from the stimulating electrodes is the lowest beam, the nearer one is the middle beam. The top beam is an extracellular monitor still farther down the fiber. Two shocks are given, 2 msec. apart. The first elicits action potentials of 98 and 110 mV. on resting potentials of 72 and 74 mV. The second elicits smaller direct spikes plus complex small potentials in the nearer penetration only (arrow), regarded as synaptic potentials from small fiber bombardment eventually leading to a spike which propagates. (There is also a lateral giant spike on the external leads, in the middle of the sweep, but this has no reflection in the internal median giant leads.) Like the preceding, the record shows the interaction of prepotentials and a complex recovery of excitability in determining firing. Time in msec. (The experiment and permission to use the record are due to the kindness of C. Y. Kao.)

former at high average frequency and the latter at low frequency of firing. But at any given average frequency there are types of neurons which are markedly regular and others which are markedly irregular in successive intervals. This suggests intracellular variables of importance in effective magnitude, such as fluctuating spike threshold, fluctuating amplitude of sinusoidal potential or of rate of rise of generator potential, fluctuating area of nonpropagated or decrementally propagated activity, and the presence of multiple loci of origin of spontaneous subthreshold activity (Wiersma, 1952a).

Besides these short-term changes in the frequency of firing there are in some cases long-term changes in average frequency on the scale of minutes. We will consider their significance below.

Eyzaguirre and Kuffler (1955) have just described a puzzling case of repetitive firing in the cell body of a neuron which has recently received an antidromic spike. Whatever the explanation—and these authors propose a tenable one based on differing local delays or partial blocks in the several dendrites—it is germane as an indication of the degrees of freedom present, and our purpose here is only to review the available ways in which the neuron can exhibit the several variables whose interaction could accomplish integration. In our present state of knowledge this means we have to include some observations whose "explanation" is less obvious than that of others. Eyzaguirre and Kuffler's interpretation of the intracellular afterdischarge in the stretch receptor cell of the dorsal muscle sense organ of crayfish may be correct; but Bullock and Turner (1950) reported a similar phenomenon in the giant fiber of the earthworm, so the phenomenon does not depend on the particular anatomy in the crayfish receptor. In the earthworm, a spike initiated at stimulating electrodes and conducted a long distance down the giant fiber arrived at a locus of partial or complete block (anode of a polarizing circuit) where it hesitated before proceeding or died out; after five or more milliseconds a burst of several impulses at high frequency originated at that locus or immediately adjacent to it.

One of the consequences of spontaneity may be *sensitivity to weak electric fields*. At any rate one can control the frequency of discharge of spontaneous integrating centers by passing a fraction of a microampere through a mass of tissue of a few ten-thousands of an ohm resistance, where the voltage drop along the length of a single cell must be a fraction of a millivolt (Bullock, Burr, and Nims, 1943, on *Limulus*; Maynard, 1956b, and Terzuolo and Bullock, 1956, on lobster cardiac ganglion; Hagiwara, Oomura, and Takagi, unpub., on citrated squid axon). The voltage drop across the membrane must be still smaller. Either an excitable mechanism not familiar to us is operating or the curve of membrane potential against firing interval is exceedingly steep, which means the threshold is very critical and constant.

The evidence against electrical transmission, based on the absence or minute size of the voltage change across the postsynaptic membrane produced by the arrival of the presynaptic impulse (del Castillo and Katz, 1954, on muscle; Bullock and Hagiwara, 1955, on squid) may be conclusive. But this does not mean, as some have supposed, that weak electric fields are without influence on poised or already active neurons. The experiments cited on cardiac ganglia, as well as many others of the same sort, classical and recent, are direct and pertinent and, when considered quantitatively, impressive in the sensitivity they bespeak.

The significance of this sensitivity is the enormous integrating potentiality, in complex centers, of the fields of current interacting among small and large groups of neurons. Here ordinary synaptic pathways give way in importance to architectonics. And synchronization and desynchronization of graded subthreshold activity of somata and dendrites take on a paramount significance both in producing fields effective upon other units and in sensitizing the somata and dendrites themselves to *effects en masse* (cf. Fessard, 1954).

Taken together with our earlier conclusion (see above under subthreshold excitability) about tonic subthreshold influence, these considerations also lead us to the suggestion that much of normal nervous function *occurs without impulses* but mediated by graded activity, not only as response but also as stimulus.

Pattern Formation in the Discharge of Groups of Neurons

As a special case of the most general interest we may examine the integrative mechanisms capable of organizing patterned bursts of impulses in which the serial order is determined centrally and in which several efferent neurons are coordinated. Since overt behavior consists in just such coordinated bursts of impulses, as far as its neurophysiology is concerned, this problem is a large segment of the problem of behavior. It is too much to expect that we can enunciate a satisfactory general solution or even a complete solution of a single case. But I believe there are some things we can say which will carry us quite a way in accounting for simple patterns with only the properties outlined above. Actually there is little difficulty in drawing hypothetical circuit diagrams of neurons with connections and properties within known limits which will produce a given pattern of output impulses in space and time. But there has been little effort to discover what actual neurons and connections are employed in real cases, perhaps because the enormous neuron pools in the familiar cases are too complex in sheer number of cells and impulses. A few cases have been studied recently in which a very small number of nerve cells control a large muscula-

ture and in which physiologically initiated movements or impulse bursts can be nearly completely accounted for in the neurograms.

In the simplest case there is a cell which has a fixed frequency of firing and this cell is simply turned on and off by input from the periphery or from higher centers. This has been found in the control of (neurogenic) sound production in a cicada (Hagiwara and Watanabe, 1956) and in the control of electric organ discharge in *Torpedo* (Albe-Fessard and Szabo, 1954.) In both the pacemaker cell is an interneuron, not a motoneuron, and the fixed frequency is high—200 per second in the former, 100 in the latter. The frequency or intensity of stimuli to sensory nerves does nothing in the cicada but determine how long the pacemaker will buzz and how promptly it will start. The system is like an oscillator controlled by a switch which can be only on or off but which can be turned on with various speeds due to the finite distance the switch must be moved before it changes its state. In *Torpedo* it does not yet seem clear whether the 100 per second frequency is independent of the input. In both cases the frequency-determining interneuron is penultimate—it controls the motoneuron directly, one motoneuron on each side in the cicada, about 70,000 on each side or 100 for each interneuron in the electric lobe of *Torpedo*. There is one other step in the cicada. Whereas the electric-lobe motoneurons follow the interneurons 1:1 after the first impulse of a series, the cicada motoneurons follow every other interneuron impulse, therefore firing the muscles at 100 per second. Moreover, the two sides are always 180 degrees out of phase, so that there must be some reciprocal inhibition of the two sides.

The only other preparation which I will discuss here is the lobster heart ganglion (Fig. 4), which is somewhat more complicated. This is largely based on the work of my former associate, Dr. Donald Maynard, but some aspects have been extended by Dr. Hagiwara and myself (Maynard, 1953 a,b,c, 1956a,b,c; Bullock, Cohen, and Maynard, 1954; Hagiwara and Bullock, 1955). Here a pattern is repeated at regular intervals, corresponding to each heart beat; and normally the heart beat, or as we shall call it the burst, is paced by the activity of certain of the four small posterior cells. Here, as in the system we have just examined, each of the follower cells responds to the pacemaker, or to some other cell triggered in turn by the pacer, with a train of impulses whose frequency is not the same as that of any other cell but is peculiar to the cell. But this frequency is not fixed. It starts high or quickly rises to a maximum and then falls along a curve characteristic for the cell over some hundreds of heart beats. This frequency/time curve could conceivably be determined entirely by the properties of the given cell since the cell can respond to a single incoming impulse by a repetitive discharge, as we have seen happen in intracellular records. A single large, slowly decaying synaptic potential can, by the

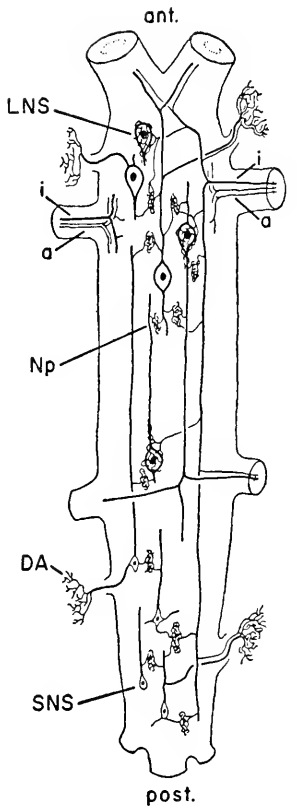

Fig. 4. Diagram of the cardiac ganglion of the spiny lobster, *Panulirus interruptus*. Not to scale; the ganglion is about 12 mm. long, the largest cells about 50μ. Neuropiles simplified. LNS, large neuron soma; i, the single inhibitor fiber; a, the two accelerator fibers from the central nervous system; np, neuropile; DA, dendritic arborization, possibly sensory; SNS, small neuron soma.

mechanism outlined earlier, elicit a short train of up to five impulses. In a normal burst it is not so simple, however.

The pacer fires repetitively. It seems probable that the pacer fires the other small cells directly and that some or all of them drive the large cells. This means there *could* result a simple "open chain" determination of pattern, as Maynard calls the type of system proposed by Rijlant for the *Limulus* heart, all the cells being controlled directly or through interneurons by the pacemaker, in direct line or chain of command (Fig. 5).

But it seems more likely that in the normal burst there is interaction. The frequency/time curves strongly suggest that two additional factors are operating: (1) great individual differences in the time course of excitability, responsiveness, or both (we have seen that these are separate properties of a cell) to the same input signal, and (2) feedback, at least positive and possibly also negative.

This feedback is probably critically responsible for normal burst maintenance. Once a pacemaker starts activity, followers build it up greatly

by positive feedback, and so intensify synaptic excitation as to bring on a compensatory depression terminating the burst. The silent period and hence burst frequency is thus determined not by any single cell but by the integrated activity of several, i.e., by the duration and intensity of the whole burst. The excitability cycle by itself may be sufficient to account for the intermittent activity patterned out of an apparent natural tendency to continuous activity, at least whenever we have several interacting units. When intermittent trains occur in single isolated neurons we must invoke a new underlying oscillatory process, but such instances do not appear to be a normal part of the activity in this ganglion.

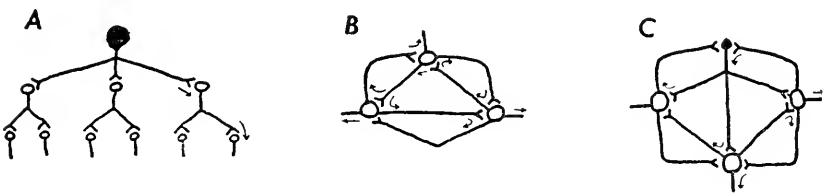

Fig. 5. Possible connections of cardiac ganglion leading to patterned burst formation. A. Multiple chain. Top cell is the pacemaker, middle row interneurons, bottom row, motor neurons as proposed by Rijlant for *Limulus*. B. Closed chain. There is no morphological pacemaker. Every cell acts as motor neuron and at times as pacemaker and as interneuron. Burst formation depends on the properties of interaction rather than on connections. C. Modified closed chain as in lobsters. Small cell acts as pacemaker, but there is feedback, and patterning of burst depends primarily upon the properties of this interaction and its sequelae in the cells. (After Maynard.)

The system just outlined is a "closed chain" in Maynard's terminology if all cells are equally effective upon each other. The actual observations are most easily understood by considering the cardiac ganglion to be a "modified closed chain" (Fig. 5) with some asymmetrical connections and specialization of function but in which recriprocal interaction and spontaneity of all units are dominant features. The largest cells are in all likelihood incapable of triggering a normal burst of the ganglion, although they are spontaneously active and in special circumstances do pace each other. Their unique role is that of motoneurons, but they act also as integrative neurons which formulate an output that is a function of, but quite unlike, their input, and help to determine burst durations and hence frequency.

One advantage of this system is its lack of dependence on any one critical value. No one cell fires at the burst frequency, i.e., heartbeat frequency. The pacemaker fires a train in each burst and is simply the first of probably four duplicates. Each of these four, having been turned off at the end of a burst by accumulating hyperpolarization from internal and external sources, develops a slow generator potential rising a few millivolts in a

second or two toward a very constant firing threshold. The burst frequency depends then not only on the rate of rise of the generator potential in the fastest pacemaker unit but also on the degree of depression at the end of the last burst, which in turn depended on the activity of many units which participated in the burst, thus on the burst duration and intensity. We are in the presence here of reverberating circuits, but they serve not to maintain activity in a continual self-re-excitation but to "provide means of spontaneously active units to undergo a slower alternating auto-excitation and depression" (Maynard). The system is capable of relative stability in the sense that removal or silence or hyperactivity of any or even several units does not greatly upset the pattern.

These are the observed properties and the inferred mechanisms. I must emphasize that we do not know the connections in detail, and it is still only the simplest inference that these properties are to be explained in terms of the properties of units as recorded from single cells in this ganglion. There is reason to believe that this arrangement is not limited to a ganglion with such a small number of cells but applies also to the large, many-celled ganglion of the heart of *Limulus*.

Superimposed upon or perhaps underlying the excitatory state which plays the rôle just described is a sensitivity to physiological degrees of stretch or inflation. We do not know whether this inheres in all the cardiac ganglion cells or only in some, but it appears to be a direct response of these cells and not due to influx from some separate sensory neurons. The effect of stretch is to accelerate or enhance spontaneity.

In addition to integrating stretch, its own spontaneity, and intramural excitation and possibly inhibition, the cardiac ganglion neurons must also integrate input they receive from two pairs of extramural acceleratory and one pair of inhibitory nerve fibers from the central nervous system, each of whose effects is differential with respect to different aspects of activity and not simply reciprocal with the other. For example, burst frequency and number of spikes per burst are decreased by inhibitory stimulation but spike frequency within a burst may go up. The time courses of development, adaptation, and after-effects are slower for acceleration and are not mirror images of those in inhibition.

We do not understand the factors that determine these effects; at times a change in the tonic level of extramural acceleration or inhibition will change the burst pattern progressively and nearly proportionally for each parameter, at other times it will affect the large followers primarily and may stop output to the muscle without gross change in burst pattern, and at still other times the small pacers are affected more, causing a slowing, for example, of the burst frequency without corresponding depression of the followers, which now escape and fire just before each burst.

Changes in the phasic rather than the tonic level of this extramural influence can lead to still more complicated differential effects, since now we have the several successive phases of after-effects, which are different between large and small ganglion cells and between acceleration and inhibition. An example of these effects is the paradoxical driving of nearly quiescent units by phasic inhibition, via the postinhibitory rebound (Fig. 2).

Signal Versus Noise and Some Specifications for a Brain

The common case of continual discharge in sensory nerve fibers may be partly understood as representing (1) reception of steady states or/and (2) a state of poise associated with high sensitivity, and also (3) a provision for signaling in one line both positive and negative changes in the stimulating parameter (e.g., increases and decreases in temperature, light, or stretch, and forwards and backwards movements of hairs in statolith organs). But it raises a serious problem in any case where rhythmicity is not perfect. What change in the output of the sensory nerve fiber constitutes a signal of environmental change and not a random fluctuation in spontaneity?

This problem has some general significance and not alone in certain sense organs but probably in the integration of signals between centers in the brain. Deferring for a moment any contributions to the discrimination between signal and noise provided by a multiplicity of parallel channels (afferent nerve fibers), it is necessary to consider first the alternatives available for analyzing the input in each single channel. Among them we may recognize certain ones, as follows. The signal could be regarded as:

(1) The actual instantaneous frequency, i.e., the reciprocal of the interval since the last impulse (Fig. 6). If this constitutes the significant signal for the central analyzing mechanism the threshold of the mechanism would be very high, since the signal has to be reliably higher than any spontaneous fluctuation in interval. In the general case the fluctuations in individual intervals are larger than any fluctuations in averaged or integrated frequency. This possibility therefore seems unlikely or at least maladaptive and wasteful of information.

(2) Frequency averaged over some period (Fig. 6). The threshold will be lower than in (1) but, if the time constant of integration is short, not much lower; and, if the time constant is long, reaction time, detection of brief stimuli, flicker resolution, and spatial localization of moving stimuli will suffer. Some compromise seems quite possible but not as advantageous as the following.

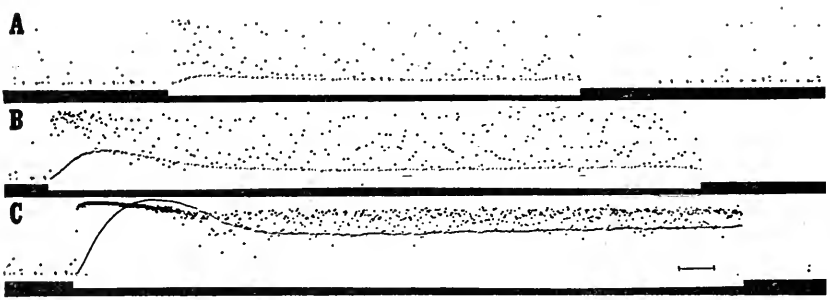

Fig. 6. Frequency-time plots of the activity of a single receptor unit in the infrared sense organ of the rattlesnake, *Crotalus*, to three different intensities of physiological stimulation. Two simultaneous plots of the same activity: the finer spots in a more nearly smooth curve are from an integrator which averages the frequency with a time constant of 2.5 seconds. The coarser spots with a wide scatter are from a pulse-interval-plotter which places the spot higher on the ordinate the shorter the interval since the last impulse. The ordinate scales are not given here as they are not important for the present purpose. Time scale at lower right is one second. The case is chosen because the wide scatter of spots indicates a very arrhythmic activity except at high frequency under strong stimulation; this is typical of this sense organ. In A the stimulus is weak though well above threshold defined as a significant change in the integrated frequency; but the unintegrated frequency record signals the environmental event very poorly and unreliably. While the integrated frequency greatly improves the detection, it is still dependent for its usefulness upon the absence of slow fluctuations in the spontaneous background; this short-term record does not show such fluctuations. B and C show the disadvantage of integrated frequency as a signal at high stimulus intensities—its slow response; the unintegrated frequency reliably reports much sooner. Note partial adaptation and postexcitatory silent period before spontaneous background returns. (From Bullock and Diecke.)

Instead of the actual frequency we may consider the ratio of, or the difference between, frequency recently transpiring and a background frequency.

(3) The ratio, frequency-integrated-over-some-short-time-just-past (F_{RC1}) to frequency-integrated-over-some-longer-period-just-past, (F_{RC2}). Sensitivity would increase as the time constants RC_1 and RC_2 increase. The greater RC_2 is, the better (within a limit depending on the relative rôle of this channel in change detection and steady-state detection). RC_2 cannot be less than several minutes if it is to avoid loss of information by an adaptation of the central nervous system more rapid than that of some receptors. RC_1 cannot be longer than a few seconds if loss of information is to be avoided, because sensory adaptation to weak stimuli already occurs in that time in many receptors. It should be, in fact, a fraction of a second in order to keep down reaction time and loss of information on brief stimuli and flicker.

Steady-state input from nonadapting receptors, like position and some temperature receptors, could not be processed by such a formula, for long

maintained high frequency would gradually raise the denominator until the ratio fell to unity. These modalities or the steady-state aspect of such input must use (2), above.

A further improvement in the extraction of information from such fluctuating signals arising from phasic input would result if the receiver could place a greater value upon changes in the ratio which occur rapidly, since these are more likely to be real stimulus-signals and less likely to be spontaneous fluctuations. This likelihood is based on the randomness of the successive intervals in spontaneous fluctuations; several short intervals in succession are highly unlikely. One possible formulation is the following.

(4) [Ratio (3)] $\times$ [Rate of change of ratio (3) integrated over RC_3]. RC_3 would probably be intermediate between RC_1 and RC_2. If F suddenly changes, F_{RC1} will be increased soon, the ratio will increase, the rate of increase of the ratio will be maximal for a short while, and a sudden stimulus will be signalled thereby. The signal meaning "strong stimulus" will then begin to decline even if the high F is maintained—first, because of the decrease over time constant RC_3 in the extra multiplying factor, and then because of the gradual fall in the ratio, as the high F gradually raises the denominator.

The possibility that, instead of a ratio of short-term integrated frequency to long-term integrated frequency, the latter should simply be subtracted from the former is available but seems less likely because of its disproportional sensitivity at low intensities.

It is possible by one further step to extract still more information from the same pattern of impulses. If the central nervous system can subtract a fixed frequency from the incoming frequency and then estimate the ratio of frequency integrated over the short term to that over the longer period, and increase the value if there has recently been a sudden rise, the effects of random fluctuations in successive intervals in the background spontaneity could be largely eliminated.

$$(5)\ \frac{[F_{actual} - F_{fixed}] \text{ integrated over } RC_1}{[F_{actual} - F_{fixed}] \text{ integrated over } RC_2} \times \begin{bmatrix}\text{Rate of change of this} \\ \text{ratio during } RC_3\end{bmatrix}$$

If the fixed frequency is higher than the extreme range of random fluctuations, no ratio is necessary since the numerator is zero, but sensitivity will not be maximal. Sensitivity will be maximal if only a few spontaneous impulses exceed the fixed level; the ratio will be more quickly altered by a real stimulus and the time constant RC_1 can now be shorter than in the last two formulae, improving reaction time, flicker, and brief stimulus detection while still smoothing enough to eliminate nearly all false signals.

Thus far we have considered the desiderata for a valid signal of en-

vironmental change in the single unit. Superimposed on these is the possibility of gaining reliability of small signals and hence sensitivity to actual stimuli by summing the activity in independent parallel channels or afferent fibers. This very probably occurs as an important normal mechanism in those organs or animals that can afford to have many channels, and permits the central nervous system to demand a high level of significance.

The problem of extracting reliable information from a continuously active background of fluctuating impulse intervals seems likely to be quite a general physiological problem. Spontaneous activity has emerged in recent years as a feature of many sense organs. Even in the best cases of rhythmicity, the constancy of successive intervals is relative and small signals look much like noise. The possibilities suggested may be difficult to test physiologically, but would be extremely interesting if they approximate the specifications for actual integrative centers. It can be anticipated that in different animals and in different modalities the relative rôles of different parameters of the integration will differ significantly. In arthropods, following the line of argument developed a few years ago by Wiersma (1952b), it may be true for some sense modalities that only a few parallel channels are available and reliability or sensitivity or resolution may be sacrificed.

We have not attempted here to extend the consideration to the problem of resolution; but it is clear that there must be a somewhat similar integration in cases like the vertebrate eye, ear, temperature, and tactile senses, where central discrimination far exceeding that of individual receptor units is achieved by comparing the firing frequencies of many channels from overlapping units of slightly different maxima. Little is known about this process in invertebrates and we shall only pause to note that it appears quite amenable to explanation in terms of the properties of units.

Expectation of New Levels of Complexity

So far we have attempted to explain or describe integrative phenomena in terms of familiar properties of units. This effort could be carried a good deal farther and might well account for a considerable part of the functioning of nervous tissue in higher animals. Certainly a treatment of the present theme must begin with, and today consists largely of, a list of such familiar properties.

The question whether the known properties of units suffice, when combined in great and intricate enough permutations, is so risky that few neurophysiologists will be caught guessing, though some have recently answered not only "no," but have implicitly or explicitly denied that physiological (matter-energy) mechanisms will be found adequate to account

for behavior and mental phenomena (Sherrington, 1951; Eccles, 1953). The gap at present is certainly staggering. It must be realized that much of our knowledge of brain physiology, while contributing to the better localization and fragmentation of the problem, actually still consists in specifying the nature of the phenomena to be explained without unequivocally helping to decide whether these will be explicable in terms of the known properties of neural units or of those plus as yet unknown properties or will not be explicable in any physiological terms.

I will confine myself here to the mere statement of my own faith—and it is just that—that no extension of the known mechanisms will be found adequate to explain higher activities of central nervous systems, but that we need not fall back on a dualism. Our position is a little like a meteorologist or oceanographer trying to account for the great events of the ocean and atmosphere from the known properties of the individual atomic species. I believe we have yet to discover fundamental new properties and relations at the level of masses of neurons—emergents in the old sense of inhering in but not readily predicted from a catalog of unit properties. One example may be the slowly traveling waves of synchronous, slow, subthreshold potential change in neuron masses.

I believe it will require such emergent mechanisms to understand, for example, complex integrations like that which must accompany the central control of afferent influx—now well known for several modalities. There must be a central correction for the control so that the world is interpreted reasonably correctly. Or again we may think of the *Efferenzkopien* of van Holst which similarly corrects for the distortions of input caused by our movements. The plausibly postulated energies, drives, appetitive behavior, releasers, and other entities of behavioral science and above all the amazing phenomenon of nearly nonlocalizable, anaesthesia- and shock-proof learning appear to me, at once, to require such still undiscovered physiological parameters and to be the stimulus to new levels of search.

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DIURNAL MIGRATION OF PLANKTON CRUSTACEANS*

EDWARD R. BAYLOR AND FREDERICK E. SMITH
University of Michigan

The behavior exhibited by certain freshwater and marine plankton in response to changes of single parameters of the environment, such as polarization, wave length or intensity of radiation, temperature, pressure, pH and redox potentials, are of increasing interest because they can be systematized and subjected to physiological analysis. In general the behavior in response to these parameters appears to be concerned with vertical migration or obtaining food. What effect, if any, these parameters may have on other behavior patterns is beyond the scope of our present efforts.

The experiments to be described have been performed by the authors and William E. Hazen with the help of students and colleagues. Our research strategy consisted of two stages of attack. In the first stage, we concentrated our efforts entirely on those qualitative observations which we felt would delineate the general behavior mechanisms under study. Thus we postponed the development of quantitative techniques, the second stage of the attack, until we had completed some preliminary analyses of a number of the behavior-pattern mechanisms. The quantitative techniques now under development are particularly important for either elegance of description or accuracy of analysis, since many behavior patterns of zooplankters are actually the end result of algebraic addition of many apparently random locomotion vectors and velocities, such as appear in orthokinesis, klinokinesis (Fraenkel and Gunn, 1938), or color dances (Smith and Baylor, 1953). The qualitative results reported here may in all cases be quantified to the following extent—that more than 75% of the population (never less than 100 individuals unless otherwise specified) must execute the same behavior pattern at the same time in response to the stimulus. The present paper is a summary of certain of our qualitative observations.

Rather than survey what many animals do in many diverse situations we thought it better to attempt a synthesis of what one animal does in many situations. This tentative synthesis is complex; for the moment its purpose is to serve as an object of criticism by this audience as a guide to more fruitful experiments. We are not at all sure that our interpretations

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are correct, but we feel that they are the simplest we can make so far. Actually, the behavior patterns may turn out to be even more complex.

In general we have concerned ourselves with two types of behavior—orientation to food and diurnal vertical migration. First, we shall take up orientation to food, a series of behavior patterns mediated by the compound eye in *Daphnia magna*, the large water flea, and in *Eubranchipus*, the fairy shrimp. We have particularly concerned ourselves with those zooplankters which filter suspended phytoplankton. I shall digress momentarily to point out how suspended phytoplankton alter certain important parameters of the environment. First, a suspension of unicellular phytoplankton polarizes a vertical beam of light so the light which is scattered horizontally is strongly polarized in the horizontal plane. We presume the mechanism operating to polarize the light is a simple reflection from the many spherical or cylindrical surfaces presented by the suspended phytoplankton or bacteria. Clearly, the sizes of particles are wrong to produce polarization by Rayleigh scattering. Second, the pigments present in many such plant cells absorb relatively more of the short end of the visible spectrum than the long end. Third, the respiration and photosynthesis of such plant cells cause a change in the pH of the water suspending them. With this information, we are in a position to consider the behavior patterns of those animals orienting to such suspensions as food objects.

Polarized Light Orientation

Daphnids orient at right angles to a vertical beam of plane polarized light by swimming back and forth in the beam at right angles to the plane of vibration of the light. When the geometry of the light beam and of the compound eye are examined, one sees that a vertical beam of light polarized in the transverse plane of the animal enters only those facets of the compound eye which are directed upward, forward, or backward. Fig. 1 shows a highly diagrammatic geometrical analysis of this optical situation. Only a fraction of the normal complement of cone lenses is shown and these are directed upward in the anterior-posterior, forward in the dorsal-ventral, and to the right in the lateral planes of the animal. The pigment mass in which the cone lenses are embedded is not shown. Polarized light vibrating in the transverse plane of the animal finds parallel internal reflecting surfaces only in those cone lenses (1 and 3 of Fig. 1) lying in or near the plane defined by the dorsal-ventral-anterior-posterior lines. Note that in this case the lateral lenses (2 of Fig. 1) do not permit internal reflection, since there is no surface parallel to the plane of vibration of the light. Since polarized light can penetrate to the inner tip of only those cone lenses presenting an internal surface parallel to the plane of vibration of

the light, then clearly the geometry of the compound eye and the plane of vibration of the light control which lenses shall have light penetrating to

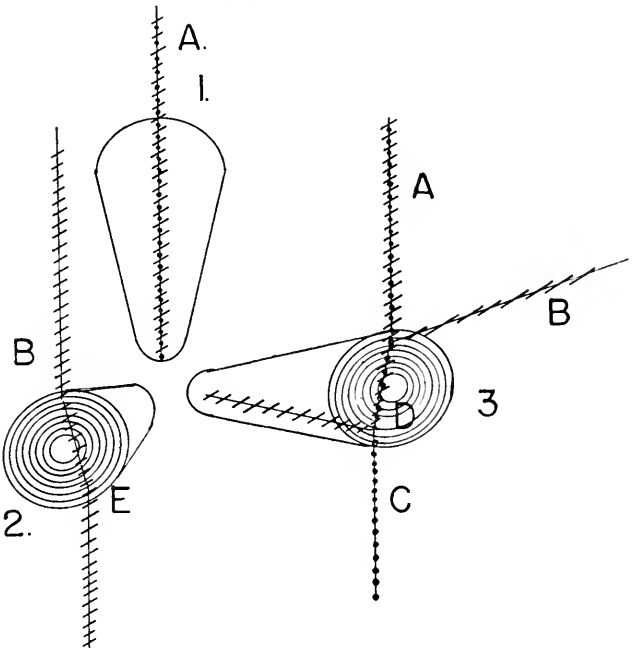

Fig. 1. A sixty-degree projection of only three cone lenses of a daphnid compound eye showing various light paths. At A light is nonpolarized. At B the light vibrates in the transverse plane of the daphnid. At C the light vibrates in the dorsal-ventral plane of the daphnid. At D a surface parallel to the light vibrating in the transverse plane of the animal permits reflection of this light to photoreceptors at the tip of the cone. At E the surface is virtually perpendicular to the plane of vibration of the light and hence the light is refracted through the lens to the outside again without reflection to the inner tip of the cone.

the tip of the cone. The important exceptions to this statement are those lenses directly facing the light source.

For a horizontal beam of polarized light vibrating in the transverse plane of the eye, the lenses permitting penetration of light to the tip are those directed anteriorly, dorsally, and ventrally. The cone lenses directed laterally do not present parallel surfaces for internal reflection and therefore are not illuminated by a horizontal beam of polarized light vibrating in the transverse plane of the animal. Operationally the behavior pattern in response to polarized light would appear to orient the animal so that the light can enter the ventral, anterior, and dorsal lenses only and would overtly exclude light from the lateral lenses.

If a horizontal beam of plane polarized light vibrating in the vertical plane is presented the animal so that the light would enter only the laterally directed lenses, the daphnid makes considerable effort to swim on its side where the light will be excluded from the laterally directed cone lenses. Further proof may be adduced by experiments described elsewhere (Baylor and Smith, 1953).

In the final analysis the response to polarized light in the natural environment is a relatively simple sort of positive phototaxis brought about by an increased intensity of the light entering the cone lenses which are directed anteriorly, posteriorly, dorsally, and ventrally compared to the intensity of light entering the laterally directed cone lenses. If a daphnid is presented with 360 degrees of light, i.e., inside an opal glass globe uniformly illuminated from the outside, movement appears completely random horizontally and vertically.

Color Dances

Color dances are statistical behavior leading to food (Smith and Baylor, 1953) and maintaining the animal within a useful range of its food once such food is found. This behavior is best seen with a light source from above giving a uniform intensity over the entire aquarium. Under red light (6,000 Å and over) the population appears calm, the individuals dancing upright in the water, with a small horizontal vector in their locomotion. The vertical vector is larger and varies somewhat throughout the population. The velocity of such locomotion is quite low, the animals occasionally appearing to be suspended in the water. Under blue light (5,000 Å or shorter) the population is distinctly agitated, the individuals leaning well forward in their dance and roaming about with a large horizontal vector to their locomotion. Velocities are estimated at three to five times the average velocity in red light.

A change of the direction of the light beam from vertical to horizontal shows that the color dances are oriented, not to gravity, but to the direction of propagation of the light. The largest vector and hence the direction of locomotion in the blue dance is always oriented at right angles to the line of propagation of the light, while the largest vector in the red dance is always directed parallel to the line of propagation of red light. When blue light is introduced from the side, a vigorous wandering occurs in all directions in the vertical plane perpendicular to the light beam; when red light is introduced from the side, the red dance remains a quiescent dance with a predominant vector, if any, in a line parallel to the direction of the light, the animals swimming slowly away from the red light and returning more rapidly at irregular intervals.

Under white light from above the dances are discrete; at any one mo-

ment an individual is either red-dancing or blue-dancing. If the proportion of energy in the short wave lengths is too high, all the animals will blue-dance all the time. Within a range of proportions specific for each species, and probably affected by several environmental factors such as presence of food or hunger, the individuals will change spontaneously back and forth from one dance to the other. The total proportion of time spent doing each of the dances is extremely sensitive to the proportional energies of short and long wave lengths. A convincing test of this hypothesis may be performed by placing, over a dish of daphnids, a shallow lucite tray having two or more compartments, one of which is filled with clear water and the other with a phytoplankton suspension. If the dish and overlying tray of water and phytoplankton is illuminated from above, the organisms rapidly gather under the phytoplankton.

Color dances have been observed in several freshwater Cladocera—*Daphnia magna, Ceriodaphnia, Moina,* and *Bosmina*—and also in a fairy shrimp, *Eubranchipus.* We have observed color dances in the following marine zooplankters: *Squilla* larvae, two pontellid copepods, and a harpactacoid copepod.

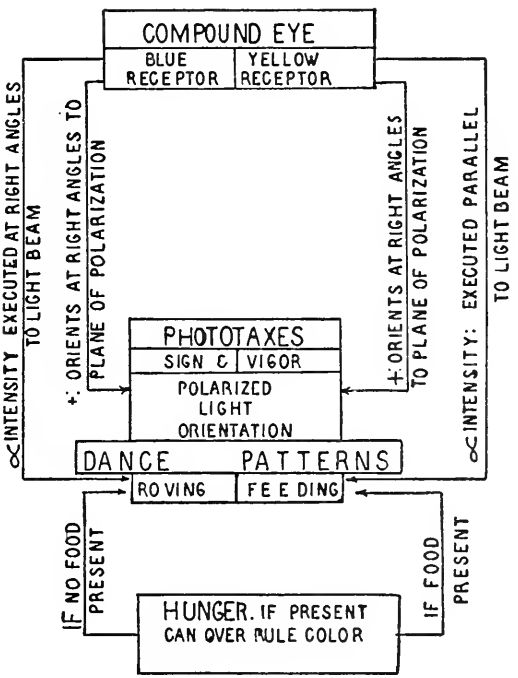

Fig. 2. Statistical behavior associated with food. Arrows indicate relationships.

If the compound eye of *Daphnia magna* is denervated and the animal is allowed to recover from this operation the color dances are completely missing.

One might be tempted to conclude that Cladocera living in water with humic stains would never blue-dance and might hence die of starvation by never reaching food which might be nearby. However, such is not the case, since the color-dance responses to incident wave lengths are overruled by hunger or by the presence of food. For example, an animal which is hungry will blue-dance in all wave lengths of the visible spectrum. If food in the form of suspended photoplankton is now added to the hungry population, there will be nothing but red-dancing observable no matter what the incident wave length.

Fig. 2 summarizes the situation with regard to color dances, showing that these two behavior patterns are mediated by the compound eye which has a receptor for long and for short wave lengths and showing that hunger or the chemical stimulation of food can overrule the light stimulus.

Vertical Migration

Vertical migration is essentially a very complex combination of geotaxis and phototaxis which is influenced by a number of parameters of the environment. In various marine and freshwater zooplankters it can be induced by light intensity or wave length, pH, redox poising compounds, temperature, or pressure. Sometimes all of these parameters are effective in a single species, such as *Daphnia magna* (see Fig. 3), and sometimes visible light has little effect, as for example certain marine zooplankters of the Inland Waterway of Florida. Since gravity is a constant force in the environment capable of serving as a behavior cue along with radiant energy which is less constant, it is not wholly unexpected that geotaxic and phototaxic behavior patterns have evolved in response to those parameters of the environment which have diffuse vertical gradients, as for example light, temperature, pressure, pH, and redox potentials. The gravitational field of force of the earth has been well exploited by the Cladocera and behavior responses to nongradient situations are oriented not to the stimulus per se but to gravity. A nongradient situation has no spatial dimensions for cueing an oriented response but the stimulus serves to set off a gravitational response. When one considers thermal, chemical, or radiant energy gradients of the environment in relation to the size of Cladocera, it is clear that the change in intensity of the gradient over the length of the animal is too small to be detected and resolved into directional information on which to base an oriented taxis. Hence, we have behavior patterns like geotaxes initiated by chemical or radiant energy stimuli. Radiant energy sources, on the other hand, may be localized if the animal has a receptor

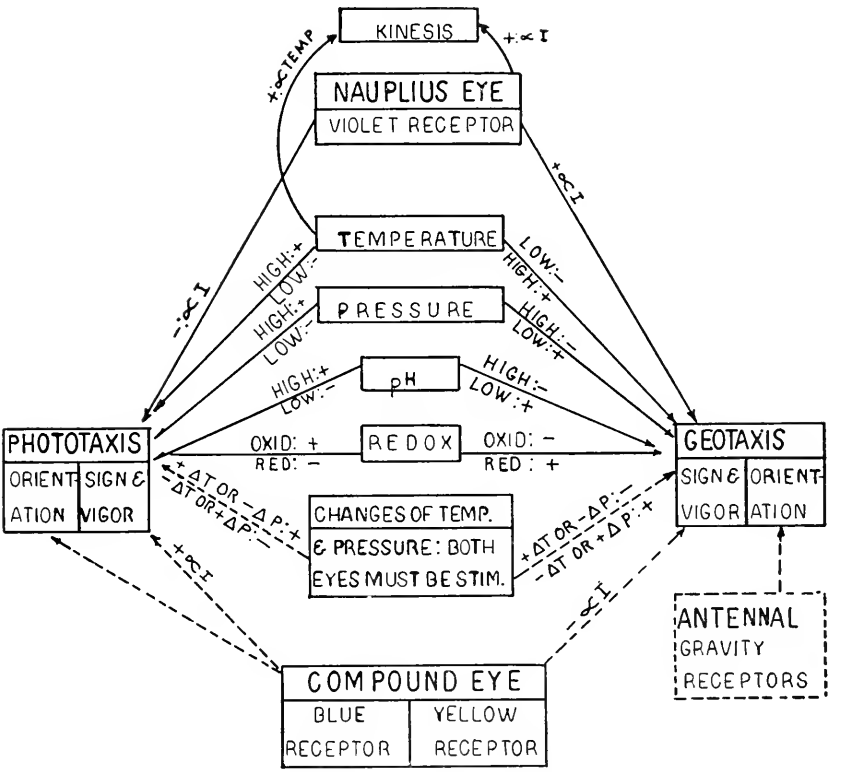

Fig. 3. Behavior patterns mediated by the nauplius eye, the compound eye, and the antennal gravitational receptors. Arrows indicate relationships. Signs on the arrows refer to the positive and negative taxes. Words on arrows indicate intensity of stimulus. Broken lines show relationships present in the Cladocera, absent in *Eubranchipus*.

backed up by an opaque curtain. Gravity receptors appear to be localized in the swimming antennae of *Daphnia magna* and monitor this force only when the animal is not swimming. (Grosser, Baylor, and Smith, 1954). Rose (1925) has obtained results with pH and temperature similar to ours.

The Nauplius Eye and Vertical Migration

The nauplius eye of adult crustacea is smaller and of less importance as a taxonomic character than the compound eye. Thus, its functional significance has been somewhat neglected. Unpublished data of Lockhead (personal communication) indicate that it has photosensitivity in the fairy shrimp. It was completely unanticipated that the nauplius eye of daphnids would be concerned with responses to pH, pressure, temperature, and redox potentials as well as being sensitive to ultraviolet and x-rays.

However, the series of behavior patterns concerned with vertical migration described below can be shown to be associated with the presence of the nauplius eye in *Daphnia magna*. Destruction of the nauplius eye by microsurgery or by needle beam x-rays results in complete disruption of the behavior pattern. Tests have been carried out in the case of each behavior pattern described for *Daphnia magna*. Only those behavior patterns associated with vertical migration are disrupted.

Ultraviolet and violet light produce positive geotaxis and a negative phototaxis which is mediated by the nauplius eye. For example, if a population of daphnids is suddenly exposed to a horizontal beam of violet light, there results an immediate movement of the population down and away from the light beam. The proportions of the down and away vectors of the locomotion depend on the wave length of the horizontal light beam, its intensity, and the state of accommodation of the daphnid population to the intensity of the beam. The down vector is greater the shorter the wave length and is roughly proportional to the intensity. A cinematographic analysis of this stimulus-response situation is being carried out. We may summarize this behavior as follows:

(1) Dimming wave lengths either shorter or longer than 5,000 Å can provoke upswimming.

(2) Brightening wave lengths shorter than 5,000 Å can provoke downswimming.

(3) Brightening wave lengths longer than 5,000 Å cannot provoke downswimming but may cause a slow spread downward.

(4) Shifting the spectrum of light toward blue without changing the intensity can provoke downswimming.

(5) Shifting the spectrum of light toward red without a change in intensity can provoke upswimming.

Fairy shrimp show behavior similar to that indicated for daphnids.

Light and Vertical Migration

The vertical response to light stimuli of the inshore plankters of the Inland Waterway of Florida[1] is considerably different from that of the freshwater plankters. An increase of light intensity may induce downswimming and a decrease in intensity may induce upswimming but the intensity changes required are very much greater. A 95% decrease in intensity (compared to 1-10% for freshwater Cladocera) is required to elicit a clear, though usually temporary response. Most species having a hori-

[1] The marine work reported here was performed at the Marineland Laboratory, Marineland, Fla.

zontal phototaxis show light-induced geotaxis; however, some species (medusae, *Mnemiopsis,* and a pteropod, *Cresceis*) which have no lateral phototaxis show light-induced geotaxis. Certain marine organisms may show a reversal of behavior outlined above. For example, Callinectid megalops larvae, usually being geopositive to increased intensity, may sometimes show the reverse pattern and swim upward in response to an increased intensity. In general, if inshore plankters are geopositive in the dark (in response to factors other than light), they will upswim to an increase in light intensity, while, if they are geonegative or show neither tendency in the dark, they will downswim to an increase of light intensity. In the presence of long wave lengths of light, the addition of short wave lengths either has no effect at all, or else the organisms downswim with the addition or upswim with the subtraction of short wave lengths.

Light and Geotaxis

There are two effects of light on geotaxis. One effect is associated with wave length while the other effect is associated with intensity. We have not determined the action spectrum of the daphnid eye accurately in the violet region and hence these effects may be identical. In our tests geotaxis is distinguished from phototaxis by the use of a horizontal light beam. Thus phototaxes will be directed toward or from the light source while geotaxes are directed up or down. The behavior pattern here is readily distinguished from blue-dancing in a horizontal beam where movement is also at right angles to the beam.

The effect of an increase of intensity is downswimming (see Fig. 3). Provided the animal is accommodated to the intensity of the horizontal beam, the effect is almost a pure downswimming in the near ultraviolet with a very small negative phototaxis. The effect of shortening the wave length of a horizontal beam of violet light from a monochromator is also downswimming. If one assumes that the peak of the violet action spectrum for *Daphnia magna* is 3,000 Å or below, then the response to either an increase in intensity with the wave length held constant or a decrease in wave length is indistinguishable operationally. Both may appear as an increase of intensity in the experimental animal. Conversely, either dimming the intensity or increasing the wave length will produce upswimming, provided the animal is accommodated to the initial intensity and wave length. Thus, geotaxis in response to light stimuli appears associated with phototaxis in the integrated behavior of *Daphna magna.* In the natural environment the two behavior patterns of phototaxis and geotaxis operate together and in the same direction in response to the same stmulus although one is oriented to light and the other to gravity (see Fig. 3).

Temperature and Phototaxis

Since the response to a vertical light beam under various conditions of temperature is up or downswimming, the following tests were all conducted in a horizontal light beam to eliminate the possibility of a light-induced geotaxis (which is discussed above). There are at least four separate and independent effects of temperature on the phototaxic response of *Daphnia magna* and *Eubranchipus* to white light, which are summarized under "Temperature" and "Changes of Temperature" in Fig. 3. Daphnids reared at 15° C and exposed to a horizontal light beam show vigorous positive phototaxis at temperatures of 0° to 5° C. The organized photopositive response is not a prolonged one at very low temperatures in the presence of light intensities of greater than 10-foot candles, since it is superseded by a somewhat violent paralytic seizure ending in death. The paralytic seizure may be induced by similar light intensities at room temperature in the presence of $1:10^7$ acetylcholine and eserine. On the other hand the paralytic seizure at low temperatures may be avoided entirely by making the water in which the daphnids swim $1:10^7$ atropine (Baylor, 1954). At the opposite end of the temperature scale for the daphnid, namely 15 degrees above its normal environmental temperature, there is a vigorous negative phototaxis in response to a horizontal beam of white light. In view of the fact that the direction of the intensity vectors of light in the normal environment of such animals is vertical and that extremes of temperature are hazardous, the positive phototaxis in cold water and the negative phototaxis in warm water may be considered to have some adaptive significance.

Small but continuous decrements of temperature can significantly increase the velocity of downswimming in response to any stimulus which will initiate downswimming. Thus a population of daphnids will continue to swim downward through a thermal gradient at high and sustained velocities in spite of the fact that the stimulus which initiated the response may have decreased in intensity. The downswimming pattern performed through such a thermal gradient will be maintained until the population reaches 10 to 15 degrees below normal environmental temperature, where the incipient positive phototaxis in response to cold temperature halts the downward migration.

Small but continuous increments in temperature can significantly increase the velocity of upswimming, once such a response is initiated by any of a number of stimuli already discussed. The pattern is now exactly the converse of that described above for decrements of temperature. Once the population of daphnids is started swimming upwards through such a thermal gradient, they will continue to swim upwards till an upper temperature is reached which will halt the pattern.

While the adaptive significance is not so obvious here, we hypothesize that the value may lie in carrying out the pattern of vertical migration (for whatever value it may have) in spite of the fact of accommodation to the light stimulus and in spite of the fact that appropriate intensity and wave lengths decrease rapidly with depth. Conversely, it allows a rapid approach to the surface waters in spite of rapidly increasing intensities of light.

In the zooplankters of the Inland Waterway of Florida vertical responses to temperature were also observed. However, the range of useful temperatures was considerably limited. At pH 8.05 a shift of minus 0.5 degrees from 31° C would induce upswimming, while a shift to 31.5° C would induce downswimming. A shift of as much as 5 degrees would irreversibly destroy the response to either pH or temperature.

Temperature and Geotaxis

In the dark, positive geotaxis results from immersing *Daphnia magna* in water 10 to 15° C above the temperature at which it was reared. This behavior pattern is a parallel of the phototaxis described in response to temperature. Small increments or decrements of temperature will cause the sustained high velocities of locomotion described for phototaxis in response to these stimuli for Cladocera only under white light.

Hydrostatic Pressure and Vertical Movement

In general, the effects of pressures on certain marine and freshwater zooplankters appear to be identical and to fall into three categories.

First, there is the effect of pressures from 750 to 6,000 psi. Pressures of these magnitudes cause paralysis followed by death within minutes, particularly at low temperatures. In *Daphnia magna* the effect appears to be on the choline esterase system, since it can be alleviated by the use of atropine in physiological concentrations. It is not known whether or not the same protection is afforded marine organisms.

Second, there is the effect of pressure ranging from 30 to 500 psi. Throughout this range, pressure can cause upswimming depending on the temperature or pH of the water within the pressure bomb. For example, the megalops larvae of portunid crabs swim downwards at all temperatures above 30° C when the pH is 8.1, but can be made to swim upwards by application of pressure. The higher the temperature the more pressure is required to induce upswimming. At 40° C the response is poor and heat death ensues in minutes. Death is not particularly delayed by high pressure.

The third category is small pressure changes in a low pressure range, from partial vacua to 10 psi. For the Florida Inland Waterway zooplankters such small changes in hydrostatic pressure were found to produce

vertical movements which compensate by a change in depth for the small pressure change. Thus, partial vacua of 6 inches of sea water produce a downward movement of approximately 6 inches. The converse operates for positive pressures unless the animal is already at the surface, in which case it still makes an effort to swim upward. The results of small positive pressure changes are shown below:

PRESSURE INCREASES REQUIRED TO PRODUCE UPWARD SWIMMING IN VARIOUS ANIMALS

Animals	Pressure Increase (psi)
Small pelagic annelids	2
Hydrozoan medusae	5-10
Temora	2
Mnemiopsis	5
Pteropods	0.1-5.0
Pontella	0.1
Sagitta	9
Pleurobrachia	9
Palaemonetes larvae	9
Acartia	9
Centropages	9
Callinectes megalops & zoea	9
Lucifer larvae	9
Penaeus larvae	9

The lowest threshold for positive or negative pressures in the Florida marine forms was 3 inches of sea water. Such a low threshold to pressure raises the question of mechanism. One possibility is that these organisms possess small gas bubbles, although none is discernable with high dry microscopy in the glass-clear medusa *Pleurobrachia,* or *Sagitta.* A test of this hypothesis is available. If the pressure is so reduced that boiling occurs, any gas bubble present would be expected to expand enormously and float the organism to the surface. In none of the species tested did this happen. The pressure decrease accompanying evacuation of a suction flask induces a strong positive geotaxis, and the populations literally grovel on the bottom while the water all around them is boiling at room temperature. Gas bubbles, in the ordinary sense, at least, are not the mechanism. No fresh-water organisms responded to this test either.

Small continuous increments of pressure significantly increase the velocity of upswimming of the Florida marine plankters until a pressure of 15 to 30 psi is reached. When the pressure was maintained at these levels for a few minutes, there resulted a destruction of the pressure-sensitive mechanism. Recovery did not occur within a week in which these animals were kept alive in the laboratory. Small continuous decrements of pressure also increase the downswimming velocity until the sea water boils.

No destruction of the mechanism results. Hardy and Bainbridge (1951) noted low-pressure responses in marine plankton.

pH and Phototaxis

The influence of pH, or carbon-dioxide-induced changes in pH, on daphnid behavior has been observed often since Jacques Loeb (1904) reported reversal of phototaxis when he profligately poured beer into his experimental aquaria of daphnids. In general we have confirmed these observations; high pH values induce positive phototaxis while low pH values induce negative phototaxis. The crossover point from positive to negative phototaxis appears to depend to some extent on the pH at which the organisms were reared. Daphnids reared at pH 8.0 become vigorously positive at pH 8.5 and vigorously negative at pH 7.0. The adults are apt to be normally photopositive to white light at pH 8.0 when fed on green algae. Conditions of culturing may change the sign of taxis; these will be discussed under redox potentials. Immature forms often exhibit the reverse photo- and geotaxis of the adults. This fact has often been observed and is believed by Skadowsky (1939) to be a function of metabolic rate.

pH and Geotaxis

Geotaxes in response to pH changes occur in the dark (vertical distribution of a population of daphnids is easily measured by a flash photograph). Results of such studies show that *Daphnia magna* swims upward in the dark at pH 9.0 and swims downward at a pH of 7.0 or below. Such geotaxis may occur in addition to the phototaxis in response to pH since it takes longer for a population to reach equilibrium position in the dark than in the light.

Probably the most startling induction of geotaxis by pH is to be observed in the Inland Waterway of Florida, where small changes of pH are of primary importance in controlling the vertical migration of the zooplankton. Special care must be taken in the collection of the plankton sample to avoid a change of more than 2° C or 0.7 pH units. Either of these will destroy the response to pH or temperature. Vertical-movement tests in response to pH were run in the laboratory on three representative species: a hydrozoan medusa, a pontellid copepod, and blue crab megalops and zoea larvae. Some effort was made to obtain relatively pure populations of the three species tested for pH responses; this was, however, impractical so that other unidentified organisms were often included in the tests. The responses of these unidentified organisms were identical with those of the carefully isolated species studied. When the water temperature was maintained at 31° C and the pH was varied through the range of 8.0 to 8.1, there was a uniform response of the species tested; a pH of 8.0 caused persistent upswimming while changing the pH to 8.1 induced persistent

downswimming. Light has little effect on this reaction. The same responses could be obtained with more marked shifts of pH, although departures of more than 0.5 units from the normal environmental pH quickly destroyed the mechanism. The daytime pH of the Inland Waterway is 8.1, the nocturnal pH 8.0. There is sufficient turbulence to prevent a gradient. The pH values were obtained with a Beckman Model B pH meter equipped with titrating electrodes. Each reading was preceded by a buffer and temperature check.

Redox Potentials and Phototaxis

Oxidizing substances to which daphnids are permeable cause positive phototaxis while reducing substances to which daphnids are permeable cause negative phototaxis. The redox poising compounds utilized for such tests are largely those intravitam stains which readily penetrate daphnids, some of which appear preferentially concentrated in the cells of the nauplius eye. Additionally, catechol and cysteine were used. Catechol (10^{-3}M) having an E'_0 of + 0.33 volts produces strong upswimming while (10^{-3}M) cysteine having an E'_0 of —0.14 volts produces strong downswimming (Smith 1954). The mid-point of the range where no effect occurs for a population of *Daphnia* reared on green algae appears to to about + 0.045 volts E'_0, while the mid-point for a culture reared on bacteria appears to be lower. The important point here is that the majority of organisms reared on algae appear to be photopositive, while those reared on bacteria having a low redox potential are photonegative. We feel that these results will largely explain the photopositivity reported for daphnids by Clarke (1930) and the photonegativity later reported for the same species by the same author (1932). A summary of our observations on phototaxis and redox poising compounds is given in Fig. 3.

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PREY-PREDATOR RECOGNITION IN THE LOWER INVERTEBRATES*

L. M. Passano
Yale University

Above the broad herbivorous base of the ecological pyramid of animals, whether one is considering the land or sea, are the lesser numbers of carnivores. Most of these animals must be below the apex, must feed on their animal prey yet in turn be fed upon by other organisms. The success of these animals means that a judicious balance between recklessness and care, effective capture of food versus preventive caution in the presence of enemies, has been achieved. The organism's behavior patterns are adapted to meet these dual needs. The animal's morphological and physiological equipment dictates the form of this behavior, its complexity, and its adaptability. Obviously, the success of this balance can be measured by the abundance of the group under consideration.

Without being able to *explain* the physiological mechanisms that are involved, nevertheless we can see in the elaborate and specialized sense organs, in the many and varied motor responses, and above all in the complexity of the central nervous systems, the physiological and morphological *machinery* that determines the success of most carnivores. But, especially to those interested in comparative physiology, a few groups of relatively simple metazoan carnivores, such as many of the coelenterates and the free-living flatworms, pose an immediate problem. These animals do not, as a rule, possess elaborate sense organs. Their tissues, as judged by such criteria as regenerative powers or specialization of function, do not appear to be completely subordinate to the entire organism. Above all, as far as the coelenterates are concerned, they have no central nervous system. We are unable to evoke any "black box" of suitable complexity to account for the behavior pattern that we can so easily observe. With the relative simplicity of both behavior and morphological equipment, it is not surprising that these organisms constitute an immediate challenge to the physiologist.

As the title of this paper indicates, only one aspect of these analytical problems will be dealt with here, the recognition and distinction of prey and predator. We shall try to determine how far, in the present state of our knowledge, it is possible to explain these behavior patterns in physiological language. Let us admit at the outset that this attempt cannot be too

* This paper is dedicated to Alexander Petrunkevitch in honor of his eightieth birthday.

successful. In spite of the work of such deservedly famous investigators as Romanes, Loeb, Pearl, Jennings, Parker, Koehler, and Pantin, we still have very little of the basic physiological information that we need before we can attempt any reasonably complete explanation. For instance, we shall see that chemoreception plays an important role in these responses; yet the receptor units detecting these environmental clues have not often been identified. But if any generalization can be made, it is that various quite simple metazoans *code* their sensory input, so that relatively weak stimulation of mechano- and chemoreceptors leads to food-capture activities, whereas strong stimulation leads to defensive reactions. Even in such aganglionic animals as sea anemones, there is no single form of stimulation that invariably leads to withdrawal or defensive responses.

While many of the invertebrate phyla include species which lead an active, carnivorous existence, hunting their food, it is often impossible to obtain from the literature sufficient information both on their natural prey and on the predators which, in turn, feed on them. One might imagine that the success of certain forms with highly specialized diets, such as the gastropod *Archidoris* which feeds on certain sponges, or many of the nudibranchs (Hunt, 1925) which feed on hydroids, is determined by the availability of their food rather than by predation. But certain of the latter group clearly exhibit withdrawal behavior when they are vigorously prodded, suggesting that their protective devices of noxious taste, slime, and hydroid nematocysts may not always be sufficient deterrent to hungry fish or crabs.

A number of the carnivorous mollusks, however—and I am thinking here of such gastropods as *Natica, Murex, Nassa,* and *Urosalpinx*— are known to constitute an important part of the diet of fish (Hunt, 1925; Hancock, 1955) and starfish. Most of these gastropods can be distinguished from their herbivorous cousins by the reduction or loss of the crystalline style (Yonge, 1930). It is interesting to note that these snails are *not* generally very restricted to certain prey; *Urosalpinx,* for example, might as fairly be termed the barnacle-drill or the mussel-drill as the oyster-drill.

From the work of Copeland (1918) it seems quite certain that chemoreception is important in guiding these mollusks to their food. He showed that in *Nassa* (= *Alectrion*) the sensory organ, the osphradium, samples the water which enters the siphon. The animal responds to dilute food extracts by increasing its rate of locomotion, extending its proboscis, and orienting the direction of movement towards the increasing chemical concentration. In addition to this "nasal" response, moreover, these snails show "taste" responses to relatively strong food solutions with tentacles, underpart of head, and anterior end of foot. There is no evidence that the eyes play any part in food capture.

It would be interesting to know whether or not tactile cues, in the ab-

sence of chemoreception, can also be used by these animals. If they can differentiate, by touch alone, bivalves or barnacle shell from a rough rock, this would seem to imply a fair degree of central integration *or* very specific sense receptors. On the other hand, these animals may be able to find their prey from the relatively low concentrations of waste products that are being excreted into the water, although this would imply that they possess the degree of sensitivity and selectivity that appears to characterize many symbionts (Davenport, 1955). It would seem most unlikely that such stimuli as were used by Copeland (extracts of fish and oyster meat) would be the naturally attracting substances for the gastropods which feed on live bivalves, or such forms as *Scaphander* which swallow whole prey (Hunt, 1925).

The best known cases of escape reactions among the invertebrates to be be considered here involve the reactions of certain mollusks in the presence of starfish (Bullock, 1953, for review). *Nassa,* which is probably more of a scavenger than a carnivore restricted to living prey, shows a classic and peculiar leaping withdrawal when touched by certain starfish. While this example has not yet been sufficiently analyzed to preclude a role of tactile stimulation of the snail by the predator's tube feet (compare Dakin, 1910, and Hoffmann, 1930), it seems quite certain that strong chemo-stimulation by substances on the surface of the echinoderm are the primary cue. Recently Heinsohn (1955), looking at this response, reported a violent twisting, followed by rapid locomotion, by the snail *Caliostoma* responding to an extract of *Pisaster.* The total response lasted at least 30 minutes, during which time the animal moved over 1.6 meters. Interestingly enough, there seems good reason to suppose that other groups of animals are equally sensitive to substances on the integument of certain starfishes. Hancock (1955) mentions the toxicity of *Solaster,* while I have observed what may be the same response in several species of ophiuroids to *Solaster* and *Dermasterias.* The latter star is particularly effective in causing the extraordinary escape response of the sea anemone *Stomphia,* to be mentioned later.

While this relative wealth of information is available concerning possible recognition and escape reactions of gastropods to starfish, apparently nothing is known about similar behavior initiated by cues from fish; yet it would seem (Hunt, 1925) that a variety of bottom fish form the chief predators of these snails. Clearly, however, the general pattern of gastropod activities—movement away from well-lit areas for some species, or crowding into the intertidal or even supratidal zones, the occupancy of crevices, and so forth—all tend to aid these mollusks in avoiding their enemies.

Two complicating factors in any physiological analysis of behavior are the possibilities that conditioning may occur or that changing "physio-

logical states" may affect the responses of the organism to specific stimuli. While I see no sound reason to exclude the possibilities of conditioning, or learning, in the gastropods, I know of no demonstration of this. But the effect of changing states seems to be present. Forty years ago Wenrich (1916), working with the fresh water mussel *Anodonta,* clearly demonstrated that a variety of internal conditions changed their sensitivity to light. Besides the unexplained but omnipresent "individual differences," the presence of eggs or embryos, of foreign material in the mantle chamber, and whether the animal had been stimulated previously or not—all these conditions had definite effects. As will be noted later, this modifiability of responsiveness is to be found throughout all the lower metazoans. For instance, Gee (1913) maintained that the internal state of leeches—whether they were hungry or satiated—profoundly influenced their food-finding behavior.

Gee emphasized the importance of "random movements" in the leech activity pattern. He postulated that the central nervous system of these annelids had two main functions: (1) the production of "spontaneity," and (2) adaptation to constant external stimuli. It is clear that he assumed that the central nervous system integrated all sensory input and that the reaction of the organism was the resultant of these concurrent stimuli. Perhaps it is even more than this; it should be remembered that Copeland (1930) and Copeland and Brown (1934) convincingly demonstrated a case of conditioning in the polychaete *Nereis,* in which the normal positive response to food extracts was duplicated by touching the anterior end of the worm, usually a withdrawal stimulus.

Copeland and Wieman (1924) earlier had demonstrated the normal chemokinetic feeding behavior of these sand worms, and it is probable that this is general in the carnivorous polychaetes. On the other hand, while defensive (escape) responses may be initiated by strong or specific chemical stimuli, it is clear that these animals normally depend on sensitive vibrational or tactile receptors, synapsing with giant fibers for this use. An easily observable example of these elegant responses can be seen in the "quick-as-a-wink" withdrawal of the tubeworm filterers such as the sabellids.

It is an obvious truism, in general résumé of the behavior patterns of these animals, that chemical and vibrational or tactile cues are the important external stimuli enabling effective prey-predator recognition. How much the disturbances engendered by prey movement, as against the demonstrated rôle of weak chemical stimuli, ensures the success of the predacious carnivores has not yet been determined. Clearly, more knowledge is necessary, both at the behavioristic and physiological levels, before specific analyses can be made. However, it does seem true of all of the organisms

under consideration that visual stimuli do *not* directly guide either their defensive or predaceous behavior. Rather, those forms which have photoreception appear to use this information to direct or modify overall activity patterns, and to keep them within the generally restricted environmental niche to which they are adapted.

This sort of résumé of the physiological mechanisms guiding such complex organisms as annelids and mollusks seems, and is, very naive. When one comes to the free-living flatworms, however, such simplifications may begin to come somewhat closer to validity. Thanks largely to the classic observations of Pearl (1902) and the careful analysis of the rôle of chemoreception by Koehler (1932), a considerable body of information is available concerning planarians. These carnivores are guided to their prey mainly by chemoreceptors, utilizing separate sensory units for weak and strong stimuli (Wulzen, 1917). As in the gastropod mollusks, these units appear to be the analogues of vertebrate smell and taste receptors, respectively. If the "physiological condition" of the animal allows it, weak chemical stimuli will initiate and orientate a positive gliding movement. In some species this movement must be against the current *as well as* along a chemical gradient (Koehler, 1932). Strong illumination may prevent this positive response. At some point when the animal is close to the source of the chemical stimulus, other chemoreceptors, generally located on the proboscis or grasping organs (Redfield, 1915; Wilhelm, 1915) are sufficiently stimulated to assume control of the behavior pattern, changing a seeking behavior to a feeding behavior. Positive-orienting reactions may also be elicited by mild tactile stimuli. The specific feeding reflex may require both stimuli concurrently, or else a strong chemical stimulus may be sufficient.

Pearl (1902) emphasized that positive responses to both chemical and mechanical stimuli were abruptly changed to negative responses when the strength of the stimuli was increased beyond some arbitrary and variable point. For instance, fairly strong unilateral tactile stimulation of the anterior end of the planarian would cause a turning away from that side, extension of the stimulated side. This extension, according to Pearl, is due to contraction of the circular, dorsoventral, and transverse muscles. A somewhat weaker stimulus, however, causes a turning towards that side, due to the contraction of the longitudinal muscles. Thus, according to this worker, neither "weak" nor "strong" stimuli lead to a crossing over of the reflex to the opposite side. Also, both chemical and tactile stimuli lead to either of these responses, Pearl's positive and negative responses, depending on their strength.

In addition to these two groups of receptors on the proboscis and the anterior lateral regions, there are receptors on the posterior end of these

planarians. Strong stimuli of either kind cause a reflex "escape" activity pattern, with the "gliding" movement changing to a "crawling" movement. This reflex appears in an all-or-none manner, not showing differential reflex behavior to weaker stimuli.

It is a common observation that there is considerable variation among individual worms to standard intensity stimuli. This feature appears to be a commonplace of planarian behavior; not only do different individuals vary, but the same animal shows opposite responses on different occasions. Pearl suggested that this variation was due to varying "physiological or tonic conditions" of the organism. He believed that the function of the nervous system is to "preserve tonus," and claimed that if the "tonus" is gone it is very hard to get positive reactions. This interpretation would give an inhibitory or depressing rôle to the central nervous system. Against this idea is the result of removal of the "cephalic ganglion" of the polyclad *Leptoplana* (Hovey, 1929), which causes it to lose all tactile reflexes entirely, becoming less sensitive rather than more sensitive to stimuli. However, the noticeable differences between triclad and polyclad nervous systems (Turner, 1946) may make this seeming contradiction meaningless.

As in the case of annelids and mollusks, it seems clear that more must be learned about the reflex physiology of these organisms before an attempt can be made to explain their behavior patterns. How much of the behavior is dependent on the "cephalic ganglion" and how much is locally or regionally autonomous? Furthermore, can the modification of stimulus threshold by changes in the internal conditions, such as previous feeding (Pearl, 1902) or lack of "symbiont" *Hydra* nematocysts (Kepner et al., 1938), be explained without recourse to postulating an integrating central nervous system? By demonstrating a conditioned reflex in *Leptoplana,* Hovey (1929) apparently showed that the "brain" of these animals has the necessary functional complexity. His localization of this conditioning in the cephalic ganglia is not convincing, however.

For three distinctly different sorts of reasons, the coelenterates differ from the other phyla that we are considering here. First of all, they have no well-defined nervous ganglia to exercise overriding control of their nervous system.[1] Secondly, we have reason to feel closer to an analysis of their behavior in physiological terms than in the case of other phyla; in part, this is simply an expression of their *apparent* simplicity, but it is also a tribute to the work of many famous biologists, among them being Ro-

[1] The marginal sense organs of scychozoans may be synaptic areas of a sort. In a series of important papers, Horridge (1955a, b) presents evidence for the independence of two conducting systems in hydromedusae, interconnected with *polarized* synapses. He also suggests that "... the ring nerves, which have ganglion cells all along their course..." cause inhibition of swimming activity during feeding activity (1956). Thus the "ring nerves" may be acting as an integrating center in these jellyfish.

manes, Loeb, Parker, and Pantin. Finally, these generally carnivorous animals are either, for our purposes of the moment, sessile forms, or *passively* floating or swimming organisms; in other words, they do not "hunt down" their prey. The well-known "fishing" behavior shown by certain medusae, the trachyline medusa *Gonionemus* (Yerkes, 1902) for example, seems more nearly related to the "slowly" waving tentacles of sea anemone or hydra than to any example of directed hunting.

Pollock (1883), a colleague of Romanes, appears to have been the first to recognize that anemones respond to weak chemical stimulation. His observations were confirmed and extended by Nagel (1892), Loeb (1918, for résumé), Jennings (1906), and Batham and Pantin (1950a,b). The

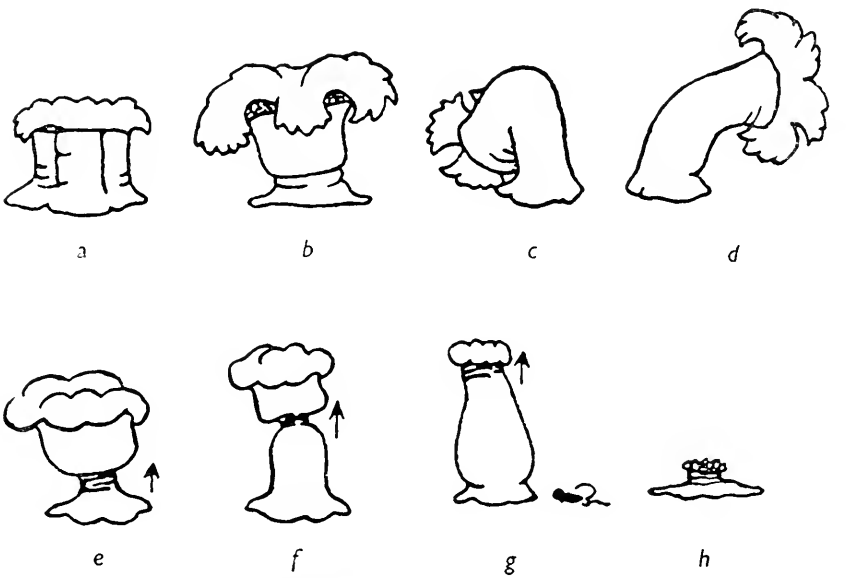

Fig. 1. Appearance of *Metridium* during various phases: a, specimen ingesting food; b, typical appearance ¼-½ hour after food ingestion; c, d, swaying movements 6 hours after food-extract stimulus; e, f, g, successive stages in antiperistaltic constriction, leading to defecation; and h, "shrivelling." From Batham and Pantin, 1950b.

latter authors, in giving us the most complete available analysis of the various activities of sea anemones we have, in *Metridium,* differentiated different "phases" of "inherent activity"[2] that may follow feeding or *stimulation by food extracts* without subsequent reward. As a result of

[2] By "inherent activity" these authors mean that "...the activity is an observed property of the animals which does not arise directly from external stimuli" (1950a, p. 299). It is clearly not a simple chain reflex.

chemical stimulation, the animal may (Fig. 1) first of all expand the disc, then sway, then show a lengthy parietal contraction, then distend, then defecate, and finally "shrivel." Here, then, we find an elaborate sequence of behavior "phases" which at their start, at any rate, show apparently purposeful food capture behavior. Is it any wonder that earlier workers often wished to endow these organisms with rudimentary intellect?

The actual capture and swallowing of prey is largely effected by the anemone's so-called (Parker) "independent effectors," the nematocysts, cilia, and mucus glands. All of these respond to chemical stimuli from the external environment (Parker, 1917a; Pantin and Pantin, 1943), although it is probable that nematocysts, at any rate, must also be tactually stimulated. Moreover, weak tactile stimulation of the tentacles may also be involved in moving food towards the mouth.

Most anemones protect themselves from forms preying on them simply by contracting into a compact mass, and sometimes by extending nematocyst-armed threads, the acontia. A few anemones will release their foothold and move slowly away if stimulated for a relatively long time by "strong" stimuli. Clearly, the terribly potent nematocysts are the chief protection these animals have against their enemies. Yet how is their behavior, and how is the discharge of their nematocysts, controlled so as to differentiate between foe and food? Ewer (1947) demonstrated that those types of *Hydra* nematocysts that are used for defense have their tactile threshold raised by food extracts, whereas the threshold of the discharge-triggering mechanism of prey-catching and prey-holding nematocysts are made *more* sensitive to weak mechanical stimuli by food extracts (see also Pantin, 1942). "Integration" of the sensory information thus occurs immediately at the sense cell-effector level.

For sensory input that goes into the anemone's nervous system there must be some sort of coding device. As in the planarians, "weak" tactile or vibrational stimuli elicit food capture behavior, "strong" stimuli cause withdrawal. On the tentacles of *Calliactis*, for example, very weak stimuli will cause a slow discharge of action potentials. Decremental conduction in the tentacle-disc region will prevent many of these spikes from reaching the synapses into the through-conduction-system, since these synapses require facilitation—that is, two or more impulses must reach the barrier within a few seconds—only an occasional impulse will be initiated in the through-conduction-system.

These occasional spikes are enough to initiate phases of "inherent activity" (Batham and Pantin, 1954), to start the expansion of the disc, or the swaying response, for instance. They are *not* enough to cause the powerful "quick-closure" protective response, since the innervation of these muscles is also protected by facilitation requirements.

Pantin and I (Passano and Pantin, 1955) recently demonstrated the great sensitivity of the disc and tentacles to a local pressure change. This is detected by the mechanoreceptors (whatever element they may be). Stimuli that are insufficiently "strong" or frequent enough to penetrate to the through-conduction-system do cause waving movements of the tentacles. On the other hand, tactile receptors of the column and foot regions are adapted for use as danger indicators. They are gastrodermal, not epidermal, and the cartilage-like mesoglea of *Calliactis* prevents them from being excited by moderate stimuli. Small crustaceans can often crawl over the column without causing any response. Strong stimuli, such as a vigorous poke by a large crab, will discharge the receptors. The resultant excitation immediately gets into the through-conduction-system (since there probably is no facilitation barrier), and, if further action potentials get into the system within a few seconds, a fast "protective" closure of the disc spincter muscle occurs.

Chemical stimuli have the same general effects on sea anemones; but, since the substances cannot penetrate the column epidermis and mesoglea, the extra-oral portions of the animal are remarkably insensitive. Only the most concentrated solutions are sufficiently strong to lead to contraction of the "withdrawal" muscles. Yet in a recent series of observations Yentsch and Pierce (1955) showed that *Stomphia coccinea* responds violently to a substance from the surface of a number of starfish, including *Hippasterias* and *Dermasterias*. I recently noted (Passano, 1955) that *Epiactis* is also very sensitive to this substance. The tentacles and disc (of both) are most sensitive, as would be expected, but stimulation of the column also elicits responses. This, of course, is immediately reminiscent of the sensitivity shown by mollusks to starfish, but it is not known whether the active agent is the same. *Stomphia* shows a striking series of motor reflexes that are initiated by this stimulus—thrashing back and forth, becoming rigidly distended, and detaching itself from the substrate. Sund (1955) has recently shown that exactly the same sequence of reflexes can be caused by a quick series of six or eight electrical stimuli, perhaps one second apart. It thus seems reasonable to conclude that some strong facilitation barrier must be broken down by a series of spikes in the through-conduction-system, but that, after that, one motor activity triggers another in a continual sequence.

Another such specific chemical sensitivity, but this time evoking feeding reflexes, has recently been demonstrated by Loomis (1955a,b). He has shown that 10^{-6}M solutions of the tripeptide glutathione cause *Hydra* to show vigorous and persistent feeding responses and, furthermore, that this substance leaks out of *Daphnia* after these Cladocera have been punctured by nematocysts. There is some evidence that glutathione is also a specific stimulating substance for other hydrozoans, but a few preliminary trials

done in the 1955 invertebrate course at Wood's Hole failed to show any obvious specificity in anthozoans. This difference might possibly be linked to the apparent differences in the nerve-net properties of *Hydra* and those other members of the phylum that have been investigated (see Pantin, 1950); on the other hand, it may be that other fresh-water hydrozoans are also glutathione sensitive.

A number of different coelenterates have "phases" of rhythmic feeding activities. According to Jennings (1906):

> ... green Hydra, undisturbed, show rhythmic activity. Every minute or two it contracts and then extends in a new direction. In this way the animal explores the region about its place of attachment and largely increases its chances of obtaining food. This motion seems to take place more frequently in hungry individuals, while in well fed specimens it may not occur.

Also Torrey (1905) and later Parker (1917b) showed that the solitary hydroid *Corymorpha* shows a "nodding" behavior every 2½ to 3 minutes, moving the hydranth down to the substrate by bending the stalk and then pulling the tentacles along the bottom. This activity is suppressed by causing water to flow over the tentacles, but is continued by both decapitate stalk and isolated hydranth, although at a *slower* rate, when these parts are isolated. Here it would look as though inherent rhythms of nervous activity, in each part, reinforce each other in the intact animal, while higher levels of activity, such as that caused by continual tentacular stimulation, inhibit the rhythmic pattern. One would expect that it would be valuable to analyze these cases more closely in the way Batham and Pantin have investigated *Metridium*.

The most pressing general problem that needs to be attacked experimentally and analytically is: How are these "inherent activity" phases initiated and modified by changes in the environment, both external and internal? The truth would seem to include both Parker's (1917b) "... behavior is chiefly determined by their immediate environment," and Jenning's (1906) "... complex movements and changes in movement may occur from internal causes, *without any change* in the environment" (my italics). Immediate environmental stimuli not only have a direct effect on actinian behavior but also lead to slow long-lasting changes in "inherent activity" and in the "phase" of activity. We must find out how these environmental changes are detected by the organism and what are the corresponding changes in the level of nervous activity. To put it another way, what slow changes in the "background level" of nerve-net-activity occur? Some coelenterates may have inherent "built in" background level maintainers, such as *Corymorpha,* the green hydra, and possibly scyphozoans with tentaculocysts; and other forms may depend on external stimuli, such as tide-pool currents (Parker, 1917a) for *Metridium*

or the "sympathetic caressing" tactile stimulation of commensal fish described by Gohar (1948).

Finally, I would like to suggest that the study of such problems is not only of interest to those invertebrate physiologists studying coelenterates, flatworms, annelids, or mollusks, but may be valuable also to the comparative physiologist and the comparative ethologist. In spite of the obvious differences in the physiological mechanisms concerned (Pantin, 1950), there are striking analogies between the behavior patterns that I have described and the innate behavior patterns studied by such workers as Schnierla and Van der Kloot in insects and Tinbergen and Lorenz in vertebrates. The latter author, in discussing the "innate behavior patterns" that he has studied so carefully, points out (1950) that, while they are superficially reflex-like in being triggered by specific stimulus situations, they are actually indistinguishable from what von Holst calls "automatic rhythms." The threshold of a starving sea anemone to external stimuli capable of initiating "phases" of feeding activity gradually decreases, eventually to the point at which such phases begin in the apparent absence of any special stimulus; this seems strictly comparable to Lorenz' cases where "Captive animals, deprived of the normal object or releasing situation . . . will persist in discharging the same sequences of movements at a very inadequate substitute object or situation."

It will be of the greatest interest if we can further analyze the different integrating mechanisms presented to us by these animals, which either have no central-nervous-system "black box," or whose "black box" is so *relatively* accessible to investigation and so *relatively simple*.

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PREY CAPTURE IN MANTIDS*

HORST MITTELSTAEDT
Wilhelmshaven

The problem of absolute optic localization is one of the earliest discussed in human psychophysiology, and one which was disregarded for the longest time in the physiology of the invertebrates. The neglect may be merely due to the fact that there are only a few cases in the invertebrates where that special question can be asked, one of these being the case of the praying mantid.

But let me explain the problem in the human case first. If you are to pick up, say, a pencil lying in front of you on a table and if you have time enough to do that without a rush, you not only see the pencil but your hand too. Therefore, you merely have to move your hand in such a way that the difference in position of these two observed things will disappear. The situation will change fundamentally if you are not allowed to see your hand or if movement goes too fast, as, for instance, in playing tennis, hammering nails, or throwing a ball into a goal. In such a case it normally will not be possible to correct the movement once started by watching the difference between its direction and that of the goal. Consequently success here depends upon information about the direction of the goal only. We certainly may assume our optic centers to be able to transmit a signal pattern representing the directional component of the retinal image concerned. Thus these centers can be expected to provide information about the direction of the goal relative to the eyeball. But, of course, that does not necessarily mean information about the direction of the goal relative to the body. For we can move our eyes and our head. It's fairly clear what should be concluded: the message steering the movement of the hand should contain information about the position of the eyeball and of the head too. How this information is gained in fact is the question—discussed in human psychology since the times of Helmholtz (1866)—with which I shall deal in the related case of the mantid.

Mantids, lying in ambush all day, detect their prey by means of their well-developed compound eyes. The prey is faced by movements of the head, the eyes being firmly attached as in all insects. If the prey is the proper distance away, it is captured by a sudden stroke of the forelegs. Two

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facts should be noted: First, the stroke has a time duration of about 10 to 30 milliseconds. Because of that short interval it should hardly be expected that the stroke is controlled by watching the difference between its direction and that of the prey. Later we shall show evidence that, if present at all, such a control at least is not effective. Second, though the animal tends to bring its head and its prothorax into one line with the prey, it is able to hit a prey which has a considerable lateral deviation from the median plane of the prothorax.

For these reasons the problem seems to be quite similar to the human case. The direction of the stroke must be determined by a message representing the direction of the prey relative to the prothorax. Consequently, that message should not only contain information about the direction of the prey relative to the head, but of the position of the head too. It may be supposed that the first information is provided by the compound eyes and the second by proprioceptive sense organs which are able to present a message about the position of the head. And indeed mantids possess a well-developed system of neck receptors, and I shall examine these sense organs first. But before going into details, I want to make some general remarks.

As already indicated by the way I have introduced the problem, it is the functional organization of a system as a whole I want to understand. Consequently, though I shall take advantage as far as possible of what is already known about sense organs, effectors, and nerve cells, the aim of this investigation is not—or at least not primarily—to learn more about single elements. In the present case, as we shall see later, we are dealing with four quite distinct functional units. My task will be to discuss how they act together and thus to explain the performance of the whole system by the properties of its parts and their functional interrelations.

This being clear, I should say a few words about the necessary limitations and restrictions. First, I shall deal with the orientation problem only and omit all ethological questions about—for instance—appetitive behavior, drives, and releasing factors. Second, though the learning problem will be touched upon, we shall be concerned with the functioning of the fully developed mechanism in the adult animal only. Third, perfect localization includes an indication of direction and of distance as well. I shall deal with the direction problem only, and only with a special part of it, namely, the orientation in the main horizontal plane.

First Series

Now we are ready to start with the first experimental series planned to throw light upon the role played by the proprioceptors. The neck receptors of mantids are of a well-known type. There are two pairs of hair plates each studded with from tens to hundreds of hair sensillae (Fig. 1).

The sternocervical plate was found by Pringle (1938), the tergocervical plate by the author (Mittelstaedt, 1952). There is another very small group of hairs at the pro-episternal wall of the prothorax which I shall not mention further, because its functional influence can be neglected.

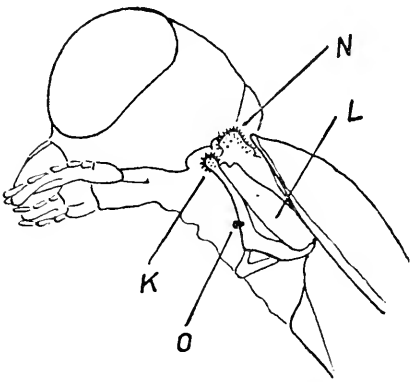

Fig. 1. Proprioceptors of the neck region (left side). K = sternocervical hair plate, situated on the anterior end of the laterocervical sclerite (L). N = tergocervical plate. The common afferent nerve of both organs is cut at the ventral border of the laterocervical sclerite. (O).

The external physical mechanism of these hair plates is easily understood. In the normal position of the head some of the hair sensillae are bent down by the posterior wall of the head. If the head is turned horizontally, say to the left, some more hairs on the left-side plates are bent down, while on the right side a correlated number are set free and erected elastically.

As Pringle (1938) has demonstrated in *Periplaneta,* bending down a hair causes an increase in the impulse frequency of the afferent nerve, which, after an initial peak rising and falling within a few seconds, remains constant in time. Thus each position of the head causes a correlated pattern of nervous activity, and the system is indeed able to take up information about the position of the head as has been demonstrated by the author in dragonflies (Mittelstaedt, 1950).

The first experimental series consists of four sets. The performance of the animal in hitting prey has been examined, (1) after cutting off the sensory nerves of the hair plates, (2) after giving the head a fixed position relative to the prothorax, (3) after a combination of (1) and (2), (4) after having loaded the head of the otherwise undisturbed animal by an extraneous mechanical force.

In all four cases a statistical method was used to get quantitative results about the hitting performance. The animals (I have worked mainly with *Parastagmatoptera unipunctata* from Argentina) were sitting free on the gauze ceiling of the cage in which they were normally kept. Then as many flies (I have used *Calliphora* and *Lucilia*) were brought in as the experimenter and his assistant could easily observe while they wrote down what

happened. The number and direction of misses, the number of hits and their bias, that is, whether the prey was hit by the two forelegs or by one only, were noted. Thus we shall characterize the hitting performance by two quantities, first the frequency of misses and the second the left or right tendency calculated from the frequency of biased misses and hits. With regard to the latter quantity it is important to note that, by definition, left tendency plus right tendency always equals 100%. Thus, if right tendency is 100%, left tendency is zero; and if right tendency is 50%, there is no bias at all. (I should add here that, in calculating the values of some of the diagrams—Figs. 2 and 5—I have used a different statistical procedure than the usual one. The difference will be less than 1% if number of strokes is 98 or more and thus will be unimportant in most cases.)

I shall begin with the effects of eliminating the hair plates by nerve section, and shall confine myself to the results of the bilateral total deafferentation. In experiments carried out with 5 individuals, the frequency of misses increased from 10-15% in the normals to 70-80% immediately after this operation. There was only a relatively small improvement in the following weeks. On an average, this may be even smaller than in the example shown in Fig. 2, where it is partly due to the chances of an accidental bias. If you are watching these animals you gain the impression that they only have a chance to hit flies sitting straight ahead of the prothorax.

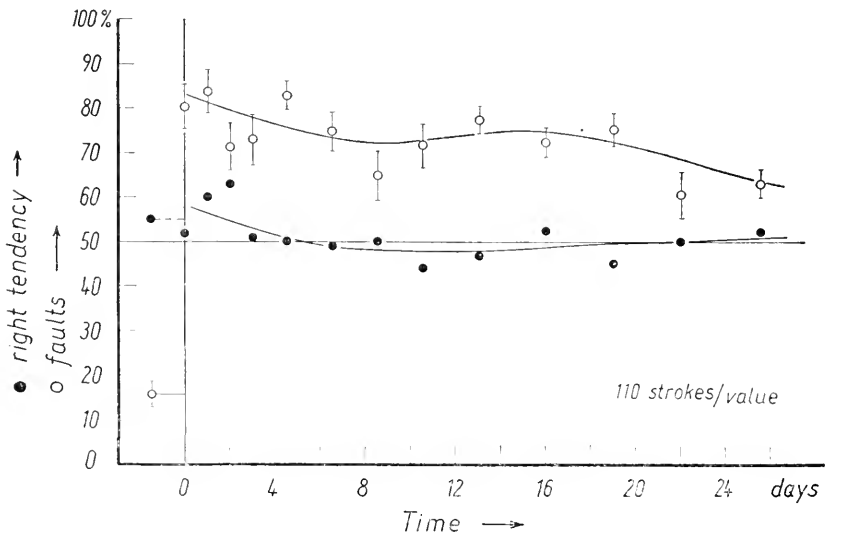

Fig. 2. Result of total proprioceptive deafferentation in two individuals. Ordinate: frequency of misses (open circles) and right tendency (solid circles), respectively. Abscissa: days after nerve section; the performance recorded before operation is shown left from the 0-line. Thin vertical lines: standard error.

Normally flies sitting on the right side are missed to the left and vice versa. I shall discuss these results later.

Next the head was given a fixed position relative to the prothorax by what may be called a little bridge of balsa wood fastened on both ends with paraffin, so that the neck region was not touched at all. Fig. 3 shows the effect of a head deviation of 10 to 30 degrees to the left (in 6 animals).

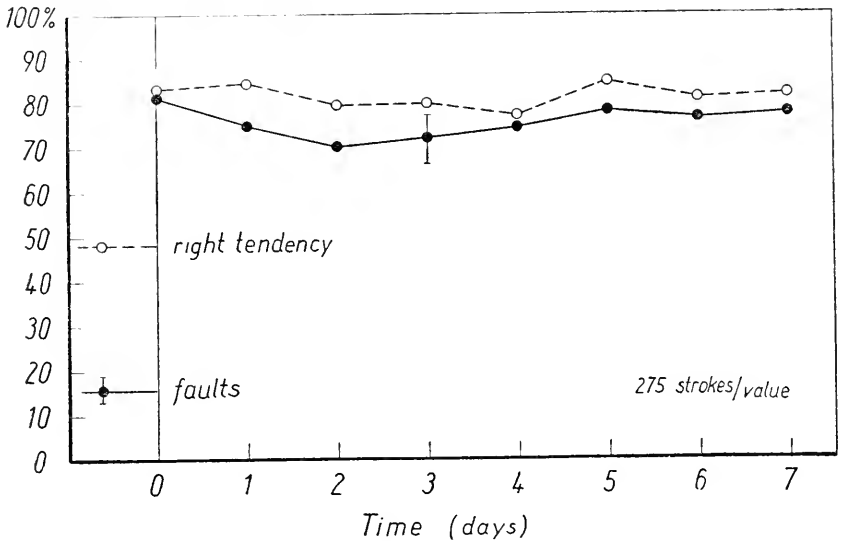

Fig. 3. Result of head fixation 10 to 30° to the left (6 individuals). Ordinate: frequency of misses (solid circles) and right tendency (open circles), respectively. Abscissa: days after fixation; the performance recorded before head fastening is shown left from 0-line. The limits of expectation, each based on a P-level of 0.025, are plotted in two of the values.

There is a large bias to the right now and a frequency of misses of about 75%, remaining constant for at least a week. There may be a slight improvement of about 10% the following week, but in general we get another proof of the small learning capacity of the mantid. If the head is fastened near its normal median position the animal has a smaller bias and a better achievement. In some cases where the exact median position was reached by chance, the bias was zero and the frequency of misses normal.

In the next set, head fastening and deafferentation were combined. The result just presented was obtained even in individuals which had undergone total proprioceptive deafferentation, suggesting that the effect of the operation is cancelled if the head is fixed. In order to get a more rigorous proof of this, the proprioceptors were eliminated on one side only. For in the controls, without head fastening, this intervention causes not only a

large decrease in hitting performance but also a strong bias, namely, a frequency of misses and a right tendency of 80-90% within 24 hours after operation on the left side. The experimentals were first tested with the head fastened only, and then operated unilaterally leaving the balsa bridge intact. The result is seen in Fig. 4. There is an effect of the additional operation and in the same direction as in the controls. The effects of the constant head deviation and of the unilateral nerve section are clearly superposed. The relative importance of the components can be estimated from the fact that the bias caused by eliminating one-half of the proprioceptors is completely compensated for by a mean head deviation of less than 20° (see left column of Fig. 4).

r-tendency caused by fixation	 20 - 40 %	 ~50 %	 60 -80 %
	before op. *after*	*before op.* *after*	*before op.* *after*
r-tendency	30,7 → (47,4)	50,0 → (78,3)	74,4 → (88,4)
faults	(39,1 → 46,8)	23,4 → 71,8	(62,5 → 95,0)
n of strokes	202 156	175 124	197 60
of animals	4	4	2

Fig. 4. Result of head fixation combined with unilateral proprioceptive deafferentation classified into three groups according to the bias caused by head fastening only. A right tendency of 20-40% (left column) corresponds to a mean head deviation of <20° to the right; an r-tendency of 50% corresponds to the median head position; an r-tendency of 60-80% corresponds to a mean head deviation of <20° to the left. The mean values obtained with head fastening alone are shown under "before op.," those obtained within 24 hours after an additional section of the left nerve under "after." Left nerve section without head fixation (controls—not shown in the table) causes a frequency of misses and a right tendency of 80-90%.

In the fourth and last set of this series I loaded the head of the otherwise undisturbed animal by an extraneous mechanical force. A small stick of balsa wood was gummed on the posterior wall of the head and then a small ball of plasticine of known weight was fastened on the stick at a distance of about 10 mm. Of course the animals had to be tested sitting on the vertical wall of their cage and even then we only counted the strokes executed while the prothorax had a vertical position.

The result is seen in Fig. 5. With the exception of one series carried out with two individuals which, even in the unloaded state, had a considerable deviation from the normal frequency of misses, a significant decrease in

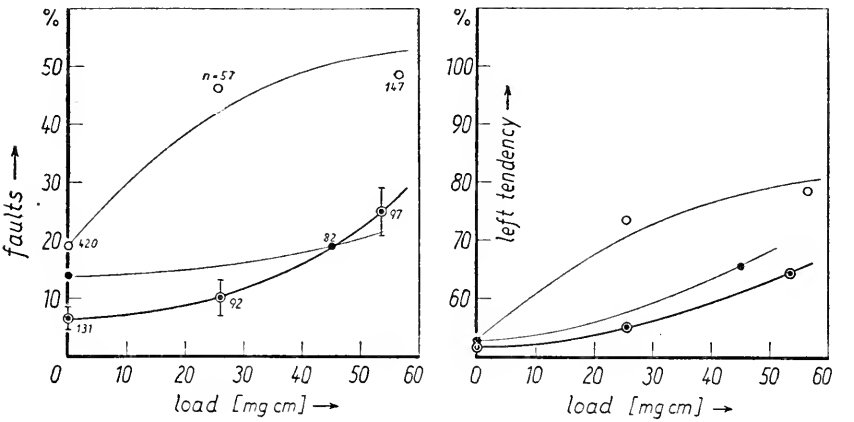

Fig. 5. Result of loading the head with an extraneous mechanical force. The angular momentum caused by the load is so directed as to turn the head to the right (for method see text). Ordinate: frequency of misses and left tendency, respectively. Abscissa: angular momentum in mg.-cm. (mg. here is a unit of force, not of mass). Solid circles, first experiment (2 individuals); open circles, second experiment (3 individuals); double circles, third experiment (3 individuals). In the last named the standard error is plotted. Figures at the values: number of strokes (n).

performance is reached not earlier than at a load of 50 mg. cm. That is a remarkable achievement if we consider that the head of *Parastagmatoptera* weighs 25 mg. and hence there is a load of twice the head weight at a distance of twice the head diameter. Thus we may be certain that loads which can be expected to occur in normal life (for instance, if the animal is catching the second fly while eating the first) are perfectly ruled out by the mechanism at hand.

Introduction of an Hypothesis

Now we have gained sufficient information to put forward an hypothesis concerning how the mechanism works. If we only had to account for the first result, of deafferentation, we would assume that the hair plates provide for the disputed additional information about head position. If we knew only the second result, of head fastening, we certainly should be advised to the contrary, namely to assume that there is no such influence at all. But it may be concluded from the third that the truth will be somewhere between; and the last result could give an idea of how this compromise is expected to work.

Evidently the mechanism is very sensitive to or easily upset by changes

in head position which cannot be corrected or, as can be said as well, to deviations from the intended head position. Removal of the hair plates causes a sharp decrease in achievement, but the effect is eliminated or even reversed simply by fastening the head in a suitable position; thus it must be assumed that these organs are involved in a system which controls the head movement—in a system which provides for the intended head position to be reached in fact.

To give a precise form to this hypothesis, so that conclusions can be derived which can be tested further by experiment, I shall take advantage of methods and theories recently developed to explain the functioning of systems like that with which we are dealing. I mean the modern theory of automatic control and of control systems in general developed during the last decades to a high level of precision and universality. I shall make an attempt to apply that concept to the system which in mantids controls the movements of the head.

If a prey comes into sight, the mantid turns its head to face it. Thus there must be a functional unit which transforms the position of the prey relative to the compound eyes into a central nervous representation of that position. The latter must be transmitted to the centers controlling the neck muscles, which then start a head movement determined by that message with regard to direction, amount and/or speed. But as soon as the head changes its position, the optic message is changed too, so that the output of the physiological pathway must necessarily influence its own input. To make that completely clear, I shall plot it diagrammatically (Fig. 6a).

I shall define all directions or positions by the angular deviations from the median plane of the organ concerned, the axis of reference for all angles being the vertical axis of the head movement. We have to distinguish (1) the angle between the prey and the head, that is the optic input ϕ; (2) the angle between the head and the prothorax μ, that is the neck motor output and the proprioceptive input as well; and (3) the angle between the prey and the prothorax σ. Then we should consider the optic control unit converting the angle ϕ into a central nervous message ϕ_c, and the neck motor unit which transforms ϕ_c into a head deviation μ. Since

$$\sigma = \phi + \mu$$

$$\phi = \sigma - \mu.$$

Thus, as indicated by the arrows, information is transmitted in one direction only, and flows within a closed loop, the output being negatively fed back to the input.

Now I shall take into account the final steady state of the system only—that is, the position after all movements have come to rest, all actions and forces being in complete equilibrium. If, for the sake of an easier introduc-

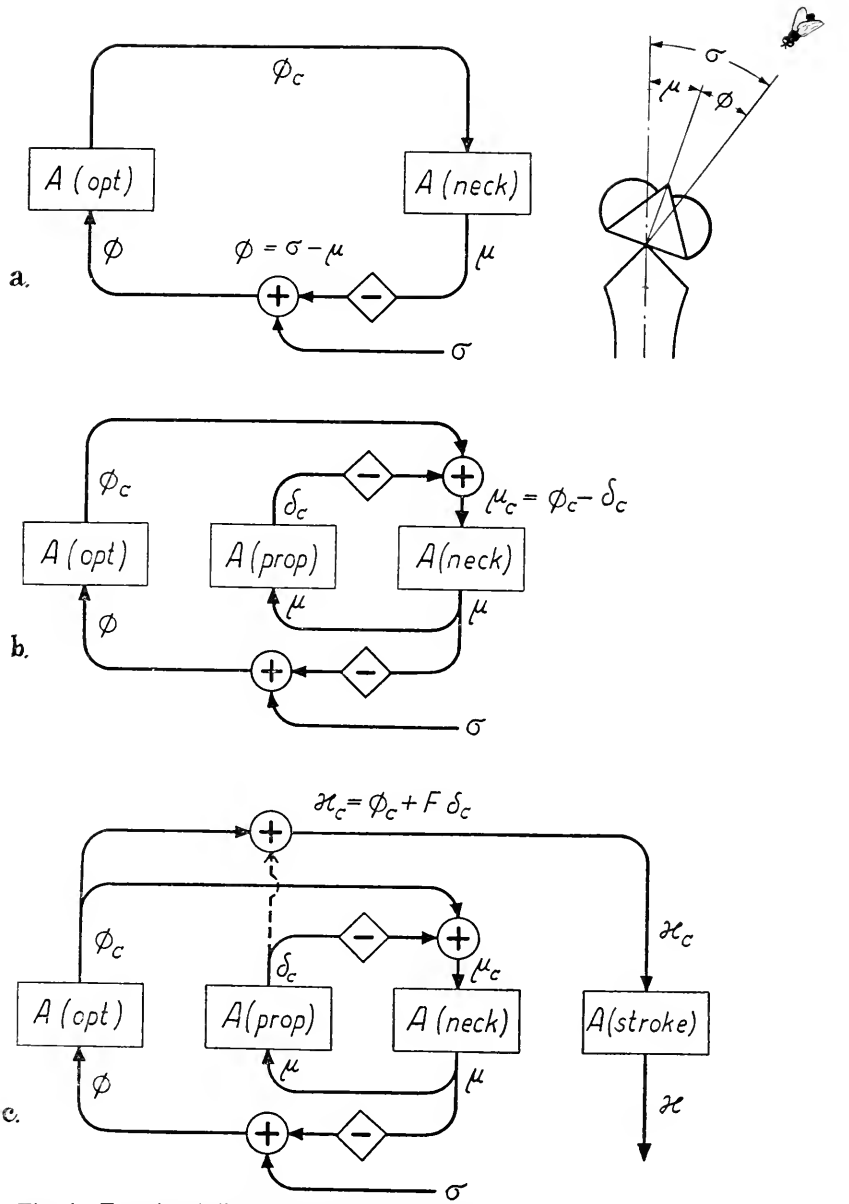

Fig. 6. Functional diagram of the mechanism underlying localization in mantids. The hypothesis is developed by steps from (a) via (b) to (c). (a) Optic feedback loop only. As indicated by the arrows, information flows from the optic unit (amplification factor: $A_{(opt)}$) to the neck motor unit (amplification factor: $A_{(neck)}$) and again to the optic unit. (b) Optic and proprioceptive feedback loops. The neck motor unit is controlled by the difference between the optic (φ_c) and the proprioceptive (δ_c) center messages. (c) Complete hypothesis. $A_{(stroke)}$ amplification factor of the central unit which determines the direction of the stroke (κ). For full explanation see text.

tion, we assume that there is a constant linear proportionality between ϕ and ϕ_e and between ϕ_e and μ, respectively, and if we define the proportionality factors as $A_{(opt)}$ and $A_{(neck)}$ respectively, then

$$\mu = A_{(opt)} \times A_{(neck)} \times \phi \text{ or}$$

$$\frac{\mu}{\phi} = A_{(opt)} \times A_{(neck)}$$

The ratio μ/ϕ will be named Ac (total), the total interior amplification of the control circuit. You may note that this is a pure number without any dimension. It may be worth while to give an example of how that works. Assume the system has an Ac (total) of 4, and a fly comes into sight at $\sigma = +20°$, that is 20° to the right. Then the fixation process will come to rest exactly at a head deviation μ of $+16°$, ϕ thus being $+4°$. For only with that optic input can the neck motor output be $+16°$, if we have 4 times total amplification. Thus in general:

$$\frac{\phi}{\sigma} = \frac{1}{1 + \text{Ac(total)}}$$

and the prey will be better centered as Ac (total) increases. The ratio $\frac{\phi}{\sigma}$ will be named the "fixation-deficit."

Now we are able to formulate the hypothesis, namely as follows: It is assumed that μ is converted by the hair-plate system into a message δ_e, and that the neck muscles are steered by the difference between the optic center message ϕ_e and the proprioceptor center message δ_e. Thus there are two circuits working together (see Fig. 6b).

In order to understand the operation of these circuits, let us watch the proprioceptive subcircuit acting in isolation. Suppose the head to be thrown out of its median position by some extraneous influence, say 20° to the right. Then the hair plates will transmit a message causing a head movement to the left. The system will come to rest at the smallest value of μ allowed for by the amount of the extraneous influence and the total amplification of the proprioceptive circuit. Therefore we may say that this circuit tends to minimize μ against all extraneous influences. Because the optic circuit—as we have just learned—tends to minimize ϕ, it can easily be seen that there must be a rivalry between the two systems, except only in the case that $\sigma = 0$, the prey being straight ahead of the prothorax—then ϕ and μ should be zero. In all other prey deviations neither the one nor the other circuit will reach its target. Thus under the conditions adopted the fixation-deficit must have a finite value, and hence, at equilibrium, there will be a constant correlation between the proprioceptive input, the optic input, and the deviation of the prey from the body axis. Consequently the optic and the proprioceptive center messages (ϕ_e, δ_e) must also have a

constant correlation to the deviation of the prey from the body axis; and this again means that *each of them—at equilibrium—contains the information which is required to determine the correct direction of the stroke.*[1]

Let us first assume that the direction of the stroke be determined by the optic center message only (Fig. 6c, without the dotted arrow). It may be useful to illustrate this by an example. For the sake of simplicity the direction of the stroke κ may be defined as the angle between the endpoint of the stroke and the median plane of the prothorax, the axis of reference being the vertical axis of the head movement. Then the prey will be hit if κ is equal to σ. Now consider the system has a fixation-deficit of 10%; that is—at equilibrium—the optic input equals $+1°$ if the prey deviates $+10°$ from the body axis, $+2°$ if the prey deviates $+20°$, and so on. Evidently the central units involved in the determination of the stroke merely have to amplify the optic input by a constant factor in order to hit the prey exactly.

Let us see now how this hypothesis fits the experimental results first presented.

(1) If the hair plates are eliminated by nerve resection on both sides, the proprioceptive circuit breaks down. Thus the optic circuit normally working against it will now be more effective in minimizing ϕ. Consequently there must be a smaller fixation-deficit than before. Because the factor by which the optic input is multiplied in order to steer the direction of the stroke is presumably not changed by the operation, the deviations of the stroke from the body axis will always be too small, except in the one case when the prey is sitting straight ahead. As we have learned, this is just what happens in fact.

(2) If the head is given a fixed position, say 20° to the left, then the two circuits are both blocked. Thus the performance will depend upon whether the animal succeeds in centering the fly by means of movements of the legs and the prothorax. If the fly is at least approximately centered that way, before the stroke is released, the optic input will be about zero, the stroke thus going more or less straight ahead. But since the fly then in fact has a mean deviation of 20° to the left, it normally will be missed to the right, quite in accordance with the facts. Yet it will be hit frequently, if the head is fastened in its median position. As we have learned, that is indeed so.

(3) The result of the combination experiment does not agree with the assumption that the stroke is determined by the optic-center message only. If the head cannot be moved, the additional elimination of proprioceptors should then have no additional effect at all. Consequently we must conclude

[1] To satisfy the rigorist, it may be added that this is true even if we leave out the linearity and proportionality conditions introduced at the beginning.

that there is yet another way by which the proprioceptors, at least in that special situation, can act upon hitting performance. The superposition effect indicates that the proprioceptive-center message is added to the optic-center message (see Fig. 6c, dotted arrow). Yet, there is strong evidence that the proprioceptive influence is smaller than the optic influence. Otherwise, if it were equal or larger, the bias demonstrated in the head fastening and in the loading experiments must be zero or even reversed in sign.

(4) Finally the loading experiment gives a good proof of the biological importance of the proprioceptive subcircuit, namely its ability to rule out disturbing forces which act upon the neck motor system. According to theory there should be no bias and no lowering of the hitting performance, as long as these forces are eliminated completely. As soon as the head begins to deviate from the intended position, say to the right, there would occur a left bias—quite in accordance with the facts. It should be noted that the subcircuit works not only against extraneous influences but also against changes in the normal state of the muscles, caused for instance by fatigue or any sort of metabolic disturbance.

Second Series

As the hypothesis put forward can be defined with mathematical precision, we should now make an attempt not merely to get knowledge about the connections between the basic units of the system as we have done up to now, but to determine quantitatively the functional relations predicted by the theory and, finally, to calculate the constants of the system. I think it is fairly clear what should be measured, namely the fixation-deficit in normals and after bilateral nerve cutting.

The plan of the procedure is simple enough; the prothorax is fastened, a fly is presented at a measurable direction σ, and, if the animal faces it, the position of the head μ is measured.

I have used two different techniques, one of which is shown in Fig. 7. The animal is sitting in its normal upside-down posture on a paper dial hanging by threads of silk. The weight of the animal is compensated by means of two pulleys and a counterweight not visible in the picture. A stick of balsa wood gummed on the head projects through a hole, loose enough to allow for rotation, and carries a pointer. Thus only the head movement was limited to rotation about the relevant axis, all joints were free, and the animal seemed to be comfortable even for weeks. In the second apparatus also the head was completely free, bearing a small thin piece of paper, which controlled the current of a photocell circuit. Finally, at the output of the device, the position of the head, of the fly, and of the thorax was registered automatically and continuously.

It should be mentioned that this experimental series is not yet finished,

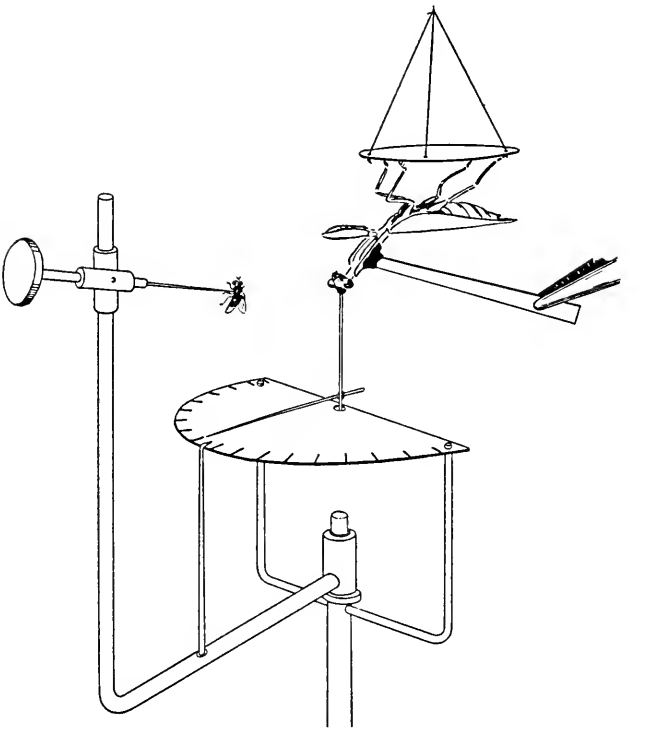

Fig. 7. Device for measuring the fixation-deficit. The animal is fixed at the prothorax, the head being free for rotation about the vertical axis. The deviation of the head and of the prey from the median plane of the prothorax are measured on the same dial.

only three animals being investigated in the first and another three in the second device. But because the result seems to be fairly interesting with regard to the problem as well as to the concept adopted, I feel I should not withhold it here.

It turned out that two quite different sorts of head movements occur in mantids, similar to the relevant eye movements in man. There are quick saccadic head movements, on the one hand, and smooth continuous movements, on the other. The first are observed if the prey appears and moves beyond a distance of about 30 mm.—that is, in *Parastagmatoptera*, nearly double the reach of the stroke. About 30 mm. distance both types are seen; if the prey comes nearer, it is followed by continuous movements only. One may be certain that, in the continuous movements, the optic control circuit is working in fact, but it must be doubted whether that be true within the short intervals of the head jerks. The time duration of the jerks

as a function of their angular size could not yet be measured exactly because of the time lag of the recording instruments. Motion-picture photographs showed a time duration of less than 80 msec. in jerks of 90°. Thus the duration of jerks smaller than 20° possibly come close to the latency of the optic circuit. If the jerk is executed without any feedback, whether optic or proprioceptive in origin, then the amount of the movement must be based exclusively upon the optic information present *before* the jerk starts. Consequently the position of the head, after the jerk has finished, should depend not only upon the position of the fly but on the initial position of the head too. Thus, if initial positions are variable, there should be no constant proportionality between the position of the fly (σ) and the position of the head (μ) at the end of the jerks, unless the jerks always

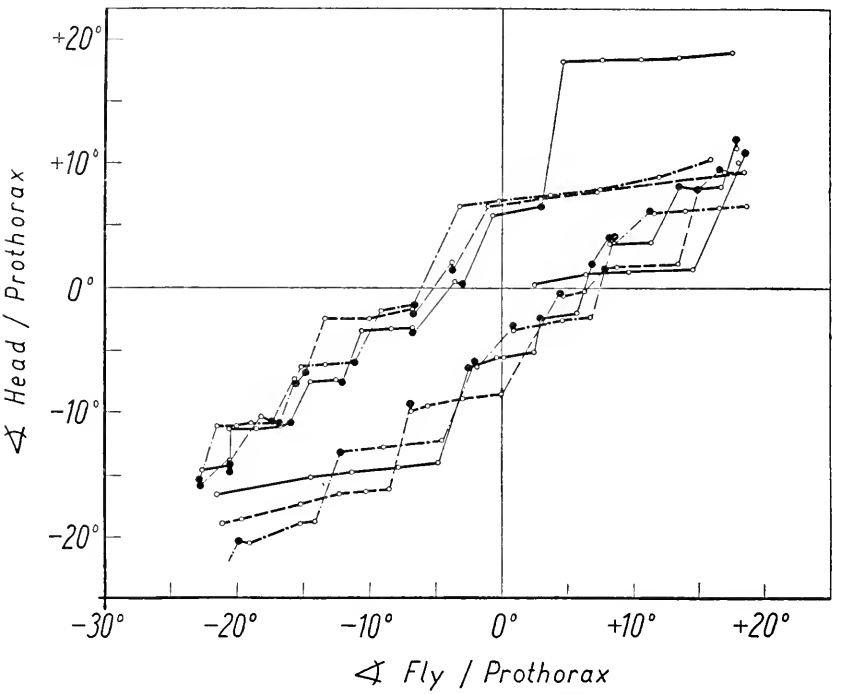

Fig. 8. Endpoints of fixation movements of the head plotted against the position of the fly which is faced. Ordinate: deviation of the head (μ). Abscissa: deviation of the fly (σ), both from prothorax median plane. +: deviation to the right; —: deviation to the left. The experimenter moves the fly in small steps from —25 to +25 and return. The procedure is repeated three times. Small circles: head positions reached by continuous movements. Large dots: head positions reached by jerks. Dots are grouped along the lower line, if the fly had reached the position concerned by moving from left to right; but they are grouped along the upper line, if the fly had moved from right to left. Distance from the fly to the head: 29 mm.

exactly reached their goal. We shall see immediately that, though the latter is not the case, there is a proportionality between μ and σ, but of a very astonishing form. In the example shown by Fig. 8, the fly is about 29 mm. from the mantis. There are both continuous movements and jerks, the endpoints of the jerks being more strongly marked. The continuous movements are weak and ineffective; but the endpoints of the jerks are grouped along two straight lines nearly equal in slope. The values lie on the lower line only, if the fly is moved from left to right, and on the upper line only, if it is moved from right to left. The distance between the two lines with regard to abscissa is about 9° (see Fig. 9). Nine degrees at a distance of

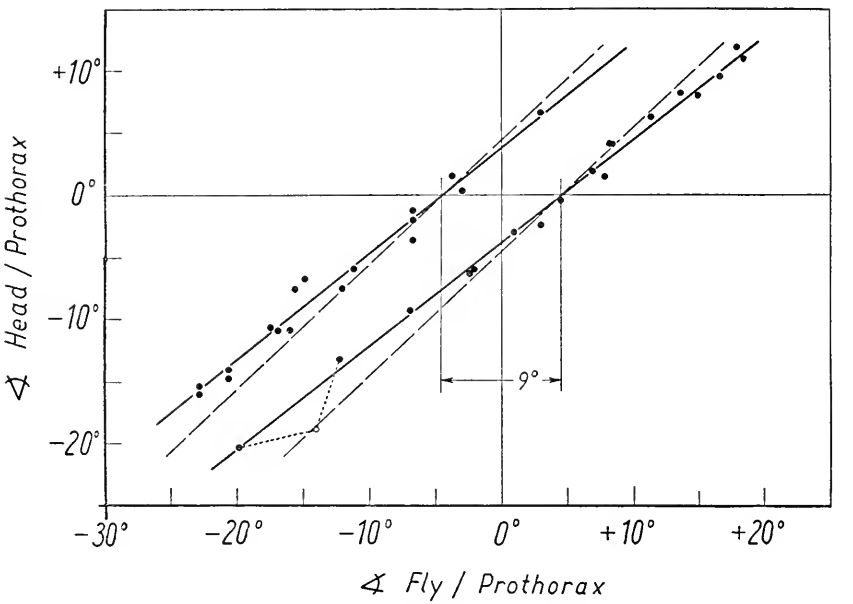

Fig. 9. The same experiment as Fig. 8 with head positions reached by jerks only. The slope of the upper full line (regression coefficient) is 0.85 ± 0.03, that of the lower full line 0.83± 0.01(6). The abscissa distance between these lines of about 9° corresponds to the angular width of the fly presented. Hence the upper and lower broken lines indicate the positions which the head would have, if the right and left sides of the fly were fixed exactly. Yet the differences in slope of full and broken lines are considerable (15 and 17%, respectively), and would be expected due to chance with a probability of less than 0.0005.

29 mm. is about 4½ mm. Four and a half mm. is about the mean horizontal width of the flies presented. Thus the simplest explanation is to assume that the animal tends to face the left side of the fly while the fly is going from left to right, and the right side if it is going from right to left. But in neither case was the side of the fly centered exactly. The slopes are 83 and

85%, the difference being insignificant. Yet the difference between each slope and 100% is highly significant. Thus I don't see how to avoid the assumption that, at the end of a jerk, the head has a position very near to that which it would have if the circuit had reached its final steady state.

In view of our problem it is particularly interesting what happens if the prey comes nearer to the reach of the forelegs. Here again, especially at the beginning of the experiment, a tendency to face the sides of the fly is observed in some cases. But in the period before the stroke is released, the picture normally changes (see Fig. 10). There are still two pathways, but

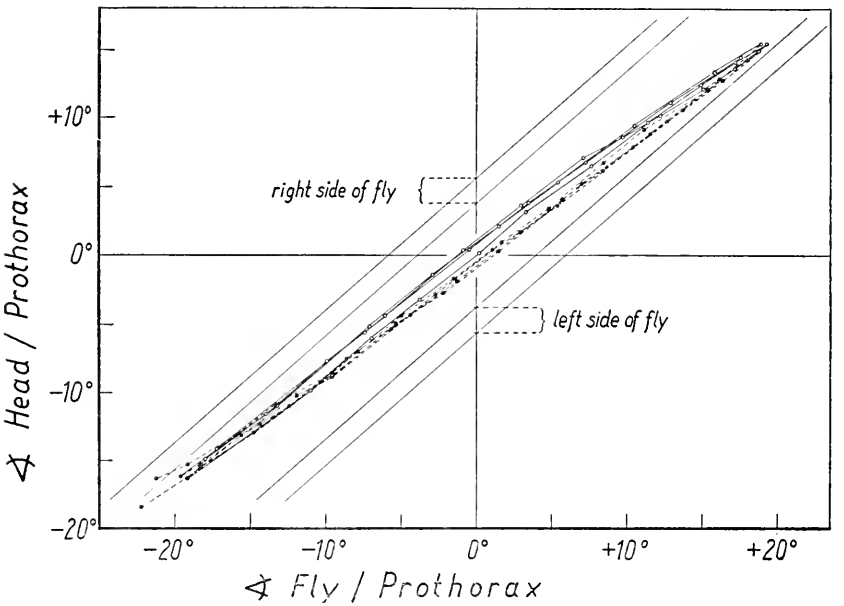

Fig. 10. Same procedure and same mapping as in Figs. 8 and 9, but the fly is presented at a distance smaller than 30 mm. The head followed the fly exclusively by continuous movements. Open circles and full lines: head positions while fly is moving from left(−) to right (+); solid circles and broken lines: head positions while fly is swinging from right (+) to left(−). The fixation-deficit is equal to 1 minus the slope of the curve and hence about 15%.

they differ by not more than about 2° and, if the prey moves from left to right, the head at $\sigma = 0$ is about one degree right from the center of the fly and vice versa, thus being slightly in advance. We get an estimate of the achievement produced here while considering the width of the fly and the fact that the best ommatidia of this insect have angular apertures of about one degree. The lines have slopes of 82 to 88%, in accordance with the first result. Thus, the fixation-deficit being about 15%, the total circuit amplification can be calculated to be nearly 6.

Unfortunately the experiments planned to give information about the fixation-deficit after eliminating the hair plates were executed before I knew the distance rule mentioned and the fact that the jerks normally are directed to the sides of the prey. For mere technical reasons the fly (in the first apparatus) was presented at a distance of more than 30 mm. and hence faced by saccadic movements almost exclusively. The head deviations (μ), if plotted against the fly deviations (σ) showed a considerable spread and, at first sight, it seemed impossible to detect whether the mantis had faced the left or the right side of the fly. The difficulty was overcome by considering the way the data were obtained. As the fly normally was moved from the median position ($\sigma = 0$) to the right or left sides respectively ($\sigma = \pm 60°$) while the reverse occurred very rarely, it should be assumed that, in the majority of presentations on the right side of the mantis, the left side of the fly (as seen from the mantis) was faced and vice versa. Hence the regression function of head deviation μ on fly deviation σ, if calculated for each side separately, should result in two parallel regression lines separated by an abscissa value corresponding to the width of the fly. Furthermore their slopes should be larger than the one calculated from the total set. This was indeed so in 3 out of 4 sets. The result is given in the following table:

SLOPES OF THE REGRESSION FUNCTIONS OF HEAD DEVIATION (μ) ON FLY DEVIATION (σ) FOR TWO INDIVIDUAL MANTIDS

Individuals	Total Slope of the Regression Function of μ on σ			Mean of the Isolated Left and Right Slopes	
	Normal	Deafferented	P	Normal	Deafferented
I	0.72 ±0.018	0.89 ±0.014	<0.0002	0.72 ±0.018	0.99 ±0.04
II	0.79 ±0.009	0.88 ±0.015	<0.0002	0.91 ±0.02	1.02 ±0.08

It can be seen that

(1) In all cases, the slopes obtained after bilateral proprioceptive deafferentation are larger than those obtained before.

(2) In both individuals tested, the differences between the total slopes before and after operation are highly significant with $P < 0.0002$.

(3) The means of the left and right slopes (with one exception probably due to an unfavorable accumulation of σ values around the median position) are about 10% larger than the totals.

(4) The left and right slopes after deafferentation are close to unity.

Discussion

It has been demonstrated in the second series of experiments that, at the time before the stroke is released, the prey is faced by a continuous optic feedback process. At equilibrium, the fixation line does not center the prey but deviates from it by an amount proportional to the angles between the prey and the median plane of the prothorax. So far the assumptions made by our hypothesis have been confirmed. All further conclusions from this series, and their interpretation in relation to the problem, depend upon whether it can be ascertained that the saccadic head movements are controlled in essentially the same way as the continuous ones or not.

The first two results (1, 2) of the last experiment obviously corroborate the assumption made before, that the saccadic movements too are controlled by feedback processes. The result not only shows that there is an effect of the proprioceptors on head movement, but it shows just that sort of influence which the theory predicts in the case of two counteracting control circuits. For, if the proprioceptive circuit be cut off, the optic circuit should be more effective in minimizing the deviation of the prey from the fixation line ϕ, and hence the ratio μ/σ (since $\phi = \sigma - \mu$) should be larger after operation than before.

Yet, if we compare the larger spread of the head positions reached by saccadic movements with the negligible fluctuations of those reached by continuous ones (cf. Figs. 9 and 10), it is fairly clear that the endpoints of the jerks are not identical with the values which correspond to the final steady state of the feedback process. Consequently, at least one restriction must be made, namely, that in the jerks the feedback is blocked before the equilibrium is reached. As an alternative hypothesis, it could be assumed that a jerk is a simple reflex initiated by the deviation of the fly (ϕ), and not at all controlled by the effects of its output. But then, as already mentioned in the last section, the linear regression functions of μ on σ obtained in all these experiments would be difficult to explain. Thus, the data available at the present state of the analysis are best fitted by the assumption that both sorts of head movements are controlled by optic and proprioceptive feedback, though the dynamic qualities of the two systems may be fundamentally different.[2]

[2] Future analysis may reveal that the continuous process operates near the theoretical optimum of velocity and dynamic stability, so that any further enlargement of speed (in the jerks) would cause overshooting and oscillation, were the circuit not interrupted from time to time. Such a dynamic dichotomy in the ways of fixation seems to be plausible from a biological point of view; the pursuit of a moving prey which is far beyond the reach of the stroke demands quickness but no precision. The reverse is true if, after a long stalking, the direction of the stroke has to be determined.

With these restrictions born in mind, I want to discuss some of the further consequences of the second experimental series. The result last presented (4) indicates that the endpoints of the jerks after deafferentation are distributed at random around the true points of fixation (the left or right side of the fly). If the same occurs in the continuous movements, so that the fixation-deficit after deafferentation is zero, some interesting conclusions can be drawn on the qualities of the system. For then either the optic or the neck motor unit must be an "integral action controller," that is, the output of the system depends on the time integral of its input, and hence only comes to rest if the input is zero. Evidently the neck motor unit operates this way and the optic unit does not; otherwise there would be no fixation-deficit in the normal mantis. Consequently, since the input of the neck motor unit is controlled by the difference between the optic and the proprioceptive center messages, both must be equal at equilibrium. And this again means that all disturbing forces acting upon the neck motor system are completely ruled out within the limitations set by the capacity of the muscles. It should be noted that this is supported by the result of the loading experiment (cf. first series). Thus, even if future research should show that the fixation-deficit after deafferentation slightly differs from zero, the following statements can be considered as good approximations, namely:

$$\mathrm{Ac\ (total)} = \frac{A_{(opt)}}{A_{(prop)}}$$

and hence the function of the deviation of the stroke (κ) on the deviation of the prey (σ) at equilibrium is

$$\kappa = \frac{A_{(stroke)} \cdot (1 + F)}{\frac{1}{A_{(opt)}} + \frac{1}{A_{(prop)}}} \cdot \sigma$$

where F is the factor which determines the additional influence of the proprioceptive center message on the direction of the stroke (cf. Fig. 6c).

Finally, the efficiency of the functional organization as revealed by the present analysis may be briefly considered from a biological point of view. Four main advantages are combined by the system:

(1) The head can be moved and hence the prey can persistently be faced by the region of the compound eye with the best visual acuity.

(2) Nevertheless the prey can be localized correctly, even if it deviates from the body axis.

(3) The hitting performance is independent of external and internal influences which act upon the neck muscles.

(4) The "calibration" of the mechanism which determines the direction of the stroke can be based solely upon the amplification factors of the proprioceptive and optic units. Thus it is at least not inconceivable that the correct adjustment is predetermined by the *structure* of the sense organs and the nerve connections involved.

Summary

The sensory-motor coordination which enables mantids to hit their prey is analyzed by recording the hitting performance under controlled experimental conditions. It is found that:

(1) Normal mantids (*Parastagmatoptera unipunctata*) hit about 85% of the flies they intend to capture. If the proprioceptors of the neck region are eliminated by nerve section, the hitting performance is irreversibly reduced to 20-30%.

(2) If the head is rigidly fixed on the prothorax in the median position, the performance is normal; but it decreases to 25% if the head deviates from the body axis by 10-30°. The prey is missed to the left if the head has been turned to the right, and vice versa.

(3) If head fastening and unilateral elimination of the proprioceptors are combined, the effects of both are superposed. The loss of one-half of the neck receptors is equivalent to an angular deviation of the head less than 20°.

(4) If the (free) head is loaded by an extraneous force, the achievement remains normal until the load surmounts twice the head weight at twice the head diameter.

It is concluded that the direction of the stroke depends upon feedback processes which control the position of the head as follows: The fixation movements of the head, which precede the release of the stroke, are steered by the difference between the optic-center message (which is a function of the angle between the prey and the fixation-line) and the proprioceptive-center message (which is a function of the angle between the head and the body axis). If the fixation movements have come to rest, the direction of the stroke is determined by the optic and (to a smaller extent) the proprioceptive-center messages, which then both contain the required information.

The hypothesis is cross-checked by measuring the position of the head at the end of the fixation movements. It turns out that the fixation line does not center the prey, but deviates from it by an amount proportional to the angle between the prey and the body axis. As predicted by the theory, this deviation is diminished after total proprioceptive deafferentation.

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NERVOUS CONTROL OF INSECT MUSCLES*

GRAHAM HOYLE
University of Glasgow

Detailed studies of the nervous control of muscles have been made principally on vertebrates and crustaceans. They have been concerned particularly with the elucidation of the mechanisms of neuromuscular transmission and have left largely unsolved many of the more general problems, such as the method of maintaining tone and the way antagonist muscles are used (see Elftman, 1941). Ideally it should be possible to give a complete analysis of an integrated movement in terms of all the events involved (for both the agonists and antagonists concerned), i.e., motor nerve impulses, transmission processes, muscle fiber contractions, activation of proprioceptors, sensory nerve impulses, and central nervous integrative processes, all stated quantitatively. Perhaps the greatest theoretical interest lies in the central processes, and it was ably demonstrated by Sherrington (1906) half a century ago that the experimentally accessible neuromuscular apparatus can be used as a window to the functioning of the central nervous system. An extension of these studies to other classes of animals may be justified on the grounds of their intrinsic interest and also because some of them may provide better experimental material for analysis of some of the general problems.

It seems likely that insects, which have been little studied in regard to their neuromuscular phenomena, offer excellent material. They certainly offer some challenging problems. In all insects there are functionally important muscles which are microscopically small, sometimes composed of no more than a dozen muscle fibers. Yet the joints operated by those muscles are moved with the precision which characterizes most insect movements, and the delicacy of action compares favorably with that encountered in the highest vertebrates, in which each muscle is composed of thousands of muscle fibers and is innervated by hundreds of nerve fibers operated by a central nervous system of immense complexity. The small size of insect limbs and the simplicity of both muscles and innervation offer peculiar advantages for a complete study of the subtler aspects of nervous control as well as the special problems of their neuromuscular transmission.

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A complete study of the phenomena of control in insects would shed light on the functioning of the insect central nervous system, on which there is at present almost no information, and hence on the properties of neuropile in general. There seems every reason to hope that a detailed study of the nervous control of insect muscle should be quite feasible and of general value.

It is to be expected that the insect mechanisms may be found to be somewhat similar to those of the crustaceans, many of which have already been fairly completely elucidated; but there is no longer any need for insect physiologists to borrow ideas from the crustacean field in interpreting the insect phenomena. Modern techniques of investigation, particularly the use of intracellular capillary microelectrodes, make it possible to give unambiguous information about the functioning of individual muscle fibers. Techniques are available for the stimulation of single nerve fibers even when these cannot be prepared separately, and they have already proved of value in insect work (Hoyle, 1955b, partly based on a method by Kuffler and Vaughan Williams, 1953).

Although this paper will be concerned with the limb muscles of insects, occasional reference will be made to thoracic or wing muscles and to the sound-producing muscles of cicadas, many of which have been evolved from muscles operating limbs; so far all the evidence shows that, although the histology and metabolism of these thoracic muscle fibers has been greatly altered in some orders, there has been no great change in either their pattern of innervation or their neuromuscular mechanisms. At the present stage of the investigation being undertaken by the author, it is not possible to go much farther than a description of the neuromuscular mechanisms, although a promising start has been made in the direction of studying natural nervous control in the body; some of this unpublished work will be described briefly. A cursory examination of the problem shows that tiny muscles cannot be satisfactorily operated along vertebrate lines, where graded tension is produced by varying the number of units each in one of two alternative states, i.e., rest or "all-or-nothing" contraction. There are just not sufficient muscle fibers available for executing smooth contractions by this method, even if space were available for the large number of nerve fibers and their cell bodies which would be required to control them. Hence an elucidation of the anatomy of the innervation must play just as important a part as a study of the physiology in contributing to an understanding of the nervous control of insect muscle.

The Innervation of Insect Muscle

Insect muscles are supplied with only a very small number of motor nerve fibers; Mangold (1905) demonstrated a double nerve-fiber inner-

vation of *Decticus* thoracic and leg muscles and *Dytiscus* wing muscles; Montalenti (1928) described a triplotomic branching in *Hydrophilus* leg. Pringle (1939), using a physiological method, showed that the extensor tibiae muscle of the metathoracic leg of *Periplaneta* receives two motor axons. The locust metathoracic extensor tibiae muscle receives three axons (Hoyle, 1955a). The homologous muscles of the pro- and mesothoracic legs only receive two (Hoyle, unpublished); many other locust muscles, e.g., the retractor unguis, also receive two. The flexor tibiae of several species, e.g., *Romalea microptera* (Ripley, 1954), *Acanthacris ruficornis* and *Zonocerus sp.* (Ewer, 1954), do however appear to receive four or even more axons, as indicated by a consideration of the number of steps which can be obtained in the tension developed by the muscles when a very carefully graded stimulus is applied to the motor nerve.[1] The flexors of the tibiae of all the legs of the locusts *Locusta migratoria* and *Schistocerca gregaria* also seem to receive several axons. I have obtained graded steps from these muscles during stimulation of the motor nerve and at the same time have recorded intracellular action potentials from various muscle fibers in different parts of the muscles. As the stimulus strength is raised, groups of fibers in different regions come into twitch activity. The action potentials in all the fibers are nearly identical electrical responses of the "fast" type (see below). Evidently the orthopteran flexor tibiae muscles are composed of four to six motor units, each of which has a separate nerve supply. From the evidence in the literature we may tentatively regard the *Periplaneta* flexor trochanteris (Pringle, 1939) and the *Dytiscus* extensor trochanteris as being similarly constructed. Graded contraction in these muscles could be effected not only by the special arthropod methods to be described, but also by the vertebrate method of varying the number of motor units in action at a time. The "fast-fiber motor unit, i.e., the complement of muscle fibers supplied by a single "fast" axon, of the flexor tibiae muscles consist of a single, coherent bundle of muscle fibers, in contrast to the vertebrate motor unit which is probably composed of fibers scattered throughout the whole muscle (data in Tiegs, 1953). The smallest insect muscles are formed of single bundles of fibers and the larger ones are constructed of several such bundles (termed "muscle units" in Hoyle, 1955a). The locust and cockroach extensor and flexor tibiae are all composed of several muscle units; those of the extensors all receive branches from the same two (or three) axons, whereas those of the flexors may receive independent nerve supplies. Perhaps this complexity of innervation imparts a greater degree of controllability to the flexors. In the pro- and mesothoracic legs of locusts and in all the legs of the cockroach, the flexor tibiae play a

[1] The same is also true of some coxal muscles of the cockroach (Becht and Dresden, 1956).

more important role functionally than their antagonists; the flexors are the main postural (tonic) as well as the main active muscles, so a high degree of control may be required for them.

In regard to the "fast" fiber, the insect motor unit may be either the whole muscle, as in the extensor tibiae, or only one or a few of the component muscle units, as in the flexor tibiae. Very recently Tiegs (1955) has demonstrated by staining methods the presence of two axons supplying the thoracic muscles of several species of insects. The sound muscles of cicadas are, however, supplied with only a single axon (Hagiwara, 1953; Pringle, 1954).

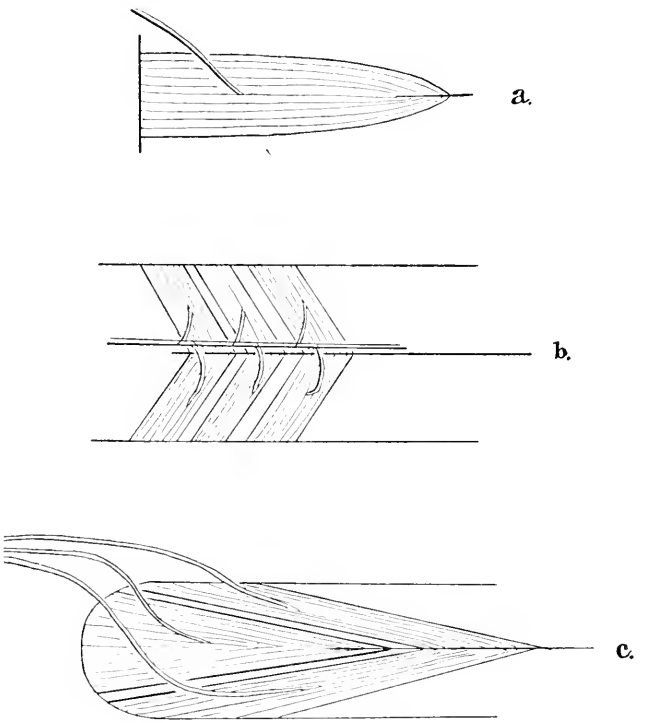

Fig. 1. Diagram to illustrate the three principal types of muscle organization encountered in insects. They are all shown here as if doubly innervated. (a) Single-unit type, e.g., retractor unguis, levator and depressor tarsi. (b) Multiple-unit, common-innervation type, e.g., extensor tibiae. (c) Multiple-unit, separate-innervation type, e.g., flexor tibiae, extensor trochanteris.

We may conclude that a double innervation is probably the common mode; in a few instances there is an additional axon and some muscles are supplied by only one axon. From the point of view of neuromuscular trans-

mission the flexor tibiae and similarly constructed muscles need not be regarded as having more than the usual double innervation, but only as compound or multiple-unit muscles, the individual units of which conform to the single- or double-innervation pattern. On this basis insect limb muscles can be divided into three categories (Fig. 1). These are the single-unit type (Fig. 1,a), e.g., the retractor unguis, levator, and depressor tibiae muscles; the multiple-unit, common-innervation type (Fig. 1,b), e.g., the extensor tibiae; and the multiple-unit, separate-innervation type (Fig.1,c), e.g., the flexor tibiae and the extensor trochanteris.

Neuromuscular Junctions

A wide variety of endings has been described for the terminations of motor axons on insect muscles. Filiform branches with no definite junctions have been observed (Montalenti, 1928; Morison, 1928) and this is the kind usually encountered in the Crustacea (Van Harreveld, 1939). The finer branches have been described as actually entering the muscle substance (Marcu, 1929; Tiegs, 1955). However, there are strong physiological grounds for not accepting this picture of axons penetrating the muscle fibers and also for being rather skeptical about the fibrillar type. This caution is strengthened by the fact that in a few instances end plates of definite structure have been clearly observed (Tiegs, 1955; Hoyle, 1955a.) There are many early references to endings of end-plate type, often large enough to warrant description as Doyère eminences. The histological observations are rendered extremely difficult by the presence of enormous numbers of fine tracheae, tracheoles, and tracheal end cells. The tracheoles often penetrate right into the muscle-fiber substance. In this case there is no doubt about the accuracy of the observations, and these diffuse surface and penetrating intracellular tracheoles with their associated cells could easily be responsible for the reports of fibrillar-type nerve endings.

The end plates are very refractory to staining. In locusts, where it is just possible to microdissect down to the level of end plates, to make out their outline, and even to pull them off the muscle fibers, no technique has been found which will stain them at all satisfactorily. The fine nerve twiglets enter the end plates at the immediate point of contact with the muscle fiber. There the nerve sheath appears to become confluent with the muscle-fiber sheath, the fine sarcolemma. At the point of contact there is often a large nucleus or cluster of nuclei of the sheath, and it may be this cluster of nuclei rather than the end plate proper which gives rise to the appearance of the Doyère eminences. A fine trachea is also usually associated with this point. The end plate is composed of about half a dozen fine tongues of granular cytoplasm. These may be embedded in a matrix of finely granular sub-

stance. There are numerous large inclusions in the tongues of the end-plate claws which are syncytial, having numerous small nuclei distributed through the claws in a random manner. The end-plate claws do not pass between the myofibrillae, merely resting on the surface of the fiber beneath the sarcolemma. The attachment must be a very loose one, judging by the ease with which the whole plate can be pulled off. The close similarity between locust endings (Hoyle, 1955a) and those of the homopteran *Cyclochila australasiae* (Tiegs, 1955) suggest that this may be a common type of ending in insects; a generalized ending based principally on the locust and homopteran endings is illustrated in Fig. 2. This bears a considerable resemblance to the amphibian endings described by Couteaux (1947). The terminations of the axons within the ending were, however, not stained.

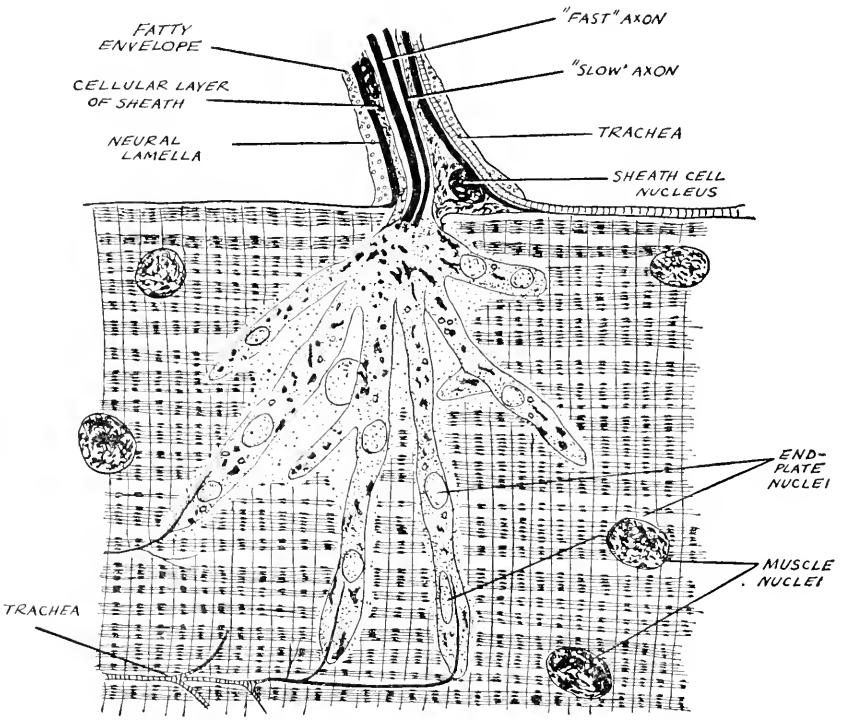

Fig. 2. Drawing to show the structure of a doubly innervated locust motor end plate. The final branches of the axons did not stain.

Multiple endings on single fibers were described clearly by Foettinger (1880). He even obtained fixed specimens showing a series of local con-

tractions under the nerve endings. Marcu (1929) counted the endings on fibers of thoracic muscles and found them at intervals of about 80μ in *Geotrupes,* 50μ in *Musca.* Weiant (unpublished), found endings at intervals of about 40μ in *Periplaneta* leg muscles though she was unable to obtain a clear picture of the individual endings. In the leg muscles of *Locusta* the endings are about 60μ apart. This kind of innervation may conveniently be called multiterminal innervation (Ripley, 1954).

The one, two, or three motor axons supplying a muscle unit travel together, bound by the same sheath right up to the point of contact, i.e., right

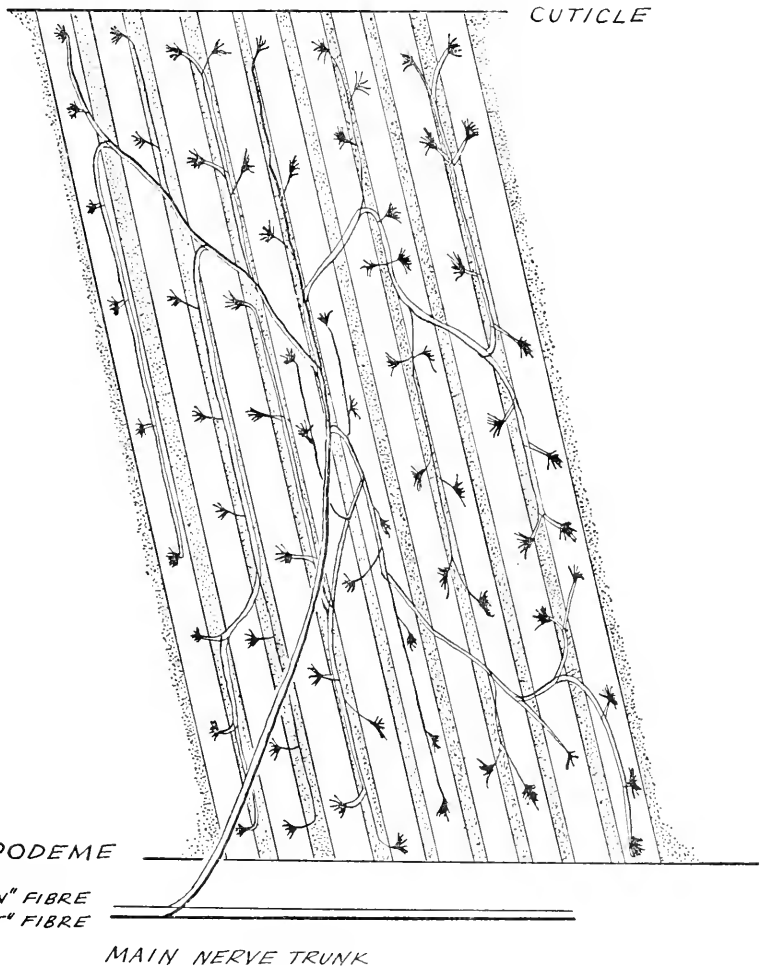

Fig. 3. Diagram to illustrate the pattern of innervation of a doubly innervated insect muscle unit, based on the locust extensor tibae unit.

into the end plates themselves. This was first demonstrated by Mangold (1905) for *Decticus* and has recently been confirmed for *Locusta* by Hoyle (1955a) and *Cyclochila, Erythroneura,* and other species by Tiegs (1955). Anatomically (Tiegs) and physiologically (Hoyle) it has been shown that, although one (usually the larger) fiber probably sends a branch to every end plate, the others (smaller ones) do not. Each end plate on a particular fiber is, however, probably similarly innervated, though there may be exceptions to this rule. This means that, whilst every muscle fiber receives a uniform supply from one axon, only a proportion of the fibers receive supplies, again of similar kind, from the others. A diagram illustrating the general pattern of the innervation of a two-axon muscle is shown in Fig. 3. Many thoracic muscles are, however, said to have only a single ending per muscle fiber, e.g., *Apis* (Morison) and *Erythroneura* (Tiegs). These will be discussed later.

Mechanical Responses

A consequence of the paucity of motor innervation is that special neuromuscular mechanisms must be used in order to effect graded contraction of the muscles. In spite of the demonstration of this economy of nerve supply and the physiological exploration of the rather similar crustacean system, workers in the insect field did not seem to appreciate this point. It was not until 1939, when Pringle studied the motor mechanisms of the cockroach leg, that the presence of special mechanisms in insect muscle was adequately realized. Earlier workers (especially Kahn, 1916; Friedrich, 1933; Solf, 1931) had treated insect preparations as if they worked like the frog gastrocnemius preparation. We may now interpret their results in the light of recent knowledge as if they had stimulated only the "fast" nerve fibers of their preparations. The adjective "fast" refers to the relative speed of the resulting contraction and not to the velocity of conduction along the motor axon. Actually the two fibers of doubly innervated muscles are sometimes markedly different in diameter, e.g., in *Geotrupes* (Marcu, 1929) and in *Cyclochila* (Tiegs, 1955), and so probably have different conduction velocities, in which the thickest and fastest axon is almost certainly also associated with the greatest speed of contraction. But the two motor axons supplying the extensor tibiae of *Locusta* and *Schistocerca* metathoracic legs are very similar in diameter, conduction velocity, threshold, etc. The corresponding fibers of the mesothoracic legs, although producing comparable mechanical responses, are different in thickness, 10-11μ for the "fast" and only 6μ for the "slow," with conduction velocities of 2.2 and 1.5 meters per second respectively. Tiegs found some cases, e.g., the dorsal longitudinal muscles of *Erythroneura,* in which the fiber diameters were about equal.

The maximum power of the locust jumping muscle is as great as 20 kg. per gm., nearly ten times better than for mammalian muscle expressed in the same terms. The only satisfactory way of comparing power development for animals as different as insects and vertebrates is to do it in terms of the mean cross-sectional area of individual muscle fibers. A rough estimate for this value compared with frog sartorius shows that each locust fiber develops about the same maximum tension as frog fibers. The difference in length of the fibers accounts for the apparent colossal strength of the insect muscles.

The summation of successive contractions during an incomplete tetanus is often very marked in insect muscle. The tetanus-twitch ratio in *Decticus* muscles at 20° C was placed at 10/1 by Solf (1931), and it has a similar value in all locust leg muscles for "fast" fiber stimulation. The ratio is increased by lowering the temperature below about 15° C. The durations of the twitches of leg muscles of *Periplaneta, Dytiscus, Tettigonia, Locusta,* and *Schistocerca* all fall within the limits of 0.1 and 0.2 sec., i.e., of the same order of magnitude as frog muscle. In *Locusta* the latent period between the action potential peak and the onset of contraction in the relatively long-fibered metathoracic flexor tibiae muscle is 2-3 msec. Peak twitch tension is reached in 0.04 sec.

The fusion frequency is usually of the same order of magnitude as that of frog muscle, i.e., about 20 per second, but some very high values have been recorded. Kraemer (1929) gives a value of over 50 per second for *Dytiscus* and Pringle (1939) over 70 per second for *Periplaneta.*

Stimulation of the second or "slow" motor axon was achieved by Pringle (1939) in the cockroach metathoracic extensor tibiae muscle. After drying out the metathoracic nerve trunk which he designated 3b and then remoistening it, he found that the response of the muscle to maximal stimulation of the nerve trunk was markedly changed. Initially there had been a twitch following each stimulus and now there was no mechanical response at frequencies below 30 per second, but a smooth extension of the tibia occurring at higher frequencies. The response occurred at a precise threshold, so was almost certainly due to a single axon. The rate of extension and final tension increased with rate of stimulation up to frequencies of about 300 per second. The results showed clearly that the requirements of tonic and slow actions could be met by a single axon, but they did not reveal the mechanism of action of this axon.

"Slow" axons supplying the pro-, meso-, and metathoracic extensor tibiae muscles of the locusts *Locusta migratoria* and *Schistocerca gregaria* leave their respective ganglia by separate nerve trunks. Branches containing the axons meet in the coxae before final branches, carrying only the extensor tibiae nerve fibers, leave to supply these muscles, as illustrated in

Fig. 4. In each ganglion the nerve trunks concerned are the branches N3b and N5. They are probably the homologues of the branches similarly designated in the metathoracic ganglion of *Periplaneta* by Pringle (1939). In Albrecht's (1953) account of the *Locusta* nervous system the trunks are designated N2 and N3, the actual second and fourth nerves not being mentioned in his description. The pro- and the mesothoracic N3b nerves carry the single "fast" axons of the extensor tibiae muscles. The metathoracic N3b carries a single "slow" axon of the metathoracic extensor tibiae, the S_1 axon (Hoyle, 1955). The pro- and mesothoracic "slow" axons travel in the N5 trunks whilst the metathoracic N5 carries the single metathoracic extensor tibiae "fast" axon. This divided origin of the "slow" and "fast" axons supplying the same muscle is extremely convenient, for it means that the neuromuscular mechanisms of the muscles can be studied during separate stimulation of the innervating axons without the risk of damaging them which is encountered when axons have to be separated by dissection. The metathoracic N3b does, however, contain an additional axon supplying the metathoracic extensor tibiae muscle. This axon (S_2) evokes no mechanical response when stimulated alone, but efforts must be made to avoid stimulating it if the effects of S_1 alone are to be observed.

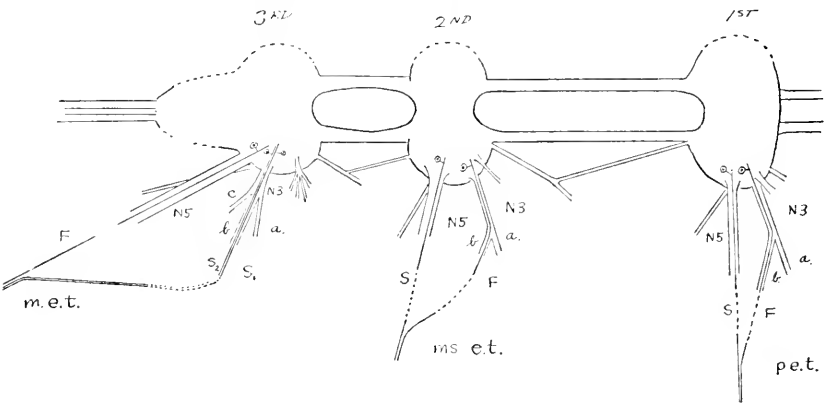

Fig. 4. Diagram of the locust thoracic nerve ganglion chain showing the origins of the "slow" and "fast" axons supplying the extensor tibiae muscles. F= "fast" axon; S_1, S_2, "slow" axons; N_3 a,b,c, N_5, nerve trunks. m.e.t. = metathoracic extensor tibiae. ms.e.t. = mesothoracic extensor tibiae. p.e.t. = prothoracic extensor tibiae.

Stimulation of the metathoracic extensor tibiae "slow" axon at frequencies up to 10 per second produces no mechanical response except in a few cases where a minute extension of the tibia may be seen to be associated with each stimulus. A slow, smooth extension starts at about 15 per second and increases in the rate of extension follow increasing frequency of stimulation up to about 150 per second. The final tension produced dur-

ing prolonged stimulation increases with increasing frequency up to about 80 per second. These frequencies are about half the corresponding ones for *Periplaneta*. The locust prothoracic and mesothoracic extensor tibiae produce final tensions at the tibial tips of barely 0.5 gm., but movement also starts at about 15 per second in the absence of load and increases in rate up to about 150 per second are evident.

The Mechanism of Transmission

Even in the smallest insects, skeletal muscle fibers are seldom less than 20μ in diameter and often are as large as 100μ or even more; this means that the intracellular recording technique can be used in studying transmission. With the recent advances in technique a new standard is required in work of this kind, so it seems desirable to lay down the principles which should be followed if the results are to contribute fully as comparative data. They must be regarded as an ideal program rather than as limiting conditions.

Anatomical Features Which Should Be Known

(1) The nature of the muscle being studied, whether multiple or single unit or diffuse, and whether the units have common or separate innervation.

(2) The number of axons supplying the units being studied; antidromic stimulation is helpful in determining this.

(3) The approximate spacing of the end plates along the fibers.

Stimulation

(1) Stimulation of separate axons. Where axons are neither separated naturally nor separable by microdissection, differences in threshold and conduction velocity should be utilized.

(2) Monitoring of nerve impulses. Tiny electrodes are particularly subject to polarization and a constant check should be made on the efficacy of stimulation. Monitoring becomes essential when differences in threshold or conduction velocity are being used to study separately axons contained in a common trunk.

Recording

Intracellular recording from as many fibers as possible from all parts of the muscle under consideration.

The "Fast" System

Rijlant (1932) found action potentials associated with vigorous spontaneous mechanical activity in the legs of *Dytiscus* and *Hydrophilus*; the potentials did not show any facilitation and were probably due to the ac-

tivity of a "fast" axon. Pringle (1939) found a similar type of nonfacilitating response when stimulating the nerve to the cockroach metathoracic extensor tibiae muscle. The "fast" responses of several muscles of *Locusta migratoria* have been studied in a series of papers (del Castillo, Hoyle, and Machne, 1953; Hoyle, 1955b,c). Similar techniques have recently been used in studies on *Calliphora vomitoria, Dytiscus marginalis,* and *Schistocerca gregaria* (Hoyle, unpublished). The "fast" responses have also been studied in cockroach leg muscles (Wilson, 1954; Hoyle, 1955c), the wing muscles of *Locusta migratoria danica, Gampsocleis burgeri,* and *Mecopoda elongata* and the sound muscles of the cicadas *Graptopsaltria nigrofuscata* and *Platypleura kaempferi* (Hagiwara, 1953; Hagiwara and Watanabe, 1954).

In the extensor tibiae muscles of all the legs of the locusts *Locusta migratoria migratorioides* and *Schistocerca gregaria* a sufficient number of intracellular insertions has been made to make it possible to state confidently that in them the single "fast" fibers innervate every muscle fiber. This is probably true of all the other "fast" fiber systems in the locusts and also applies to each unit of the locust flexor tibiae muscles and no doubt also to the extensor trochanteris of *Dytiscus* (Kraemer, 1932) and others.

The responses of each muscle fiber are substantially similar. In some instances a microelectrode has been used to record the responses in different parts of a single muscle fiber, and these investigations showed that there is very little difference between the responses recorded at the different points. The fibers on which these studies have been made are all ones which receive multiple nerve endings at intervals of less than 0.1 mm. along their entire length.

The intracellularly recorded resting potentials average 60 mV (50-65 mV) in all *Locusta* and *Schistocerca* leg muscles in good condition when bathed in a saline containing 10 mM K per liter, which is the lower limit of their haemolymph potassium content. In zero-5 mM K per liter saline the resting potentials approach 70 mV. In *Gampsocleis* wing muscles and *Platypleura* sound muscles the average resting potential is 60 mV in 3 mM K per liter saline, whilst in *Mecopoda* and *Graptopsaltria* the recorded values were only 42 mV (Hagiwara and Watanabe, 1954). In *Calliphora* and *Dytiscus* values of 60 mV are common in 5 mM K per liter saline. In *Periplaneta* flexor tibiae Wilson (1954) obtained resting potentials in 2.7 mM K per liter saline averaging 45 mV (30-70 mV). There are reasons for being skeptical about the lower means of 42 and 45 mV, since these result from several very low values of resting potential which were recorded and included in the analysis. In most insect haemolymph the potassium concentration is rather high; in the omnivorous cockroach it may reach 30 mM and in the grass-feeding locust nymph 40 mM. Values

even twice these have been found in some species. Now the resting potential is directly related to the potassium gradient across the muscle-fiber membrane and an increase in external potassium effects a reduction in the resting potential (Hoyle, 1953b; Hagiwara and Watanabe, 1954). At 30 mM K per liter the mean resting potential of locust muscle fibers is reduced to 35 mV, half the maximum possible value. Low resting potentials may therefore be due to inadequate mixing between the haemolymph and the saline in certain regions of the muscle. Theoretically there should be little difference between the resting potential values of healthy fibers bathed in the same saline, and so higher values of the range are probably more nearly the correct ones. Fibers with a low resting potential may be aging, fatigued, damaged, or partly depolarized by high local external potassium. Results obtained from them must be examined in the light of these possibilities. When Wilson (1954) found that the responses obtained in the low-resting-potential fibers differed in several respects from those obtained in the higher ones, he claimed that there were two different sorts of fiber and that these should be associated with the "slow" and "fast" systems. He did not attempt to stimulate the "slow" and "fast" nerve fibers separately, or even let the preparation do this for him, i.e., by leaving the connections with the ganglion intact and recording during spontaneous activity or reflex stimulation. Although he suggested that a high local external potassium concentration might be leading to the low values for resting potential, he did not carry out the obvious test and raise the potassium level whilst recording from the large-resting-potential ("fast") fibers. Had he done so he would have seen that this treatment converted his "fast" fibers into "slow" ones. In other words, all Wilson's observations were probably on "fast" fiber responses recorded from both high- and low-resting-potential muscle fibers.

The "fast" responses of both the locust and cockroach muscles consist of large depolarizations which often overshoot the zero potential base line. Overshoots up to 20 mV may have been recorded from locust fibers having large resting potentials. In many cases, however, the potential fails to overshoot or even quite reach the zero level; this is almost always associated with a low resting potential and so possibly with poor conditions. Hagiwara (1953) and Hagiwara and Watanabe (1954) found similar responses in the wing muscles of *Oxya* and the sound muscles of *Graptopsaltria* and *Platypleura*, though overshoots were rarely observed except in *Platypleura*.

When locust muscle is gradually cooled, the time course of the response lengthens and an obvious step appears in the rising phase. This step is sometimes noticeable in the rising phase at ordinary temperatures, particularly with a fast time base. As the temperature drops to about 12° C

the step is not only very much more marked but the final hump of the action potential, the part occurring after the step, is greatly reduced. At about 8° C it is completely absent. There remains a potential with a smooth, unstepped rise and an exponential decay. This potential is similar in shape to the vertebrate end-plate potential and may be similarly designated in insects. There is, however, a marked difference between the insect and the vertebrate muscle. In the former the end-plate potential is not just confined to a single site as in vertebrate muscle, and the latency of the response does not differ in different parts of the fiber. These observations reflect the nature of the innervation, i.e., the distributed end plates of the insect muscle; and the logical interpretation is that in the insects end-plate potentials analogous to the vertebrate end-plate potentials are produced nearly simultaneously in the several end-plate regions of the fiber. The area involved is so large that depolarization occurs synchronously over the whole surface of the fiber.

If the membrane potential is raised or lowered by passing polarizing or depolarizing current across the muscle-fiber membrane through a second intracellular electrode inserted in the same fiber, the magnitude of the end-plate potential is correspondingly raised or lowered. There is a simple linear relationship between end-plate potential and resting potential, the line passing through the origin. This evidence strongly suggests that the end-plate potential is due to the formation of a temporary short circuit of the resting membrane, probably produced by a chemical substance released under the end plate and increasing the permeability of the membrane to several ions (cf. Fatt and Katz, 1951). The effect of reducing the temperature is to show that the normal response has two components, a primary junctional response or end-plate potential and a secondary, spike-like response. The end-plate potential is probably due to a partial short circuit of the membrane in the several areas on each muscle fiber in the immediate vicinity of the end plates. If no further response occurred there would be a simple exponential decay of the end-plate potential after the peak of the transmitter action. Instead the depolarizing action affects the properties of the resting membrane. This leads to a transient additional change in potential which sums with the end-plate potential, thus generating the secondary response. Typical responses from *Calliphora, Periplaneta,* and *Schistocerca* muscle fibers are illustrated in Fig. 5. The time courses of pure end-plate potentials are indicated by dotted lines.

The secondary responses can be studied independently of the end-plate potentials by passing depolarizing current across the muscle-fiber membrane. They appear at and above a critical level and consist either of oscillatory responses of small amplitude with frequencies from a few to over 100 per second, or spike-like responses reaching a height up to 25 mV

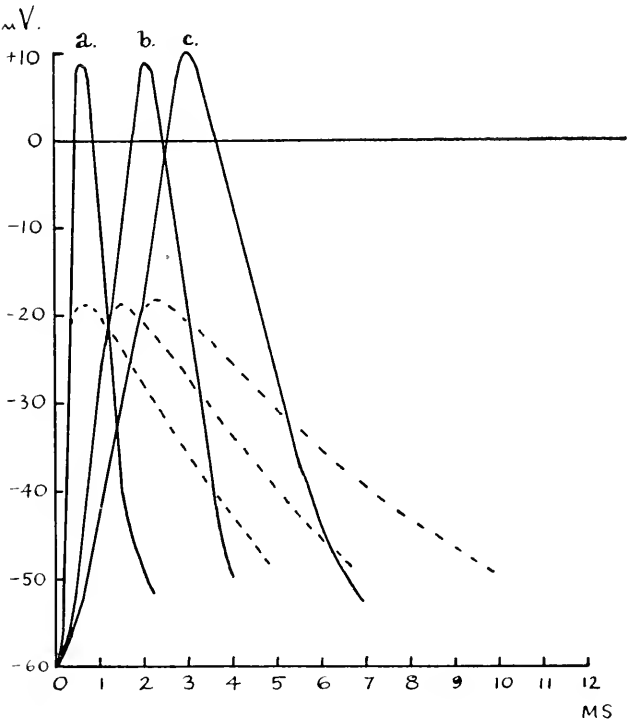

Fig. 5. The time course of typical insect muscle action potentials recorded with an intracellular electrode at 20° C during "fast"-fiber stimulation. Traces taken from fibers with 60 mV resting potential from: *a, Calliphora vomitoria; b, Periplaneta americana; c, Schistocerca gregaria.* The probable time courses of the pure end-plate potentials are shown with dotted lines to emphasize the magnitude of the secondary responses and their effect on recovery.

with a duration of 1-6 msec. Only the latter are comparable to those evoked by the natural stimuli, the end-plate potentials. They may be evoked by depolarization of about 20 mV. The surprising thing is that the spikes are never larger than about 25 mV as measured from the depolarization plateau, which is usually about 14 mV depolarization (45 mV membrane potential level). Also they are very variable in both magnitude and duration even when recorded from the same site. Theoretically the largest ones might just be capable of exciting the resting membrane and so setting up propagation, but electric pulses of similar magnitude and duration to the spikes rarely give rise to equally large responses. This means that the insect secondary responses are only local events; there is no evidence of their being propagated, as Fatt and Katz (1953) have demonstrated propa-

gation of the somewhat similar though larger responses of some fibers in Crustacea, and as occurs always in ordinary vertebrate skeletal muscle. In vertebrate muscle the spike response may reach a height of 70 mV, three times that of the locust.

Further information on insect transmission can be obtained by studying the effects of potassium, calcium, and magnesium on the process. Raising the magnesium or lowering the calcium in the bathing fluid has an effect similar to cooling, in that the step in the rising phase of the action potential which is probably due to a delay in the start of the spike responses is markedly increased. This effect is partly due to a reduction in the magnitude of the end-plate potential. If the magnesium is raised or the calcium lowered sufficiently, only the pure end-plate potential remains since it becomes too small to evoke any spike response. The pure end-plate potentials show considerable facilitation and summation. Potassium in excess lowers the magnitudes of both the end-plate potentials and the secondary responses. Its effect is partly indirect, due to the reduction of the resting potential.

A typical locust response from a fiber with a 60 mV resting potential has an overshoot of 13 mV. The total action potential of 73 mV is composed of 48 mV junctional response (peak height) and 25 mV spike response. A similar fiber not showing an overshoot might have an action potential of 58 mV. This would be composed of about 40 mV junctional response and 18 mV spike response. The presence or absence of the overshoot is not an important matter functionally, for fibers without an overshoot undoubtedly twitch quite vigorously. When treatment with high magnesium, high potassium, or calcium-free saline has reduced the response to a pure end-plate potential of no more than 20 mV, there is still a small twitch contraction. On the other hand, a single muscle fiber shows a brisk local twitch when current sufficient to evoke only a local response is passed. Evidently either the end-plate potential or the local spike can activate the contractile mechanism. It has not yet proved possible to affect the secondary response experimentally without at the same time affecting the magnitude of the end-plate potential, so an exact estimate of the part played by this response in eliciting contraction is not possible at present. It seems probable that in arthropods generally the link between membrane depolarization and tension is direct and progressive. A wide range of tension can be produced in the same fiber, provided its membrane potential is lowered in graded steps. Even the large "fast"-fiber action potentials, which are all-or-nothing events, may nevertheless be required in quick trains before the muscle contracts fully. Otherwise it is difficult to account for the high tetanus/twitch ratio encountered even in short-fibered insect muscles where there is virtually no connective tissue and the tendons are inelastic.

The "Slow" System

So far the "slow" systems have only been demonstrated in the extensor tibiae muscles of the orthopterans *Periplaneta, Locusta,* and *Schistocerca* (Pringle, 1939; Hoyle, 1953a and 1955b). A detailed investigation has only been undertaken in the case of the *Locusta* metathoracic extensor tibiae and the *Schistocerca* prothoracic and mesothoracic extensor tibiae (Hoyle, unpublished). Using external recording, Pringle (1939) showed that the action potentials of the "slow" system may facilitate considerably, by as much as sixfold. In the locust metathoracic extensor tibiae the externally recorded action potentials are extremely variable, depending on the electrodes used and on their position (Hoyle, 1955b). Two types of record may be observed, small spikes and slow shifts of potential. With balanced input there is seldom any sign of facilitation, but with focal external recording, using a suitably small electrode, small spikes can be obtained from many sites which clearly resemble the characteristic shape of end-plate potentials. Some of these show considerable facilitation. At frequencies above about 60 per second there is also summation. In other positions the potentials cannot be regarded as end-plate potentials, and show neither facilitation nor summation. Under these circumstances intracellular recording offers the only possible method of resolving the situation. With this technique many surprising features have become apparent which could not even have been suspected from an analysis of records from external electrodes.

Since every muscle fiber is innervated by the "fast" fiber, it follows that "slow" activity must involve contractions of some or all of the same fibers. The first surprising feature is that there is a response to "slow"-fiber stimulation in only a fraction of the muscle fibers. In the *Locusta* metathoracic extensor tibiae the fraction is about 30%. Partial innervation of this kind has recently been recorded in crustacean muscles (Furshpan, 1955). The innervation in the *Locusta* and *Schistocerca* prothoracic extensor tibiae muscles is 40-50% and in the mesothoracic muscles about 40%. The second surprising feature is the extremely wide range of magnitudes of the responses (Fig. 6). This is the case even when fibers with similar resting potentials and similar "fast" action potentials are compared. In the *Schistocerca* mesothoracic extensor tibae the responses vary from 2 to over 50 mV in different fibers of the same preparation. The smaller responses are pure end-plate potentials, the larger ones compound. The largest of the compound responses are almost as big as the "fast" responses of the same fibers.

The largest responses show virtually no facilitation and do not summate; they are so similar to the "fast" responses that the same transmitter sub-

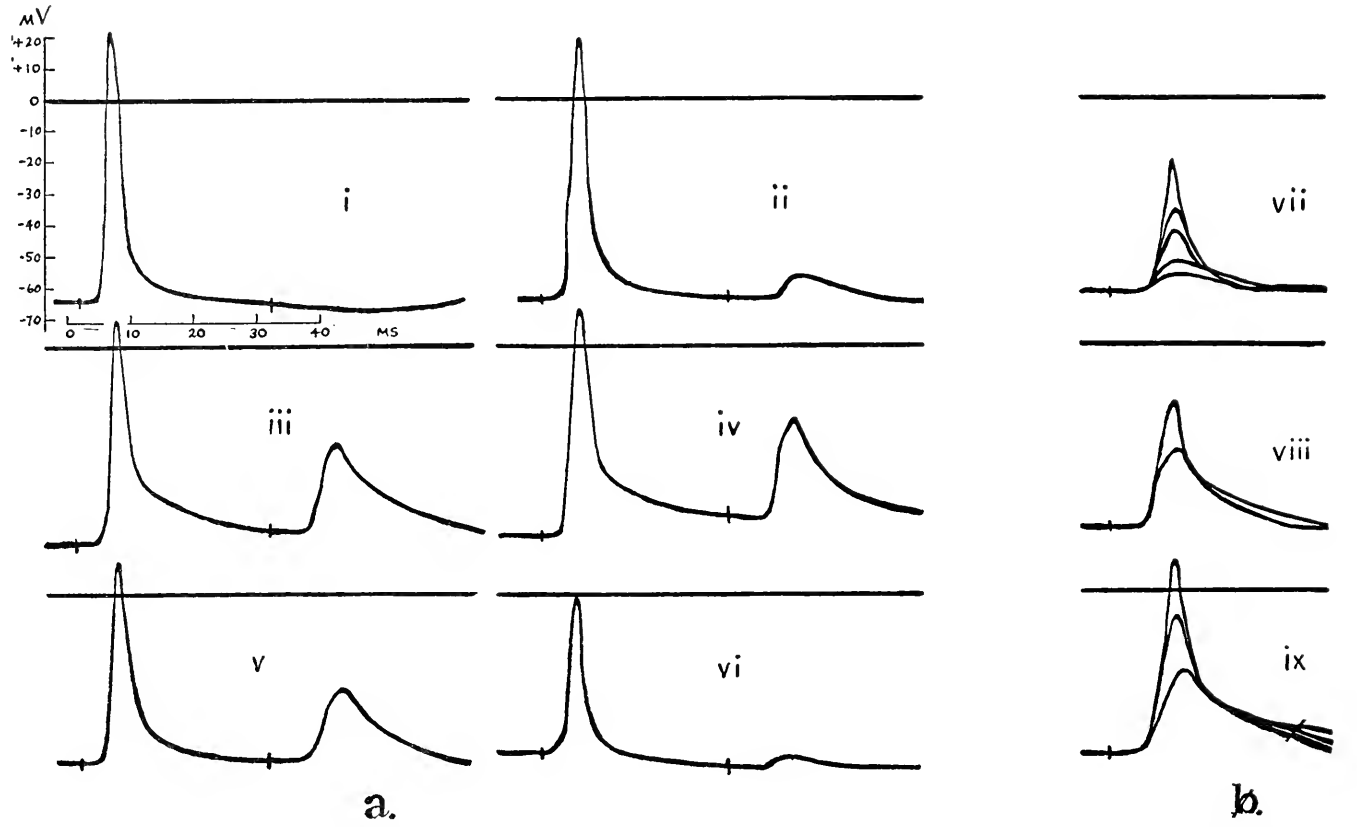

Fig. 6. Action potentials recorded with an intracellular electrode from various muscle fibers of the extensor tibiae muscle of the mesothoracic leg of *Schistocerca gregaria*. (a) "Slow" and "fast" responses recorded together. The first stimulus artifact in each trace indicates stimulation of the "fast" axon, the second stimulation of the "slow" axon. The situation in *i*, with no response to stimulation of the "slow" axon, is that found in more than 50% of the fibers. The others are typical examples from the remainder. (b) Traces obtained during "slow"-fiber stimulation at about 30 per second to show the extent of facilitation in different fibers. The upper trace in each record shows the maximum height observed in that fiber. The calibration scales in (a), *i* apply throughout.

stance might be involved, just released in smaller quantities. The smaller responses often show quite marked facilitation. In a few instances this has been as great as sixfold. The larger end-plate potentials show facilitation of only a fraction, say 1/3, of the initial magnitude, but this, combined with variation in magnitude of the secondary response, may lead to a doubling of the total response, including occasionally an overshooting of the zero base line. The magnitude of the "slow"-fiber end-plate potential can be raised or lowered by raising or lowering the resting potential with the aid of polarizing or depolarizing current. This is consistent with the view that the end-plate potential is due to the release of a chemical transmitter substance which raises the permeability of the membrane in the end-plate regions to some or all ions.

The situation in the extensor tibiae of the jumping leg appears to be considerably more complex. The end-plate potential type of response is obtained from only about 10% of all the muscle fibers. In about 20% there is a response of a different kind. The response consists of a slow depolarization rising to a height of not more than 1 mV in about 50 msec. and declining in about 800 msec. These potentials show almost no facilitation, but they summate during repetitive activation to produce a depolarization plateau. The two types of responses were designed S_{1a} and S_{1b} respectively (Hoyle, 1955b). The slow depolarizing fibers (S_{1a}) contract smoothly at all frequencies. Many of the larger end-plate potential-type fibers (S_{1b}) show small twitches in response to each stimulus and only contract smoothly at higher frequencies characteristic of tetanus.

Ordinarily the process of mechanical excitation is linked to the multiterminal anatomical arrangement, but the local response evoked by passing current through an intracellular electrode produces quite a marked local twitch. It remains possible, therefore, that under tetanus even a single end plate could elicit an appreciable mechanical response from the whole fiber, especially in short fibers, and so a single end plate might be the mode of innervation in some short-fibered insect muscles. Morison (1928) and Tiegs (1955) have claimed that many insect muscles receive only one end plate, at one end of the fiber; Tiegs showed that they may nevertheless receive two axons. Intracellular recordings from fibers of this kind would be of considerable interest.

These results also raise the question as to whether or not insect muscle can be excited directly. Many earlier workers, e.g., Heidermanns (1931), Solf (1931), thought that in their experiments they were exciting the muscle directly in the same way that frog sartorius or gastrocnemius can be excited. The effectiveness of direct stimulation in frog muscle is due to the fact that an adequate depolarizing current readily sets up an active membrane response which is progagated as a spike of all-or-nothing char-

acter along the whole length of the muscle fiber. Since no insect muscle has yet been shown to be capable of producing a propagated spike action potential, the claims for direct excitation must be viewed with caution. The presence of nerve branches throughout an insect muscle makes it likely that external electrodes will excite these nerve fibers at a lower threshold than that at which the muscle could be excited, and the presence of simple "steps" in the response records of Solf (1931) and Kraemer (1932) suggest that they were only stimulating the nerve. Roeder and Weiant (1950) found that a muscle of *Periplaneta* became completely inexcitable after the motor nerve had been cut and allowed to degenerate, although the muscle fibers appeared to be still in good condition. Several insect muscles cannot be made to contract unless a deliberate attempt is made to stimulate them via the nerve (e.g., Tiegs, 1955).

Inhibition

Friedrich (1933) claimed to have demonstrated the presence of an inhibitory nerve in *Dixippus* leg. Actually all he obtained was a slight "relaxation" of the resting tibia during stimulation below the threshold of the exciting nerve. Ripley and Ewer (1951) argued that his effect could have been obtained with a loosely held preparation by contraction of the coxal muscles. No doubt other explanations are possible; the phenomena have certainly never been confirmed. Pringle was unable to find any evidence for peripheral inhibition during his studies on cockroach preparations. Ripley and Ewer (1951) described a relaxation of the levator tarsus muscle of *Locusta* when they raised the stimulus strength (applied to the whole nerve trunk in the thorax) by threefold. I have studied the same preparation in detail, monitoring nerve impulses and using intracellular electrodes to record from the muscle. All the fibers in the nerve which supply the tibia are excited between the limits threshold to threshold plus 25%. At around three times threshold an apparent inhibitory effect appears, but this is due to failure to excite the nerve (Hoyle, 1955b), probably because of polarization at the stimulating electrodes. These observations emphasize the need to monitor nerve impulses in this kind of experiment. It is unlikely that observations on inhibition will be accepted at the present time unless they are supported by experiments along the lines indicated earlier in this paper.

There remains, however, the problem of the function of axons like the third axon (S_2) supplying the locust jumping muscle (Hoyle, 1955a). Since this is only about half as thick as the "slow" axon which travels in the same trunk, differences either in stimulus strength or in conduction velocity can be utilized to stimulate it separately. At the same time the "fast" axon can be separately stimulated and to an incomplete extent the

effect of interference between the third axon and the "slow" one can also be studied. So far no combination of stimuli has been found which leads to an inhibition of either the "slow" or the "fast" systems. The effect of stimulating S_2 is to increase rather than to decrease the mechanical response. Intracellular recording reveals that stimulation of S_2 effects a small hyperpolarization in those muscle fibers which have a low resting potential. The hyperpolarizations summate to raise the resting potential up to but never beyond the value of 70 mV, which is probably near the potassium equilibrium potential. Hyperpolarization of the resting membrane is a property of the crustacean inhibitory nerve. The locust hyperpolarizer axon is only present in the metathoracic leg, and it may perhaps be an evolutionary relic of a once common inhibitor axon. Now its hyperpolarizing function alone seems to remain; this could be of value to insects in which there is a fluctuating, often high, value of haemolymph potassium as in grass-feeding locusts. The potassium tends to depolarize the muscle fibers; and any agent acting against this tendency would ensure a larger end-plate potential and active membrane response, which both depend on the resting potential, and in turn greater activation of the contractile mechanism would follow.

This is merely speculative, but the presence of the locust S_2 axon is significant. It must be taken to indicate that somewhere in the class there may be fibers possessing the property of peripheral inhibition.

Locust Muscle: The Overall Picture

The locust pro- and mesothoracic extensor tibiae muscles are small and functionally nonspecialized. They seldom support the weight of the body, so are probably not called upon to produce prolonged activity. They are concerned with providing some of the thrust needed during walking and running and in checking quick movements of the antagonist flexors (which normally take the weight of the thorax). Their "slow" fibers are well suited to most of these tasks. The graded end-plate potentials in the various fibers ensure that some muscle fibers are ready with near-maximal twitches for immediate work against the inertia of the system. Other fibers producing little tension at the start of a train of excitation come in later as facilitation and summation develop, to reinforce the first-acting fibers. A smooth development of tension is always assured even when excitation is at high frequency. Evidently the "slow"-fiber peak tension of 2/5 maximal "fast"-fiber tension is adequate for all slow movements and tonus. The tension can always be reinforced by calling in the "fast" system and so utilizing the other 3/5 of the fibers. Perhaps in many movements the "fast" system is used alone.

The metathoracic extensor tibiae muscles are specialized for the function

of jumping. So great is the power developed during a twitch due to a single "fast"-fiber action that we can say at once that the F axon is used only in jumping. All ordinary movements must utilize the S_1 axon alone. Ten per cent of the available muscle fibers can produce a peak tension of 80 gm. in the muscle, representing a thrust of 2 gm. at the foot. This is more than adequate to shift the locust, itself weighing barely 2 gm., and 10% is the total number of fibers innervated by the S_{1b} end-plate potential-type endings. What then is the value of the S_{1a} slow depolarizing endings? Do these endings represent some sort of evolutionary degeneration? This is not as fantastic as it may seem. The jumping muscles have been evolved from muscles resembling the prothoracic and mesothoracic extensor tibiae muscles where the "slow" system utilizes 2/5 of the fibers. Activation of 2/5 of the jumping muscles produces considerable tension, enough to give a small leap if developed rapidly, so a still smaller proportion of the muscles would be quite adequate for the "slow" system. Only 1/5 of the tension can actually be produced at all rapidly by the S_1 axon, since the S_{1a} fibers are activated so slowly; an effective reduction in innervation has therefore been achieved. The metathoracic extensor tibiae is an important tonic muscle, unlike the prothoracic and mesothoracic muscles; it supports the climbing and vertical resting locust and raises the abdomen during walking. The S_{1a} type ending is probably well-suited to the tonic control of the muscle.

In making these deductions we must realize that the evolution of the powerful jumping apparatus required not simply an enlargement of the limb and a habit of tucking the tibia under the femur. It was also necessary to develop compatible neuromuscular mechanisms from the existing ones, and the much more subtle but equally important problem of altering the central nervous synapses appropriately had to be overcome at the same time. The requirements of tonic and slow activity had to be met at all stages and effected by a muscle which acquired the functions of its antagonist whilst increasing its power by 20 times.

The Natural Control of Insect Muscle

The study of neuromuscular mechanisms is only a part of the much broader field of the whole functioning of the motor apparatus, itself a branch of the physiological study of animal behavior. Consequently, if we can advance from a purely descriptive study of the events associated with transmission to a study of the natural functioning of the muscles, we enter in effect the realm of the central nervous system and make contact with the automatic control systems associated with proprioceptive feedback.

With a view to obtaining information about the natural functioning of locust muscles I have developed a technique which I shall describe briefly.

It consists of implanting small fixed electrodes through the cuticle, with fine, insulated copper wires attached. The animal is allowed the freedom of a 2 ft. sq. "Perspex" double-walled cage within which it trails the leads along with it. This makes possible the study of the muscle impulses (electromyogram), which are amplified and recorded on moving paper by an electroencephalograph machine, and observation of the animal and the electrical activity in a particular muscle or muscles at the same time. Two pairs of leads are usually possible without seriously disturbing the animal and recordings can be made from antagonistic muscles.

Records from the metathoracic extensor tibiae muscle are particularly easy to interpret, since they consist of a single series of action potentials due to S_1, except in the rare event of jumping. They show that impulses effecting tonic contraction occur at the low rates of 10-20 per second but in very irregular trains. Bursts of firing at rates up to 50 per second are associated with maneuvering, and even in vigorous marching they do not exceed peaks of 100 per second.

Records from the antagonist flexor tibiae muscle are complex, reflecting the compound nature of this muscle. Activity consists probably of "slow"-fiber activity in one or more units reinforced by bursts of "fast"-fiber activity, again with one or more units in action.

The mesothoracic extensor tibiae muscle shows tonic activity at about 20 per second due to the "slow" fiber and occasionally increases up to about 100 per second during movement. The bursts of high-frequency activity during movement are usually reinforced by brief bursts of 4-8 impulses due to the "fast" fiber. The antagonist flexor muscle, like that of the metathorax, reflects its multiple-unit nature by giving records of a similar, complex nature.

Antagonist muscles are frequently used against each other, as Elftman (1941) has calculated that they must be used in human walking, in order effectively to decelerate a rapid movement, and in other ways.

Conclusion

Most of the work described had been done on locust limb muscles, but evidence has been presented which indicates that the mechanisms utilized in locusts are possibly present universally in insects in the skeletal muscles. This is particularly likely to be true of the "fast" system, and there seems no reason why the locust "slow" systems could not be utilized by other insects; in fact it does not seem necessary to require much modification of the locust machinery to work say a *Drosophila* leg.

The "slow" system utilizes the same muscle fibers as the "fast" by bringing a proportion of them into contraction, each in a progressive manner. There is no justification for accepting Wilson's scheme of separate "slow"

and "fast" muscle fibers having different properties and separate nerve supplies. The "slow" system works by virtue of the distributed end-plate innervation which ensures a uniform depolarization of the muscle fiber and an extensive, though graded, contraction in response to this. It is this same distributed end-plate supply which enables insect muscle to function in the extraordinary mineral environments which insect haemolymph often presents, media in which the mechanism for establishing the vertebrate propagated action potential would be immediately paralyzed.

Up to the present time no rational explanation has been offered as to why there are these "slow"- and "fast"-fiber mechanisms in the large Crustacea. The answer may lie in a consideration of the insects; for, as has been pointed out, the highly economical "slow"- and "fast"-fiber system is admirably suited to the special problem of controlling a very small muscle composed of few fibers. If we suppose that the ancestral arthropods from which the Crustacea and insects were derived were very small animals and that the "slow"/"fast"-fiber mechanisms were evolved to meet their needs, then it is readily possible to explain their persistence in the larger decapods. On the grounds of performance there is little to choose between vertebrate and arthropod.

Summary

(1) The mechanism of production of smoothly controlled movement by the muscles of insects is discussed in relation to their innervation. The small number of muscle fibers available makes it necessary to seek some different phenomena from those familiar in vertebrates.

(2) Each muscle in an insect is usually supplied by two axons and itself constitutes a complete, single motor unit. Some muscles are however composed of a few units attached to a common apodeme, each unit of which is served by separate axons.

(3) The axons usually supply several end plates to each muscle fiber, evenly distributed along its length. In many cases individual end plates receive branches from each of the two axons.

(4) One of the two axons, the "fast" axon (F), evokes a large, twitch-like response following each impulse. The other, the "slow" axon (S), produces a mechanical response only when impulses in excess of about 15 per second pass down it. "Slow"-fiber responses always consist of smooth and relatively slow contractions; there is a very large increase in the rate of contraction of the muscle with increasing frequency of stimulation of the nerve and also in the final tension reached.

(5) The "fast" fiber innervates every muscle fiber and produces large electrical responses. These are formed of large end-plate potentials and

brief, local, spike responses. In some fibers there is an overshoot of the zero base line.

(6) The "slow" fiber innervates only a proportion of the muscle fibers, sharing end plates with the "fast" fiber. Neuromuscular transmission is effected by end-plate potentials of various magnitudes showing considerable facilitation and summation.

(7) In the execution of natural movements there occur combinations of long trains of "slow"-fiber activity with occasional bursts of "fast"-fiber activity.

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MYOGENIC RHYTHMS

J. W. S. Pringle
Cambridge University

Rhythmic muscular activity is the most characteristic effector action in higher animals. Historically, concentration of attention on the limb muscles of land vertebrates and on other easily isolated muscles in the limbs of arthropods has tended to produce a picture of muscle physiology which may not be valid for the animal kingdom as a whole. This review, while concentrating mainly on insect fibrillar muscle, will concern itself also with other mechanisms by which rhythms of activity can be generated in muscular tissues.

The most convenient physiological classification of muscles is one based on the ideas of Bozler (1948), who first distinguished "long-fibered" muscles, in which each fiber is innervated by branches of a controlling nerve fiber, and "short-fibered" muscles with more diffuse innervation and the possibility of conduction of excitation from muscle fiber to muscle fiber. Within each class there occur striated and unstriated examples, correlated in general with speed of action. Thus, the somatic muscles of most higher animals fall in the first class, whether they are striated as in vertebrates and arthropods or unstriated as in the byssus retractors of lamellibranch mollusks, and vertebrate visceral muscles fall in the second class, whether they are striated as in the heart or unstriated as in the ureter.

Until recently the occurrence of a myogenic rhythm of activity (that is, a rhythm in which nervous tissues play no essential role) was recognized only for the class of short-fibered muscles, and in particular for the vertebrate heart. In 1949 I called attention (Pringle, 1949) to the unusual neuromuscular mechanism which appears to be present in the indirect flight and haltere muscles of the higher Diptera, in which a high frequency of muscular contraction is not accompanied by the synchronous muscle potentials which are characteristic of the activity of other somatic striated muscles. The evidence for the existence here of a novel type of rhythmic mechanism was much extended by Roeder (1951), and at the same time Boettiger and Furshpan (1950, 1952) were demonstrating that the skeletal mechanical system of the fly thorax has by no means the simple lever action described in most entomological textbooks. By the time of the appearance of Chadwick's (1953) chapters on insect flight in Roeder's textbook, it was clear that there was something very unusual in the biophysics of the flight motor in certain higher insects.

Attempts in Cambridge to isolate an indirect flight muscle in order to elucidate the mechanism of the rhythmic activity were unsuccessful, and attention was therefore directed to the sound-producing muscle (the tymbal muscle) of cicadas, which appeared to be histologically similar to the indirect flight muscles. The results of this work (which was done in Ceylon on *Platypleura capitata*) have now been published (Pringle 1954a,b). The hypothesis there put forward is somewhat different to that suggested by the flight-muscle work in 1949; it is that these fibrillar muscles do not differ from ordinary striated muscle in the way in which they develop tension and can shorten after the arrival of motor nerve impulses, but that, when they are connected to a nonlinear elastic system in the skeleton which produces sudden shortening at a critical tension, a process takes place in the contractile machinery ("de-activation by release") which changes its properties for a short time to those of the unexcited muscle. In this "de-activated" condition the muscle can be passively extended by small forces such as the residual elasticity of the tymbal skeleton, and the redevelopment of tension after the deactivation interval (aided perhaps by a sudden stretch at the end of the interval) continues the activity in a myogenic rhythm. It was suggested that Boettiger's click mechanism in the dipteran thorax makes this explanation adequate also for the myogenic rhythm of the indirect flight muscles.

It may perhaps be useful to discuss these ideas in more detail in order to clarify some of the terminology used. Fig. 1 shows diagrammatically the course of events which are supposed to take place when a long-fibered muscle is excited through its motor nerve. The impulse travels along the nerve fiber (1) to the neuromuscular junction (2) where it initiates the junctional phenomena; we are not here concerned with the nature of neuromuscular transmission. The junctional processes lead to a local depolarization of the surface membrane of the muscle fiber (3), which in turn may produce a propagated action potential. In arthropods this appears to be rare, but instead a multiplicity of nerve endings on each muscle fiber produce local depolarizations over a wide enough area of surface to have much the same effect; in any case the depolarization appears to be the event which initiates further changes inside the muscle fiber. These surface changes lead to events of largely unknown nature (4) which finally induce activity in the contractile machinery (5) and may move the skeletal elements (6) and the external environment.

In this complicated and only partially understood sequence of phenomena it is easy to introduce confusion by inexact terminology. I would like to suggest that we should agree on a definition of words. *Excitation,* by analogy with the similar phenomenon occurring in nerve fibers, should be limited to the processes taking place in the muscle surface membrane;

stimulation is already, by custom, the thing done to the excitable tissue and *excitation* is its response. *Excitation* of the muscle fiber leads to an *active state* in the muscle fiber as a whole; this is the unknown process labelled (4) in Fig. 1. Normally the *active state* produces, in turn, an

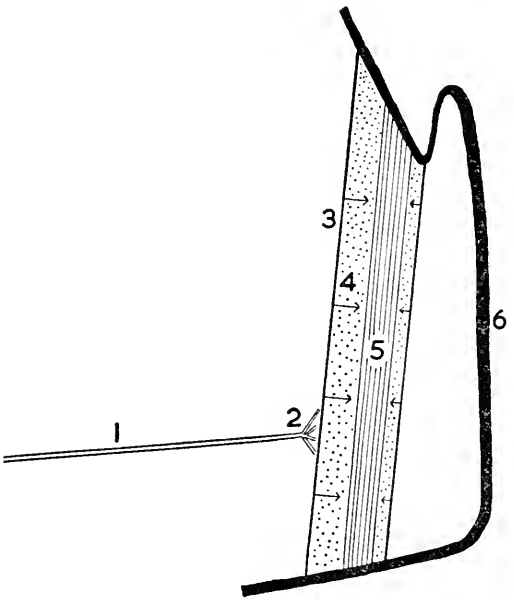

Fig. 1. Hypothetical course of events in excitation of long-fibered muscle through its motor nerve.

activation of the contractile mechanism (5), with development of *tension* and/or *contraction* depending on the nature of the load. A corollary of these definitions would be that *tetanus* means a maintained contraction or a maintained tension and not a maintained *active state* which must be referred to as such. It is unfortunate that the terms used for processes (4) and (5) should be so similar, and process (4) might perhaps better be called *priming* of the contractile machinery by some form of *coupling* to the surface events. The original terms as defined above will, however, be used in this review. Clearly the distinction between *active state* and *activation* is only essential at present for insect fibrillar muscle, in which deactivation by release affects the contractile mechanism but not processes (3) and (4). It may well prove, however, that such a distinction would be useful also for other muscles.

The Evolution of the Myogenic Property of Insect Fibrillar Muscle

Boettiger (this symposium) has evidence that the myogenic type of flight motor occurs in Diptera, Hymenoptera, and Coleoptera and in the smaller Homoptera (but not in cicada flight muscle); it also occurs in the tymbal muscle of cicadas of the genus *Platypleura*. The flight muscles of other insects show the normal 1:1 ratio between nerve impulses and muscular contractions (Roeder 1951). Hagiwara, Uchiyama, and Watanabe (1954) and Hagiwara (1956) have shown that a 1:1 system occurs in the tymbal muscles of certain cicadas, and in a survey of some of the larger Japanese species they have found the fully developed myogenic system only in *Platypleura kaempferi*. In my paper on the physiology of sound production (Pringle, 1954b), I suggested that the tymbal muscle might be an evolutionary development of the metathoracic flight muscles, but I have more recently made a detailed investigation of the anatomy of the sound-production system in the Homoptera Auchenorrhyncha (Pringle, 1956) and am forced to revise this opinion. There is a primitive genus of cicadas, *Tettigarcta*, the two species of which are found in the mountains of Australia, and I have been able to obtain well-preserved specimens of both sexes of each species. In the anatomy of its sound-production system, *Tettigarcta* is intermediate between true cicadas and cercopids, which have been well discribed by Ossiannilsson (1949). The whole sound-production system appears to be morphologically in the first abdominal segment, as correctly stated by Myers (1928); and I have now concluded that sound production has evolved not from the flight system but from a movement made during copulation, which has become converted into a noncontact communication system by the development first of a click system in the first abdominal tergum (cercopids and *Tettigarcta*) and then by the addition of a sound-frequency carrier as the damping of the clicks is reduced by the presence of internal resonant air sacs (true cicadas). There is a correlated development of the receptor organ in these insects, *Tettigarcta* having chordotonal organs, probably sensitive to the click vibrations, in the same morphological position as the tympanal chordotonal organs of true cicadas.

This re-interpretation of the homologies, coupled with Hagiwara's evidence that the myogenic system is present in the tymbal muscles of only some cicada genera, makes it clear that the development of the myogenic rhythm property in connection with sound production is an independent evolution from its appearance in the flight motor system. Probably here also it has evolved independently in the four insect orders in which it occurs. This at once poses two questions:

(1) How is functional continuity preserved in the change over from a 1 :1 to a myogenic system in flight and sound production?

(2) Is deactivation by release a potential property of all muscle, realizable whenever a suitable arrangement is developed in its skeletal attachments, or is there a fundamental difference between the contractile mechanisms of fibrillar and nonfibrillar muscles?

Let us examine the first question, dealing first with sound-production. Functionally, it is not difficult to suggest reasons why a higher pulse frequency is advantageous in the cicada system. Insect ears respond to sound pulses with impulses at the pulse frequency; and, if excitation of the female through this nerve has a sexually exciting function, more intense excitement should be produced by a higher pulse frequency in the male song. Hagiwara (1953) and Hagiwara and Watanabe (1956) have described an alternative method of at least doubling the pulse frequency in the song of *Graptopsaltria nigrofuscata,* where the tymbals on opposite sides click exactly in antiphase, the motor nerve discharge being driven by a ganglionic pacemaker. Physiologically, the requirement for effective deactivation by release appears to be a sufficiently rapid movement of the tymbal at the *in* click; since there is no antagonistic muscle whose contractions must be coordinated with those of the tymbal muscle, extra clicks above the 1 :1 ratio with nerve impulses introduce no difficulties in the mechanism as the system evolves toward greater speed of tymbal movement.

With the flight motor the position is different. The orthopteran case may be taken as typical of the primitive flight systems in insects, and fortunately we have a considerable knowledge of the dynamics and physiology of flight in locusts from the work of Ewer and Ripley (1953) and of Weis-Fogh and Martin Jensen (1956), and in *Periplaneta* from Roeder (1951) and Sotavalta (1954). The physiological picture of locust flight muscles has been much confused by the experiments of Voskresenkaya (1947), discussed by Chadwick (1953). This worker gave electrical stimuli to a locust thorax in which the motor nerves were still attached to the intact ganglion, and reported various types of rhythmic activity from the flight muscles, not always correlated with the stimuli. Ewer and Ripley (1953) were able to show that some of Voskresenkaya's results were due to after-discharge from the ganglion, but they confirmed that there is not an exact correlation between stimulus and contraction frequencies even in the isolated nerve muscle preparation. Electrical recording from the tergosternal muscle showed, however, that the most important phenomenon is a long relative refractory period in the motor nerve so that, with near-threshold intensities of stimulation through platinum wire electrodes, impulses may be generated at only every second or third stimulus as the stimulus

frequency is increased, and the frequency of impulses actually reaching the muscle is always sufficiently low to produce discrete contractions. Only with stimulus intensities well above threshold could the nerve-impulse frequency be raised to the level which produces a smooth tetanus in the muscle. There is thus no evidence in the locust tergosternal muscle of any inherent muscular rhythmicity.

It is probably useful to try to start right from the beginning in a consideration of the evolution of insect flight. There is fossil evidence that the first winged insects were large creatures with well-developed locomotor habits and that their wings first arose as lateral expansions of the terga of the thoracic segments. Wings thus first appeared in insects as new structures and not as a modification in function of an existing appendage as in the birds. At first the lateral thoracic expansions of insects must have provided lift as fixed aerofoils, and a control of their incidence is therefore the first requirement in a system of control. Chadwick (1953) has argued and Weis-Fogh (1956, Pt. IV) has now demonstrated in the locust that the main control of lift in flight is by a reflex system adjusting wing incidence in certain portions of the stroke. This then may be thought to be the primitive control mechanism, deriving from the time when the wings were fixed aerofoils and preserved in the make-up of insects ever since. Weis-Fogh has pointed out to me that a wing-folding mechanism may have been the second feature to appear, since large lateral expansions must have been difficult to manage under all conditions; this also is preserved in modern flapping forms. Only later, perhaps, did the flapping machinery arise. Birds, by contrast, probably flapped their wings from the start, using the locomotor muscles of the fore limbs of their running reptilian ancestors.

The next problem concerns the nature of the rhythmic mechanism. The more primitive insects have flight muscles which give a normal single twitch for a single motor nerve impulse (Roeder's 1:1 muscle), and if the early insects were large there is initially no requirement for a very high frequency of wing beat and for a very short muscle twitch. A number of workers have studied the effect on wing-beat frequency of alterations in the loading and inertia of the wings; in 1:1 systems these also provide evidence about the nature of the neurogenic rhythmic mechanism, since the frequency of motor nerve impulses corresponds to that of the wing beat. The problem is whether the rhythm-generating mechanism is innate in the ganglion or involves reflexes from wing sense organs. Roeder (1951) reports that in *Periplaneta* and in *Agrotis* (Lepidoptera) amputation of the wings produces little change or a decrease in frequency. Sotavalta (1954) confirms that there is no change in *Periplaneta,* but records a slight increase (up to 20%) in Lepidoptera. Tiegs (1955) also finds an increase in various noctuids, Neuroptera, and Isoptera (all 1:1 systems).

Weis-Fogh (unpublished) tells me that in *Schistocerca* the usual result of amputation is a pronounced irregularity in the wing beat, but that some rhythmic activity can still occur when the wings are reduced to mere stumps. One difficulty in intepreting results of this sort on otherwise intact insects is that we now know that the incidence-control reflex must have been operating; and, since loading as well as inertia must affect the magnitude of the forces at the wing base to which sense organs must respond, it is not easy to predict the nature of the change produced by amputation on the form and intensity of sensory excitation. It appears likely, however, that there is, at least in some of the 1:1 groups, a reflex effect from the wing sense organs on the rhythmic mechanism, but that something in the nature of a pacemaker is present in the ganglion capable of discharging rhythmic bursts of motor nerve impulses even in the absence of sensory feedback. It is relevant to note here that in Diptera (with a myogenic rhythm in their indirect flight muscles) amputation produces a fall in the frequency of motor nerve impulses (Roeder, 1951), although it leads to a large rise in wing-beat frequency. In all 1:1 insects it is necessary to suppose that there is a considerable measure of inherent organization in the flight motor centers in the thoracic ganglion producing coordination of activity in the various muscles which move the wings; Tiegs (1955) has emphasized that the evolution of the flight mechanism has resulted in a reduction rather than an increase in the number of muscles involved.

The first rhythmic mechanism in the insect wing system was thus probably of a nature similar to that found in the rhythms of swimming and locomotion, where there is an inherent pattern of coordination in the central nervous system, but where reflex feedback is always very important and may sometimes be essential (toad; Gray and Lissmann, 1946). As some insects got smaller, the frequency of action demanded from this system increased to the point where it became difficult for the flight muscles to perform one complete contraction and relaxation during a single beat. This seems to be the present condition in some Orthoptera, Lepidoptera, and Odonata. Tiegs (1955) has recorded a normal wing-beat frequency of 57/sec. in the hawkmoth *Hippotion*. By direct observation he finds that electrical stimulation at 30/sec. produces a partial tetanus, although complete fusion of twitches is found only at 70/sec. Heidermans (1931) reported a similar condition of partial tetanus in Odonata. Weis-Fogh (unpublished) tells me that in *Schistocerca* stimulation experiments suggest a similar result, but that this is misleading, since there is a rise of temperature of about 8° C in the thorax of a flying locust, and that at the real temperature of the muscles during flight there is time for a complete contraction and relaxation; twitch duration is known to have a high temperature coefficient in frog muscle (Hill, 1951).

It is probably at this point in the story that the click mechanism, so beautifully demonstrated by Boettiger in Diptera, begins to be important. Weis-Fogh (unpublished) has recently obtained some results which help greatly for an understanding of the origin of this peculiar feature of the wing articulation. He has shown that in the thorax of *Schistocerca* the relationship between applied force and wing displacement is not linear in either wing, and that in the hind wing there is a range of instability which amounts to a click mechanism; the fore wing shows what may well be an earlier evolutionary stage. This demonstration of a click mechanism in an insect whose flight muscles are of the 1:1 type suggests that even here we may have some measure of deactivation by release in the flight muscles. The instability has the obvious function of increasing the velocity of wing movement above that obtainable with a simple lever action, but it may also assist muscular relaxation. If it could be confirmed that there is a similar instability in the lepidopteran wing articulation, the difficulty of a partial tetanus in the flight muscles would be resolved; for the deactivation process could remove the tetanic tension during the phase of elongation of the muscle, and the redeveloped tension once deactivation had worn off would assist the true twitch tension in the next stroke.

The evolutionary picture is thus of an increase in the neurogenic frequency of wing beat beyond the limits set by tetanic fusion in the muscles, with partial deactivation by release ensuring separate contractions. From this to a myogenic rhythm is a small step. Once the myogenic alternation is assured, the motor nerve impulse frequency can drop back to a low level, with a continued evolution of high-frequency muscular activity. The articulation mechanics now continue to evolve in some orders to allow increasingly isometric contraction of the flight muscles, apparently a necessity for the very high-frequency wing beats of the smaller Diptera and Hymenoptera. In the higher Diptera the indirect flight muscles do not shorten when detached from the exoskeleton (Tiegs, 1955), and give the appearance of being inexcitable by electrical stimuli applied in the mutilated thorax whose elastic properties have been disturbed. The Homoptera have not evolved so far in the direction of isometry; Tiegs (1955) finds that in the jassid *Eurymela* faradic stimulation produces an easily visible shortening of the tergosternal flight muscles, although they have the typical fibrillar structure of myogenically rhythmic muscles.

Parenthetically it is interesting to consider the cicadas, whose flight muscles, unlike most of the Homoptera, are of the 1:1 type. Tiegs (1955) emphasizes that in *Cyclochila* (Cicadidae), as in *Siphanta* (Flatidae), the flight muscles are intermediate in histological structure between normal insect muscles and the fibrillar type. Cicadas and other Homoptera are known (Snodgrass, 1927) to have a peculiar anatomical arrangement of

their mesothoracic tergal muscles. Part of this muscle has the normal longitudinal arrangement and is functionally a wing depressor. But another part, more laterally situated, has an oblique orientation due to the carrying down of its posterior attachment on the very well-developed mesothoracic phragma; this muscle is the main wing levator. Such a reversal of the function of a muscle would be almost impossible to understand if functional continuity is to be preserved in a 1 :1 excitation mechanism; it would imply a change over of ganglionic connections of an unprecedented nature. If, however, the cicadas have passed in their evolution through a myogenically rhythmic stage, there is no difficulty in understanding such a change in the timing of the muscular contractions; in a myogenic system the timing of contractions is determined by the mechanical conditions, not by the ganglionic connections of the motor nerves. The fact that Homoptera have not evolved very far towards an isometric contraction of their flight muscles has thus allowed the cicada muscles to revert to the 1 :1 excitatory mechanism after the oblique tergal muscle had assumed its modern orientation and role.

Tiegs (1955) has described and illustrated some further features of the histology of fibrillar flight muscle which are relevant to this evolutionary story. He has resolved the problem of the sarcolemma, described by recent workers as being absent in the bee and in *Drosophila*. It is, in fact, present in all cases and is a true cell membrane; the discrepancy has arisen from the fact that many apparent "cells" in the transverse section are merely areas delimited by intracellular tracheae within a very large cell. He has established that the fibrils which can be isolated even from fresh muscle are bundles of myofibrils for which he proposes the term "sarcostyle"; each sarcostyle in Diptera is formed ontogenetically by the incorporation of a nucleated myoblast into the syncytial muscle cell. Finally he has shown that there is an important difference in the histology of the motor nerve ending as between normal insect muscle and the dipteran fibrillar type; in normal muscle the motor nerve ends in "Doyère cones" (apparently analogous with the vertebrate end plates) on the surface of the fiber, but in dipteran fibrillar muscle the nerve actually penetrates the giant muscle cells and there are no end plates. How this last observation is to be correlated with the muscle spikes recorded by Pringle (1949) and Roeder (1951) is not clear, but it may represent yet another peculiar feature of these very remarkable tissues.

Myogenic Rhythms in General

The occurrence of a myogenic rhythmic system in many different orders of insects suggests, as has already been stated, that it has evolved many times. It is always, so far as is known, correlated with the presence of large

sarcostyles, a sarcoplasm of low viscosity and with numerous large sarcosomes. Perhaps the most striking case, for which we have at present only histological evidence, is in the delphacid *Perkinsiella* (Tiegs, 1955), where a tergal abdominal muscle appears to have been drawn into the complex of flight muscles as a wing levator and to have become fibrillar in structure. It seems clear that in insects, at least, there is a potential deactivation-by-release mechanism present in all striated muscle. Is this true of all muscles in the animal kingdom?

It is important to emphasize first of all that this type of myogenic rhythm has little in common with the well-known myogenic rhythm of the vertebrate heart. In insect fibrillar muscle, the rhythm derives from a property of the contractile fibrils in the interior of the muscle fiber, and there is little or no synchronous change in the permeability properties of the external membrane as measured by electrical potentials across the cell surface. In heart muscle, on the other hand, the rhythm of mechanical activity is accompanied by a rhythmic change in membrane potential, and it seems to be established beyond reasonable doubt that the rhythm is determined by these membrane properties. Del Castillo and Katz (1955) and Hutter and Trautwein (1955) have independently demonstrated that the cardio-inhibitor and cardio-accelerator fibers in the vagus and sympathetic nerves produce their effect by modification of the membrane potential of the heart muscle fibers in the usual directions; the spikes of activity originate at a critical level of spontaneous slow depolarization during diastole. At least in the mammalian heart it is also clear that the mechanical stimulus of stretch produced by an increased venous return does not affect the frequency of beat in the denervated heart; control of frequency of beat is a reflex phenomenon from mechanoreceptors in the great veins and aorta.

In some vertebrate smooth (short-fibered) muscles we also have a myogenic rhythm (Bozler, 1948), and here mechanical conditions do affect the frequency of contractions. Our knowledge of the physiology of these tissues has lagged seriously behind that for striated muscle, but it seems probable that here, as in the heart, the rhythmicity derives ultimately from rhythmic properties of the cell membranes; the form of action potentials is similar to those of heart muscle. Conduction of excitation can take place through the "muscle net" without the involvement of nerves, but is not all-or-none as in the heart; whether the effect of mechanical stimuli is mainly on the membrane properties of the individual muscle cells, or on the intercellular conduction mechanism, remains to be elucidated.

Myogenic rhythms also occur in the hearts of some arthropods (Krijgsman, 1952) and mollusks (Krijgsman and Divaris, 1955); we have no knowledge of the membrane potential changes in these tissues, but their

rhythmicity is affected by stretch. Pharmacological evidence suggests that the rhythmicity originates in the surface membrane.

A somewhat different type of myogenic rhythm occurs in the embryonic skeletal muscle fibers of various lower vertebrates (Harris and Whiting, 1954; Harris, 1955). In the embryo dogfish the myotome muscle fibers show rhythmic contractions before motor or sensory nerve fibers reach them. The contractions are regular and synchronous down the whole column of one side, but there is no correlation in the timing of contractions on the two sides. At this stage acetylcholine accelerates the rhythm. When nerve fibers first reach the muscles they have what Harris calls a "neurocratic" action; the frequency of the myogenic rhythm is increased, still without bilateral coordination. Finally true coordinated movements occur at about the time the sensory nerve supply develops. Electrical recordings have not been made from these delicate tissues, but Whiting (personal communication) reports that they are unexpectedly inexcitable by electrical stimuli. Were it not for this observation one would be tempted to conclude that here again the rhythmicity comes basically from the cell membranes; the influence of acetylcholine and the exact synchrony of contractions down the whole column of one side are difficult to explain except in terms of an impulse mechanism, propagated from muscle cell to muscle cell; there is a significant overlap of fibers across the myotome boundaries at the myogenic rhythm stage, which disappears later.

Is there, then, anywhere in the animal kingdom other than the insects any sign of a rhythmically contractile tissue in which the rhythm does not derive from properties of the cell surface? Rhythmic movement in the lower animals is usually required for swimming, and Gray (1953) has recently reviewed his work on this type of propulsion. We know virtually nothing about the neuromuscular physiology of these movements in any invertebrate and it is dangerous to argue too closely by analogy from fish and snakes. Gray (1951, 1955) has shown that even in bacteria and spermatozoa there is a great similarity in the dynamics of swimming to the movements of higher animals, and there is thus probably a strong functionally conditioned isomorphism between all propulsive mechanisms of this type. In bacteria and spermatozoa it is hardly possible to conceive of anything akin to a reflex responsible for rhythmicity or coordination, and here at least there is a strong prima facie case for looking in the mechanism of contraction for the origin of the rhythm. There is no device here for any sudden release of tension in an active tissue, and if the rhythm is myogenic the effective mechanical stimulus must be a relatively slow one. The possibility of a nonnervous origin for the rhythm of swimming should not automatically be excluded even for Metazoa; a lot could be achieved by a myogenic rhythm with "neurocratic" control.

The Mechanism of Deactivation

In Pringle (1954a) I discussed briefly the possible relationship of the phenomenon of deactivation by release in insect fibrillar muscle to the mechanical properties of vertebrate striated muscle. It is well known that a sudden release given to an excited muscle leads, if its amplitude of release is sufficient, to the complete disappearance of tension, followed by its redevelopment at a rate comparable to that at the beginning of excitation. A. V. Hill has interpreted this and other results in terms of a "series elastic component" in the muscle which goes slack when there is a sudden release. In the experiments of Gasser and Hill (1924) on the frog sartorius muscle, a release of 10% of the resting length was required to produce a tension drop to zero, but Hill (1950) states that a smaller figure would have been obtained if due allowance had been made for the elasticity of the suspension. This disappearance of tension on quick release resembles that found with the cicada tymbal muscle, but the fact that the tymbal muscle is thereupon re-extended to its initial length without immediate reappearance of the original tension makes it impossible to explain the result simply in terms of a series elastic component. The deactivation process here occurs with a release of 1.5% of the resting length, showing that any series elastic component present is very small, a result perhaps understandable in terms of the different histology of the muscle attachments in insects and vertebrates.

Any hypothesis about the nature of deactivation by release must take into account the normal features of muscular contraction (Wilkie, 1954), the extensive studies of glycerinated muscle fibers (Weber and Portzehl, 1954; Morales, Botts, Blum, and Hill, 1955), and the pecularities of fibrillar muscle (Tiegs, 1955), including the enzymatic properties of the large sarcosomes (Watanabe and Williams, 1951). In the absence of any general agreement on the nature of the biochemical and biophysical machinery involved in contraction, in spite of intensive current study, hypotheses are particularly vulnerable but may nevertheless be attempted in the hope that the properties of this tissue may throw light on the general problem.

A generalized if somewhat naive view would be that contraction and tension development occur in a muscle because excitation in some way allows access to certain sites in the actomyosin complex of a high-energy substance liberated by the sarcosomes. It is already clear that maintenance of tension in striated muscle involves the continuous expenditure of energy, and the sites are therefore presumably occupied and vacated cyclically, with breakdown of the high-energy substance. The fact that the additional heat of shortening is proportional to the distance shortened could be explained

if the rate of vacation of the sites is directly proportional to the distance through which the muscle has shortened; in addition to the number required in any unit of time to maintain tension, a further number of high-energy molecules would then be needed to achieve shortening.

One hypothesis to account for deactivation by release can be stated as follows. If a sudden shortening occurs in a muscle due to the sudden release of tension, a large number of sites on the actomyosin complex will be suddenly vacated. Supply of high-energy molecules by diffusion from the sarcosomes may be inadequate to provide for their re-occupation immediately following this large demand, and the state of the muscle would then become temporarily similar to the inexcited condition. This would produce deactivation by release. Whether or not the deactivation phenomenon will occur depends, according to this hypothesis, on the relative rates of vacation of active sites by rapid shortening and of resupply of high-energy molecules by diffusion from the sarcosomes. If the maximum possible rate of vacation is low and the diffusion pathway small, complete deactivation can never occur. If the rate of vacation is large and the diffusion pathway relatively long, deactivation may last for sufficiently long to allow the myofibrils to be re-extended to their initial length and a myogenic rhythm of activity becomes a possibility. A rough calculation of probable ATP diffusion rates over the distances involved shows that a time lag of a few milliseconds in re-establishing the local concentration in the myofibrils is a possibility.

Consistent with this hypothesis is the histological observation that the "fibrils" are thick in insect muscles showing a myogenic rhythm. Tiegs (1955) has shown that the fibrils which can be isolated from these fibrillar muscle are, in fact, sarcostyles composed of several myofibrils, but this does not alter the fact that the large sarcosomes are situated between the sarcostyles with a relatively long diffusion pathway. The diameter of the sarcostyles in fibrillar muscles of Hymenoptera, Coleoptera, and jassids is from 3 to 5μ, by contrast with values of less than 1μ for orthopteran wing muscles, insect limb muscles, and vertebrate striated muscle. This difference and the large sarcosomes are the only known histological features consistently associated with the myogenic rhythm property.

A hypothesis of this sort demands no sudden change in the basic mechanism of contractility in the change from the 1:1 state to the myogenic rhythm, and there is thus no difficulty in the occurrence of rhythms in many different evolutionary lines in the insects. It makes no suggestion about the nature of the *active state* in the muscle fiber during which access to the sites on the actomyosin complex is possible by the high-energy molecules. Pringle (1954a) showed that the duration of the active state following the arrival of a single nerve impulse is not markedly different, whether

the mechanical response is a single twitch (isolated muscle) or a train of myogenic contractions.

This first hypothesis has been stated in terms of a complex between actomyosin and the high-energy molecule leading to contraction or the development of tension. According to Weber and Portzehl (1954) the evidence from the glycerinated muscle model is that tension is produced when ATP bound in the fibers is split; ATP whose splitting is prevented by —SH poisons is the most effective plasticizing agent for the fiber model. The resting state is with ATP bound to the actomyosin and the fibrils fully plastic and extensible. In the fiber model ATP splitting is inhibited at the concentrations above a certain value but still in the physiological range. Weber supposes that, when the muscle is brought into the active state, ATP splitting starts because of a shift in this critical inhibitory concentration and tension then develops.

A second hypothesis may now be stated consistent with the view that tension development accompanies the splitting and not the binding of ATP. Again it must be supposed that the rate of splitting depends on the rate of shortening. A sudden shortening on quick release therefore produces a large and nearly synchronous splitting of ATP, and the re-occupation of the sites gives an initial plasticizing effect; the muscle therefore behaves as if it was relaxed for a short interval of time until splitting again occurs. Possibly the sudden stretch of the *out* click of the tymbal or the opposite stroke of the wing again initiates splitting and the development of tension. It is necessary in order that this cycle of events shall occur that the splitting of ATP with the development of tension does not immediately follow the binding of ATP on the actomysin complex, so that the re-occupation of the sites by ATP shall have time to produce a plasticizing effect before splitting has again proceeded far enough to produce an appreciable tension. There is here no diffusion lag producing the oscillation; the necessary condition is that the splitting shall follow the binding of ATP with a finite time lag even in the active state.

A lag between binding of ATP and splitting with development of tension is not inconsistent with the result of experiments on normal striated muscle. When a vertebrate striated muscle is excited by stimulation of its motor nerve, the twitch tension does not appear for several milliseconds. Hill (1949) has explained this lag in terms of the stretching of the series elastic component, and has shown that, if quick stretches are given in addition to stimulation, a change in the mechanical properties of the muscle can be detected much earlier; he concludes that activation starts almost immediately upon excitation. It would be equally possible to interpret this experiment as indicating a normal slow development of activity in the contractile mechanism, but that quick stretch accelerated the activation. There

is, so far as I am aware, no other evidence that activation occurs in vertebrate muscle more rapidly than is indicated by the development of tension. Experiments by Weber and others on the glycerinated fiber model are unable to produce evidence about the initial rate of splitting of ATP and the development of tension since the time course of events is here always limited by inward diffusion of ATP into the model.

The structural peculiarities of fibrillar muscle would be correlated, on this type of hypothesis, with a higher degree of lateral association of muscle elements than in normal muscle, so that plastic behavior is reduced to a minimum; mechanical events are thus transmitted more completely to the contractile machinery and synchronization of the energy-yielding cycles is more perfect after quick release. Boettiger's evidence (this symposium) of a high coefficient of elasticity in passive flight muscle may point to the correctness of this correlation. Clarification of the biochemical nature of the deactivation cycle must, however, await further quantitative studies.

Summary

(1) Myogenic rhythms of activity have been described in the heart and certain visceral short-fibered muscles of vertebrates, in the hearts of mollusks and some arthropods, in the striated trunk muscles of the dogfish embryo, and in the indirect flight and tymbal muscles of certain insects (fibrillar muscles). They may also occur in the swimming movements of micro-organisms and spermatozoa.

(2) In vertebrate short-fibered muscles the rhythmic property resides essentially in the surface membrane of the fibers; potential changes are observed synchronous with the contractions. In insect fibrillar muscles myogenic rhythmicity normally occurs in the presence of a nonlinear elastic ("click") mechanism in the muscular coupling, and depends on deactivation of the contractile fibrils, with no synchronous potential changes in the fiber membrane. The mechanism of rhythmicity is unknown in dogfish embryo muscle and in the contractile filaments of micro-organisms and spermatozoa.

(3) Fibrillar structure is correlated with myogenic rhythmicity in insects, but has evolved independently several times in the motor systems for flight and sound production. In Homoptera, whose sound-production mechanism in the first abdominal segment may be derived ultimately from movements during copulation, the course of the evolutionary change from a neurogenic to a myogenic rhythm can be understood without difficulty. In the flight system it is suggested that the deactivation phenomenon was first useful as a method of achieving a high-frequency rhythm of neurogenic wing beat and later led to a myogenic rhythm in several different orders.

In cicadas the flight muscles have possibly evolved through a myogenic stage back to a 1 :1 system.

(4) Two tentative hypotheses are put forward to explain the phenomenon of deactivation by release. One depends on a longer diffusion pathway between sarcosomes and the interior of the sarcostyles in fibrillar muscle; the other is based on Weber's hypothesis that the splitting of bound ATP provides the immediate energy source for the contraction of the actomyosin complex in the myofibril, and postulates a lag between ATP binding and ATP splitting to account for the deactivation interval.

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THE MACHINERY OF INSECT FLIGHT*

EDWARD G. BOETTIGER
University of Connecticut

Nature, in the course of evolution, has experimented with the problems of heavier-than-air flight. Three different solutions have proven successful, and today birds and insects share with man the control of the air. Once in possession of a supplementary power source, man required only knowledge of the principles of flight to develop the airplane. The flying machinery of birds and insects could furnish necessary clues, and so during the latter half of the nineteenth century, many studies of animal flight were undertaken. These provide the background of our present knowledge.

Among birds, the mechanical aspects of the flight mechanism are quite similar, while insects exhibit a variety that is obvious even to the casual observer. The presence of an exoskeleton makes possible different mechanical arrangements to couple the driving muscles to the wings and gives to insects the remarkable features of their flight: rapid starts and changes in direction, hovering, and in some cases even backward flight. Insect flight machinery includes many tiny structures moving in intricate designs, and the muscles that move them. Our knowledge of this complex mechanism is still only fragmentary, but already new and exciting physiological adaptations have been found. The intent of this paper is to consider in some detail the most interesting of these—after a brief introduction to the general features of insect flight. The appearance in recent years of an excellent review by Chadwick (1953) relieves me of the necessity of dealing with the earlier work. As a source of information on power output of insects in flight, one should study the beautiful experiments of Hocking (1953) and of Weis-Fogh (unpublished); and for flight muscle histology, morphology, and development, the recent work of Tiegs (1955).

CLASSIFICATION AND DISTRIBUTION OF FLIGHT MECHANISMS

To sustain an insect in flight at its normal cruising speed requires the consumption of relatively large quantities of fuel by the driving muscles. Only 3-5% of the food energy can be used to give momentum to the air flowing through the wings (Hocking, 1953). That the air flow can be

* The original work discussed here was begun and carried out through several summers in collaboration with Dr. Edwin Furshpan. Other students who have participated in various phases of the work include Frances McCann, Richard Baranowski, William McEnroe, and Rudolph Pipa. The generous financial assistance of the National Institutes of Health made the study possible.

maintained during 85% of the wing cycle (Williams and Galambos, 1950) results from the very complex pathway the wings traverse, as was noted by many earlier investigators. The movement cycle, producing the necessary precise changes in angle of attack, depends upon the proper and constantly changing relations among the structures of the articulation in their horizontal and vertical movements. The mechanical features are intimately related to the physiology of the flight muscles; for they determine the limits of length change and to some degree the tension and the rate of change of tension in the muscles. Each type of flight machinery encountered in insects is therefore an integrated, well-adapted system.

The following components constitute the flight machinery: (1) the wings; (2) the wing articulation, including the direct muscles that control the setting of the articulating parts, the base of the wing, and the structures that relate it to the major portion of the thorax; (3) the thoracic component or special parts of the thorax that serve to couple the driving (indirect) muscles to the articulation; and (4) the driving muscles. Since it is not possible to treat here all of these adequately even for one type, the wings and the aerodynamic problems of insect flight will not be discussed.

On the basis of the presence or absence of the thoracic component, the flight mechanisms may be divided into two types. In the more primitive type, the muscles that furnish the power to move the wings are attached directly to the articulation, as in the Odonata or dragonflies, where each of the four wings possesses a set of elevators and depressors (Sargent, 1951). Although it is known that the fore and hind wings move in opposite phase (Chadwick, 1940), there is no information on the nervous control of the muscles or on the details of their movement. The muscle is reported to be in incomplete tetanus when stimulated at flight frequency, indicating that only part of the possible tension change is useful (Tiegs, 1955). The tension in a tetanus is little more than in a twitch. As found in a number of other insect muscles, the protofibrils are organized into sheets or lamellae separated by mitochondria and sarcoplasm. No true fibrils are present and so these muscles are termed lamellar muscles.

All other flying insects possess the thoracic component, coupling special indirect muscles to the wing articulation. These muscles, especially in the higher insects, almost completely fill the thorax and may be of three general types: (1) lamellar; (2) microfibrillar in which the protofibrils are organized into fibrils which have a diameter of about 1.5 μ in the freshly teased preparation; and (3) fibrillar with large fibrils averaging about 3.0 μ in the fresh state (Pipa, 1955). This classification is certainly not a rigid one but is useful for purposes of discussion. In some flight muscles, in fact, the protofibrils appear to be organized into both lamellae and microfibrils in the same cells (Tiegs, 1955).

Lamellar muscle is found in Odonata and certain Orthoptera (Blattidae, Mantidae) which do not have typical longitudinal muscles attached to a well-developed phragma. This muscle type apparently appeared early in the insects. In many softer-bodied insects, as the Lepidoptera, and in those with two sets of longitudinal muscles, as the Ephemeroptera and Locustidae, microfibrillar muscle is found. The insects with the most spectacular flight ability have harder cuticles and fibrillar muscle (Hymenoptera, Diptera, Coleoptera, Hemiptera, and many Homoptera).

The present evidence supports the idea that two very different flight mechanisms have evolved among the insects, one associated with presence of microfibrillar or lamellar muscles and one with fibrillar muscle. In the former type, little is known of the mechanical aspects of flight or of the physiology of the driving muscles. Where studies have been made (Roeder, 1951), it is evident that the wing stroke is synchronous with nerve stimulation of the muscle. This stimulation is often a single impulse but may be multiple (McCann, unpublished). The flight muscles show little summation and each stroke is either a twitch or brief tetanus. This mechanism we have termed the synchronous type.

The existence in insects of some sort of peripheral control of wing movement was suggested by the experiments of de Geer (1776), who found that on removing the wings of some insects, thereby unloading the muscles, the wing beat frequency greatly increased. Pringle (1949) demonstrated that the action potentials appearing in the thorax of flies during flight were not correlated with the wing movements. By simultaneous recording of these potentials and of thoracic movements, Roeder (1951) confirmed this observation for several species of Diptera and Hymenoptera. More recently this behavior has been found in Coleoptera, Homoptera, and Hemiptera. This second type of flight mechanism we term asynochronous.

Only in the Homoptera is there evidence for the existence of both synchronous and asynchronous types in one order. The extensive study of this group by Tiegs (1955) suggests a step in the evolution of the asynchronous mechanism. He finds that in the cicadas the synchronous flight muscle arises by the multiplication of a few rudimentary muscle fibers. In the other Homoptera studied, formerly free myoblasts become incorporated into the young fibers. The myoblasts extend along the growing fiber, adding new fibrils. In jassids each myoblast adds one new fibril. In cercopids the muscle starts to develop by cleavage of functional nymphal fibers or of rudimentary fibers arising in the early instars. Then the free myoblasts become incorporated into these young fibers.

With this information it is tempting to speculate that it is only from the free myoblasts that fibrillar muscle can be formed. The apparent presence of both mechanisms in the cercopids might then result from the fact that

this group shows an intermediate condition with respect to the formation of the muscle, the flight muscles developing from both preformed fibers and free myoblasts.

The asynchronous system presents a basic problem in muscle physiology, for excitation and contraction appear to be dissociated. The key to the solution of this problem lies in the peculiar mechanical system in which the muscle operates. We must therefore begin our analysis with the mechanics of the flight.

The Mechanics of Flight

When flies are placed in a jar containing CCl_4 vapors, flight movements are induced at a certain level of anaesthesia. A number of other volatile compounds will produce a similar result, but only in CCl_4 does the flight tone sharply increase in frequency. At a certain level the movements become erratic and sputtering, suddenly coming to a stop with the wings either up, as at the end of a normal up stroke, or down, as at the end of a down stroke. All intermediate positions are unstable. Considerable resistance is encountered if one attempts to move the wings from the stable positions. However, when a critical point is reached, the wings snap without further aid to the end of the stroke (Boettiger and Furshpan, 1952). Because of its similarity to the action of a common noise maker this was called the click mechanism (Boettiger and Furshpan, 1950).

Movements of the wings under CCl_4 can best be achieved by pressure upon the scutellum. The remarkable feature of these wing movements is that the wings, on being snapped up and down, appear to follow the normal flight path with proper changes in angle of attack and in direction during the stroke. Evidence for a similar snap action during flight was obtained, and so it was suggested that CCl_4 by its effect on the flight muscles, direct, indirect, or both, sets the articulation as in normal flight. Therefore, if the positions of the structural components are studied in CCl_4-treated flies with the wings in the up and in the down positions, the mechanics of flight may be worked out (Boettiger and Furshpan, 1952).

The mechanical system moving the wings in flight is composed of the articulation and the thoracic component. As these are bilateral structures, their movements will be described on only one side. The thoracic component shown in Fig. 1 consists of the scutellar lever and the tergopleural elements that move it, the anterior notal process with the parascutum to which it is attached, and the mesopleural process.

Scutellar Lever: The tergum or upper part of the thorax is hinged at *j-k* and rests at its posterior end on the joint at *i*, Fig. 1A. On the down stroke contraction of the longitudinal indirect muscle (5, Fig. 2) pulls the articulation *i* of the scutellum *a* forward and slides the tergum posteriorly,

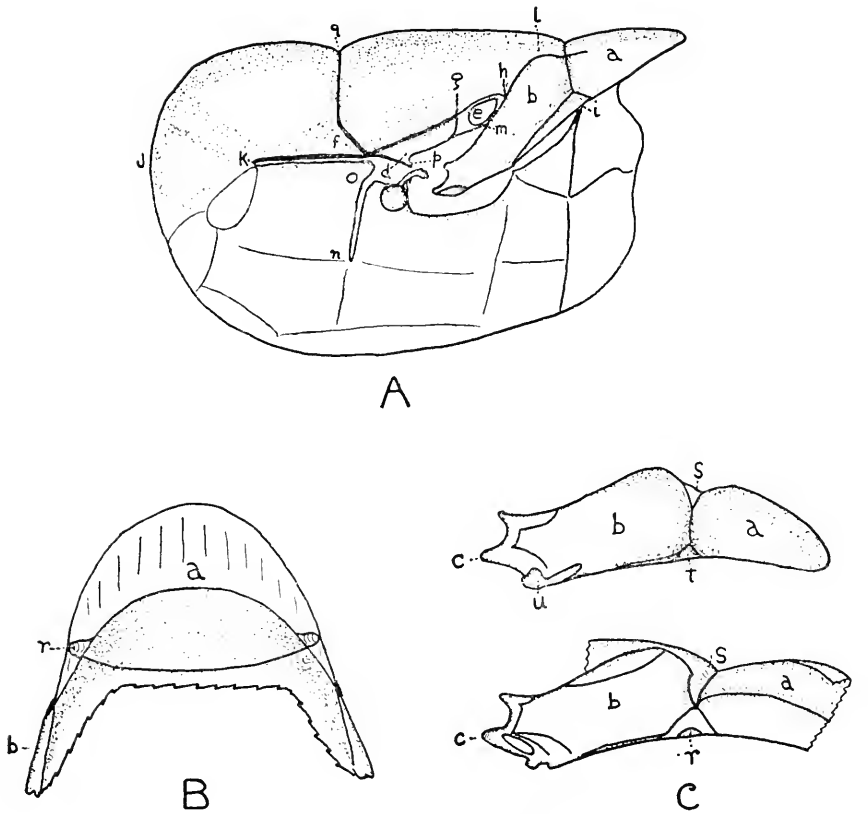

Fig. 1. A. Left side of thorax of *Sarcophaga bullata*. B. Ventral view of scutellar lever dissected free from the thorax. C. Detail of lever arm and attachment to scutellum, outside above and inside view below: a, scutellum; b, lever arm; c, process articulating with the first axillary sclerite; d, anterior parascutum; e, posterior parascutum; f, junction of prescutal ridge, transverse ridge and parascutal hinge; g, end of parascutal hinge; h, point in line of attachment of notum with lever arm; i, point of rotation of the scutellar lever on the postnotum; j-k, fulcrum of the notum lever in the action of vertical muscles; l-m, line of attachment of notum with lever arm; n-o, lateral vertical cleft; p, anterior notal process; q, transverse ridge; r, articulating groove for attachment of postnotum; s, scutellar bridge; t, triangular structure supporting articulation of lever and postnotum; u, groove for a process of first axillary sclerite.

thus rotating the scutellum downward. The forward-projecting arms *b* of the scutellum act upon the wing articulation to force the wings down. The shortening of the vertical muscles (1, 2, 3, Fig. 2) pulls the tergum down and forward, forcing joint *i* of the scutellum posteriorly. By this action the longitudinal muscles are restretched, the scutellum is rotated upward, and the arms of the scutellum move down, thereby forcing the wings up.

This lever-like structure, moved by the action of the indirect muscles on the tergum and on the joint of the scutellum, is termed the scutellar lever. By the action of this lever, the wing-tip movement is amplified about 20 times. The two wings move together since the lateral arms are quite rigidly connected through the scutellum. Reducing this structural rigidity, by simply removing the soft cuticle forming the top of the scutellum, destroys the ability for sustained flight.

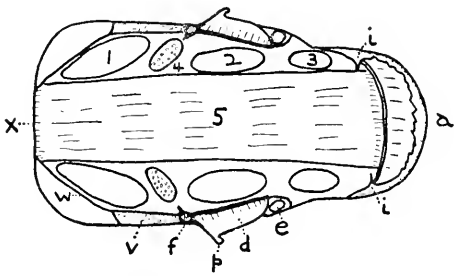

Fig. 2. Horizontal section through the notum. Structures labeled as in Fig. 1, with the following additions: v, prescutal ridge; w, chitinous supporting structure; x, anterior hardened plate; 1, first dorsoventral muscles; 2, second dorsoventral muscles; 3, oblique dorsal muscles; 4, tergal remotor muscles; 5, longitudinal muscles.

Anterior Notal Process: The anterior notal process (*p*, Figs. 1, 2) in flies is supported by and moves in conjunction with the parascutum (*d* and *e*, Fig. 2) and so these must be treated together. The anterior notal process is strongly supported by the termination, *f*, of the prescutal and transverse ridges. The parascutum is hinged to the rest of the tergum between *f* and *g* so that the anterior notal process can be freely rotated up down but cannot twist. The presence of this hinge makes it impossible for the tergum to produce up and down movements of the anterior notal process except through the scutellar lever.

Mesopleural Process: The mesopleural process is a pleural derivative to which is attached one end of the second axillary sclerite (Fig. 3). It serves as a fulcrum for the rotation of the sclerite. At the end of each stroke it moves in closer to the tergum, thereby increasing the wing-stroke amplitude.

The second mechanical component is the wing articulation, consisting of the 1st and 2nd axillary sclerites, a number of other structures of secondary importance for wing movement, and the direct muscles of flight. The articulation contains elements for folding the wings as well as those concerned in the wing cycle. The basic features of its operation, however, depend upon the movements of the 1st and 2nd axillary sclerites and their relations to the elements of the thoracic component described above. These relations are illustrated in Fig. 3. The space between the hinge *h*, connecting the parascutum to the lateral border of the tergum, and the mesopleural process *a* (Fig. 3) is bridged by the parascutum (including the anterior notal process) and the 2nd axillary sclerite. At *k* the radial vein of the

wing is tied in with the anterior notal process and the 1st axillary *e*. The wing cycle reflects primarily the movement of this point of union. On the down stroke, *k* is moved up by the action of the scutellar lever on the 1st axillary sclerite, and moved posteriorly, due to the movement of the tergum transmitted through the anterior notal process and the parascutum. As a result the wings move down and forward with proper changes in tilt. On the return stroke the reverse action of the parts brings the wings back to the up position. The union point *k* moves in a cycle as it follows different paths on the up and down strokes.

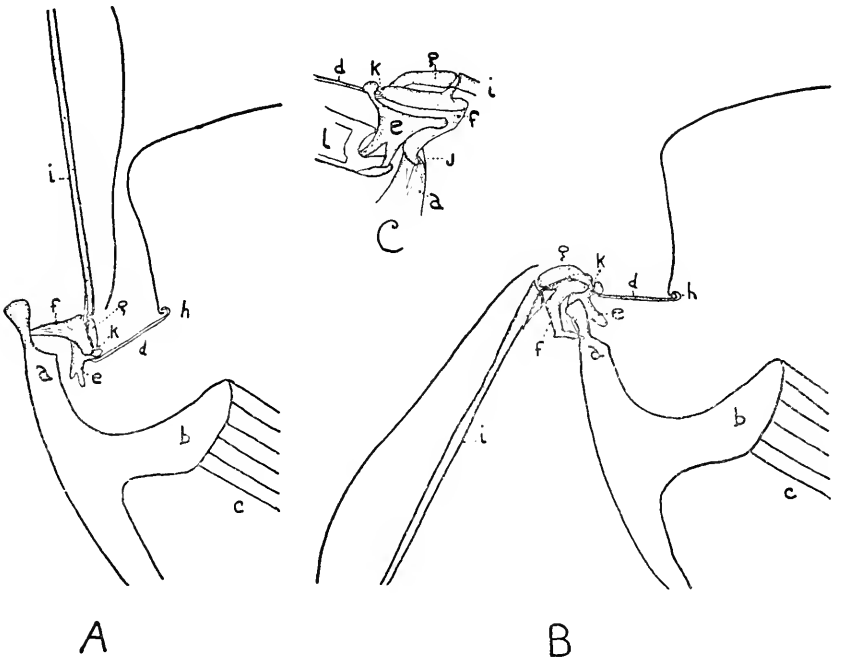

Fig. 3. Cross-sectional view of thorax showing details of the articulation of the right wing. A. Wing in up position, anterior view. B. Wing in the down position, anterior view. C. Posterior view of the axillary sclerites of right wing showing their relation to the mesopleural process, the lever arm, and the anterior parascutum: a, mesopleural process; b, pleural apophysis; c, anterior pleurosternal muscle; d, anterior parascutum; e, first axillary sclerite; f, second axillary sclerite; g, base of radial vein; h, hinge; i, radial vein; j, hook articulation; k, point of articulation of anterior notal process, first axillary sclerite, base of radial vein, and second axillary sclerite; l, end of the lever arm.

When the articulation is set for flight or by the action of CCl_4, the hinge of the parascutum and the mesopleural process are brought closer together. This results from an outward thrust at *f* (Fig. 1), the junction of the prescutal and transverse ridges, produced by tension in the indirect muscles,

and an inward force on the mesopleural process resulting from its structural connections and the contraction of the pleurosternal muscle. At the beginning of the next stroke, whether an up or a down stroke, *k* is moved by the action of the scutellar lever acting through the 1st axillary sclerite. The hinge of the parascutum and the mesopleural process are forced apart to accommodate the full width of the parascutum and 2nd axillary, storing potential energy by straining the tergum and the mesopleural process. At the critical point, where *k* moves past the direct line between the hinge *h* and the hook articulation of the 2nd axillary sclerite on the mesopleural process, the union point *k* is forced strongly toward its extreme position by the recoil of the strained elements, releasing the energy stored at the beginning of the stroke. By the wing articulation and the thoracic component, therefore, changes in length of the indirect muscles are magnified 400-600 times and the muscle can operate almost isometrically.

The foregoing is a simplified and brief statement of the mechanical events of the wing cycle of *Sarcophaga bullata* Parker as analyzed in CCl_4-treated flies. Other factors, such as the secondary wing articulation, the posterior notal process, the basalar and the subalar, may also play important roles.

To obtain further information and to check the conclusions made on CCl_4-treated flies, studies were made of the flight mechanism in action. If the description given above holds for normal flight, the following can be expected: (1) the anterior notal process must move in and out for each up stroke or down stroke; (2) the mesopleural process must also move in and out each stroke; (3) the movements of the scutellum should accurately reflect, as a built-in isotonic lever, the changes in length of the driving indirect muscles during flight.

Tiny pieces of mirror silver were fastened to the thorax of flies with wax, one on the side of the mesopleural process, one on the tergum just above the parascutum, to indicate the in and out movement of the anterior notal process, and one on the side of the scutellum to show wing position. A light beam, reflected by the three mirrors, was brought to focus on moving film in a Grass kymograph camera. By careful adjustment the three spots could be brought close together, but not in a vertical line. Therefore, some means of obtaining simultaneous ordinates in the three records was necessary. A two-bladed fan driven at high speed was mounted to cut the light beam before it reached the mirrors. Blanks appearing in the records could then be lined up and the instanteous position of each structure during a cycle determined. A typical result is shown in Fig. 4A. The dotted lines indicate the positions of the blanks made by one blade of the fan on the three records. It is apparent that not only are there in and out movements of the tergum and mesopleural process but also anterior-

posterior rotations evident in the anterior notal process record, where for part of the cycle the light is moving forward faster than the film.

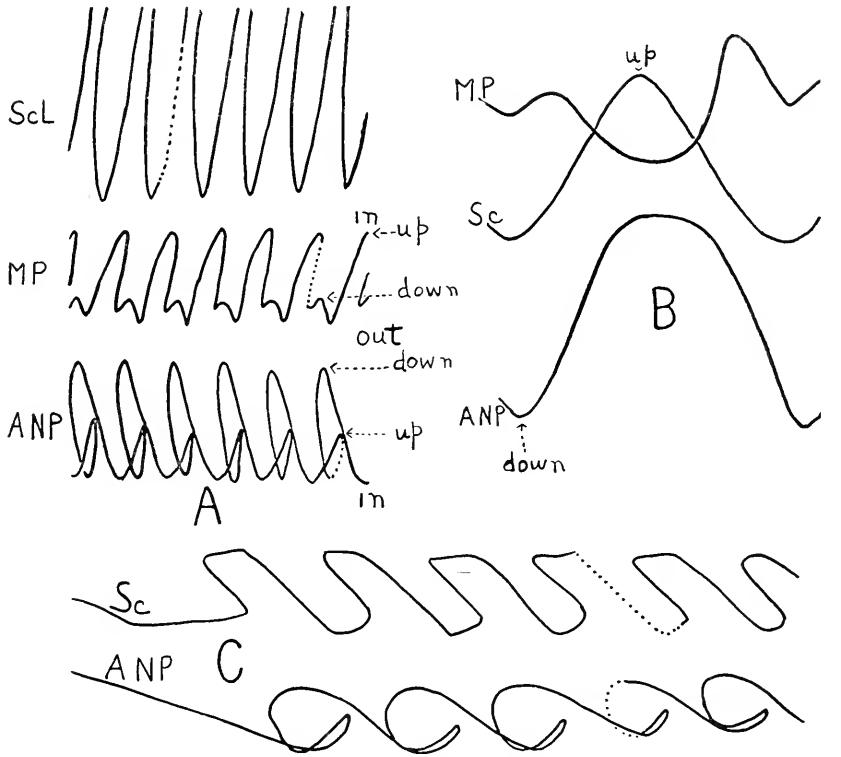

Fig. 4. Movements of the thoracic structures. A, fly, *Sarcophaga*; B, wasp, *Polistes*; C, fly, *Sarcophaga*. Records of optical levers from mirrors attached to the scutellum (and moving with the scutellar levers ScL), to the mesopleural process (MP), and to the tergum just above the parascutum where in-and-out movements of the anterior notal process (ANP) are effective. In A and C the original records are traced and movements are tilting the mirrors not only in the vertical axis but to various degrees horizontally, with or against the time axis. In B the record has been redrawn to eliminate anterior-posterior movements and the three beams shifted so that simultaneous movements of time are vertically under each other. In A and C the time correspondence is indicated by the dotted segments. The terms *up* and *down* refer to wing position; *in* and *out* to lateral movement of the part relative to the thorax.

From the static analysis (Fig. 3) based on CCl_4-treated flies, it was predicted that, at the beginning of a stroke, the anterior notal process moves in (light beam down) while the mesopleural process moves out (light beam down). After the critical period, the mesopleural process moves in (light beam up) and the anterior notal process out (light beam up). The record

(Fig. 4A) shows that these movements actually do occur during tethered flight in each stroke, up or down. The relative amount of movement is seen to be different in the two strokes, showing that they are not symmetrical. For the wings to operate always at the best angle of attack, the up and down movements of the wings must take different paths. These paths are determined by the setting of the articulation. The setting cannot be altered during the course of a single cycle, for the direct muscles cannot significantly change their tensions within the very brief cycle of operation of the indirect muscles, 6 msec. in *Sarcophaga.*

The agreement between the movements predicted from a study of CCl_4-treated flies and those found in flight are sufficiently good to give strong support to the above analysis of the mechanics of flight in Diptera. As will be indicated later, it is probably that all insects with the asynchronous mechanism have some sort of snap action at the articulation, for it plays an important role in assuring full amplitude of wing movement. In Fig. 4B are presented results obtained on the wasp *Polistes* sp. The record has been redrawn to eliminate anterior-posterior movements and the three records

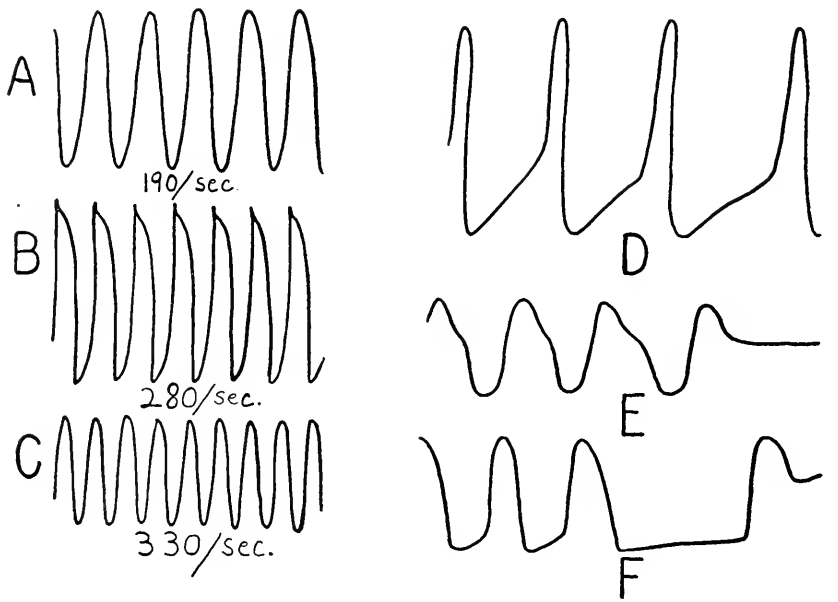

Fig. 5. Movements of the scutellar lever recorded by optical levers from mirrors on the scutellum. All records on fly, *Sarcophaga bullata.* A, normal; B, wings removed; C, wings removed and articulation damaged. A, B, and C are taken on the same fly, with the same magnification and time scale. Wing frequency is stated on the record. D, erratic flight movements in CCl_4-treated fly. E and F, parts of the same record showing the development of a fast stop on the down stroke (E) and on the up stroke (F).

shifted to the same axis. The complete cycle took 8 msec. The anterior notal process does not show double movement each stroke, but is rigidly connected to the tergum and consequently moves with it, there being no hinged parascutum. Removal of the wings in wasps reduces the amplitude of muscle movement to about one-half, while in flies there may be little or no change (Fig. 5B). This suggests that wing inertia plays a role in maintaining wing amplitude in wasps, while in flies the snap action in the articulation is more important.

If the various articulating parts are not held in the proper relation, erratic flight movements result. The sputtering flight under CCl_4 already referred to is an example. Frequently a mounted fly will show similar behavior, apparently trying different settings in an effort to attain free flight. In Fig. 4C movements of the scutellum and anterior notal process were caught in a moment when the articulation was not properly adjusted, and so the relation between these erratic movements and the mechanics of normal flight can be determined. In this case the scutellum moved little at the beginning of the down stroke. Not until the anterior notal process moves inward, to allow the union *k* of this process and the 2nd axillary sclerite to attain the critical point, does the scutellum move rapidly as the result of the recoil of the strained elastic elements. The fast movement begins at the instant the anterior notal process reverses direction. When the inhibition to movement is somewhat greater, the wings may be brought to a sudden stop, as shown by the scutellar movement record of Fig. 5E,F. The stop may last the duration of several cycles. The inhibition to movement develops gradually, being greater each cycle until more force is required to move the articulation past the critical point than is generated by the indirect muscles. Movement then stops until balance is again achieved. On the down stroke the stop appears at a different point than on the up stroke. These unusual movements can, therefore, be readily understood with our information on the mechanics.

One of the simplest ways to reveal the snap action is to study scutellar movements with the wings removed. After the removal of the wings, the only resistance to movement is that in the articulation. Once this is overcome at the beginning of each stroke, the movement accelerates to the end, driven by the stored elastic energy (Boettiger and Furshpan, 1951). Normally the wing acts as a governor to smooth out the stroke. The accelerating movement, beginning at the critical period, is brought to a sudden halt by mechanical stops. The articulation allows only limited movement of the scutellar lever and consequently of the driving muscles. Mechanical limits to the movement in such a vibrating system may be necessary to prevent the tearing of the muscle by the build up of amplitude due to inertial forces. That the setting of the articulation may be altered by injury to

secondary elements of the articulation is shown in Fig. 5C, taken on the same animal as records A and B. Sensory elements of the base of the wing which regulate loading in the articulation may have been injured. In one case the articulation was placed into a unique state by CCl_4 (Fig. 5D); for, after a delay on the up stroke, the critical point was passed but the moving parts apparently hit an elastic stop, rebounding back past the critical point immediately, without locking into the up position. The mechanical action here is similar to that which accounts for the ringing of the tymbal in the cicadas studied by Pringle (1954).

To complete this survey of the kinds of movements that may be brought about by the unique mechanical system and its equally unique driving muscles, a few normal starts and stops are illustrated in the records of Fig. 6. In A the fly was making a series of starts and stops but there was

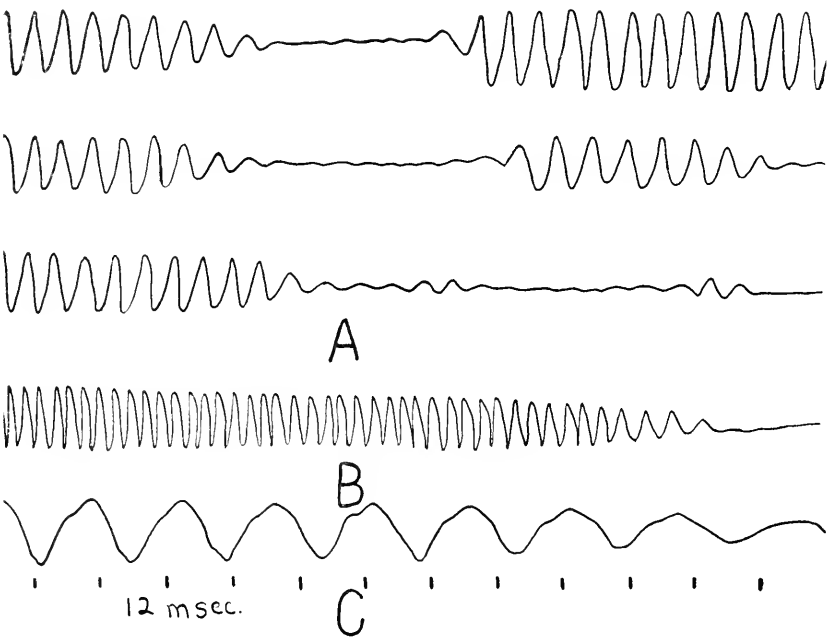

Fig. 6. Scutellar movements during starts and stops recorded on fly, *Sarcophaga bullata*, with optical levers. A, normal; B, wings removed; C, wings loaded with wax. Time scale applies to all records.

still evidence of a continuous rhythm of very small amplitude between one stop and the next start. The articulation is set very rapidly in a normal start, for full amplitude is attained almost immediately. The slight vibrations in the record suggest that the indirect fibrillar muscles are excited before the articulation is set. A stop with wings removed, B, is contrasted

with one occurring when the wings are loaded with wax. The movements decrease after the cessation of excitation much more rapidly in the fly without wings. The slow decrease found when the wings are loaded shows the effect of inertia. The muscle loses its tension relatively slowly after excitation is stopped, but will continue to vibrate only with an inertial load (see below).

In addition to improving our understanding of the mechanism moving the wings, the foregoing examples give much information on how the driving muscles must operate: (1) they shorten very little because of the large mechanical amplification factor; (2) vibrations of high frequency with practically no amplitude are possible; (3) the snap action is not necessary for the operation of the asynchronous mechanism; and (4) a shortened muscle may be easily relengthened for some time after it has shortened.

The Physiology of Fibrillar Muscle

Having discussed in some detail the wing articulation and the thoracic component of the flight machinery in one group of insects with the asynchronous system, we may now turn to a consideration of the driving muscles. These consist of two sets acting in opposition upon the thoracic component, as described above. The evidence in the literature suggests that these fibrillar muscles have unique physiological properties. They are capable of very high frequency operation, up to 2,200 cycles per second (Sotavalta, 1953). In flies with thorax open, electrical stimulation produces no visible shortening. That the action potentials from the thorax do not correlate with wing movements has already been noted, as has the increase in frequency when the wings are removed. Do these special properties of fibrillar muscle represent a modification of the excitatory or of the contractile mechanism?

If the excitatory process is basically modified in fibrillar muscle, the electrical properties of the membrane might show differences when compared with other muscles. To test this possibility, microelectrodes were inserted into the fibers of the dorsal longitudinal muscle of flies and both resting and action potentials recorded by the method of Nastuk and Hodgkin (1950). The resting potential was usually about 60 mV and the action potential 80-100 mV, although values as high as 120 mV were observed (Boettiger and McCann, 1953). The form of the action potential is shown in Fig. 7D. Several species of wasp gave very similar action potentials. From the fibrillar muscle of Coleoptera, however, large action potentials were not easy to obtain and did not show clear evidence of overshoot. In the best preparations, resting and acting potentials of 60 mV were found. The microfibrillar muscle of a few species of moths pro-

duced action and resting potentials similar to that found in beetles, though usually smaller. Qualitatively it appears that the electrical manifestation of the excitatory processes of fibrillar muscle fibers are similar to those of other muscles. Different groups of insects have fibrillar muscle with quantitatively different electrical properties. These variations may eventually be correlated with the different patterns of nerve innervation of fibrillar muscles indicated by the work of Tiegs (1955).

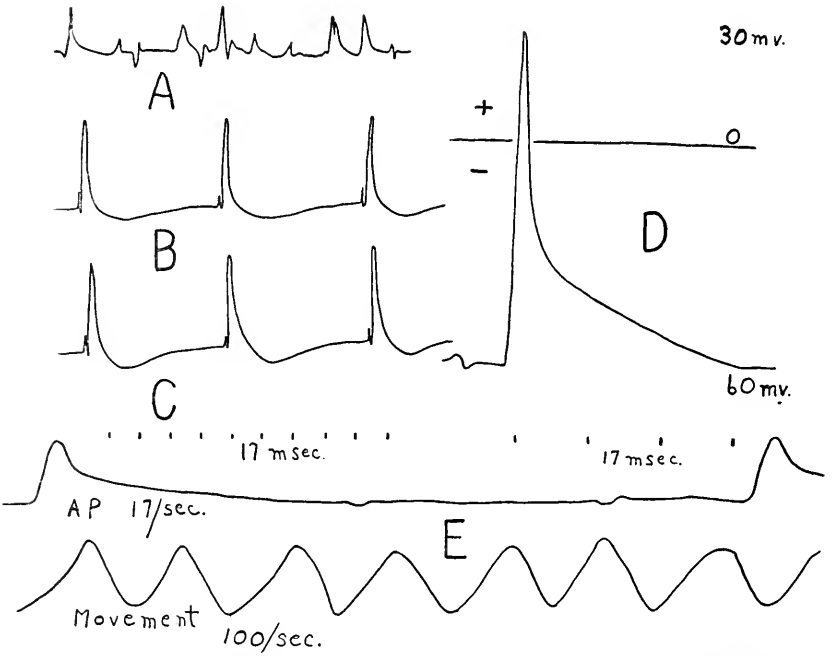

Fig. 7. Action potentials of the indirect flight muscles of the fly, *Sarcophaga bullata*. A, Normal discharge recorded with external leads; B, during stimulation of the ganglion, the action potentials of all fibers recruited to stimulus frequency, flight continuing; C, same after cessation of flight movements. Time scale applies to records A, B, and C. D, single-fiber action potential of longitudinal flight muscle recorded with internal microelectrode on stimulation through leads directly on the muscle; + and − refer to the sign of the internal electrode. Stimulus artifact shows at the beginning of the record. E, single-fiber action potentials recorded with internal electrode during spontaneous flight movement compared with thoracic movements. Small movements in the action potential record are probably action potentials in adjacent fibers.

When transmembrane potentials of a muscle fiber are recorded during flight, one observes a beautifully regular discharge, apparently in all fibers. Identical discharge patterns can be elicited by the vapors of ether and a number of other compounds. With CCl_4 the discharge frequency rises

rapidly, the spike height decreases, and the membrane does not completely recharge between responses. The regular discharge patterns arise in the thoracic ganglion. A record of the action potentials of a single muscle fiber and of thoracic movements is included in Fig. 7E, to show beyond all doubt that there is no phasic relation between the membrane events and the mechanical movements produced by the muscles.

Response to Electrical Stimulation

It is possible to drive the flight mechanism of a fly through stimulating electrodes pushed into the vicinity of the thoracic ganglion (Boettiger, 1951). In the best cases a series of wing beats follows each stimulus, making the sound of a short buzz. With increasing frequency of stimulation, these fuse into continuous movements, though the buzz is louder and higher in pitch immediately following each stimulus. At 15-20 stimuli per second in *Sarcophaga*, the movements are of normal amplitude and frequency without modulation. As the frequency of stimulation is increased still further, the flight tone sputters and suddenly the wings stop in either the up or down position. The fly now shows the same responses as one treated with CCl_4.

A record of the thoracic potentials with external leads is shown in Fig. 7 before (A) and during (B) driven flight. The normal irregular firing of the muscle fibers gradually changes to a synchronous one as the muscle fibers are recruited to the driving stimulus frequency. During the recruitment there was no noticeable change in flight movements. After a short period of driven flight the fly stopped and folded its wings back but the action potentials remained (C).

These experiments with driven flight prove that, even with simultaneous excitation of the muscle fibers of both sets of antagonistic muscles, the shortening and lengthening cycle of the contractile elements are not altered. Also we may conclude that the high-frequency behavior of these muscles is not the result of an alteration in the number of fibers responding, since the excitatory processes of all fibers were synchronized with the stimuli.

Under the conditions of driven flight the direct muscles must also be excited. In certain cases it seems that the indirect driving muscles are activated while the direct muscles are not. In Fig. 6A the small vibrations indicate activity of the fibrillar muscle, the rapid rise in amplitude at the start resulting from contraction of the direct muscles. Movements of the scutellum and muscle potentials were recorded in an unusual start under external stimulation (Fig. 8). The stimulus is shown to control the firing of the indirect muscle fibers, for only one large action potential follows the stimulus artifact. At the beginning of the record, each stimulus produced only a small slow movement of the scutellum due to its action on some direct

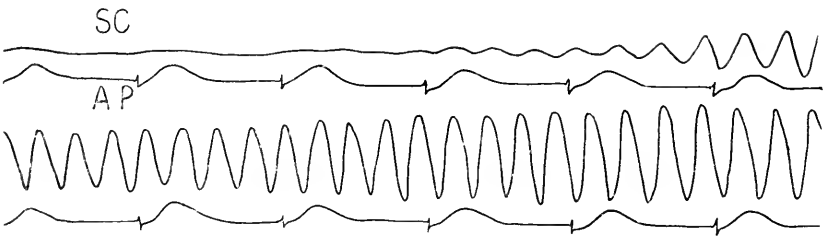

Fig. 8. A start during stimulation of the thoracic ganglion of the fly, *Sarcophaga bullata*. AP is a record of the action potentials obtained with external leads, each potential preceded by a shock artifact. SC is the simultaneous record of movements of the scutellum recorded by an optical lever.

muscle. After one of these movements, a very slight vibration is noted. The next stimulus increased the oscillatory response, which, with continuing stimulation, gradually built up to normal amplitude. This start contrasts with normal starts (Fig. 6). The wings were held straight out and the vibrations began from this position. Although some of the direct muscles must have been excited, the articulation was apparently not set. The medial positions of the wing are unstable in an articulation set for flight. The beginning of the vibrations may be correlated with the shortening of the direct muscles, principally the pleurosternal, but under conditions where the indirect muscles were already under tension and resisting the shortening. Excitation of the opposing fibrillar muscles without the proper mechnical situation results in each muscle preventing the shortening of the other. To get out of this impasse, movement must be initiated by an accessory agent, in this case the direct muscle. Once one muscle is shortened slightly, and its antagonist necessarily lengthened, vibrations begin and with proper setting of the articulation grow to full amplitude. The smallest vibrations in the record of Fig. 8 are about one-twentieth normal amplitude but at very nearly normal frequency. As the shortening of these muscles during flight is 0.02-0.04 mm., the shortening in the vibration is less than 2 μ or 0.04% of muscle length. In wasps, similar vibrations have been noted. In one case the ring stand to which the wasp was fastened could be felt to vibrate while the animal appeared to be perfectly still.

In these very small vibrations one cannot believe that any snap action occurs. All that is necessary is for the opposing excited fibrillar muscles to be moved in opposite phase, and this movement need not be very fast. The system having a negative resistance, once started, will then tend to build up. For the attainment of full amplitude with a damped load, such as the wings moving through the air, the snap action may be necessary.

Although much useful information can be obtained by the study of intact insects, the correctness of our conclusions and the details of the

physiology of the fibrillar muscle must be studied in an isolated muscle preparation by the classical methods.

The Mechanical Properties of Fibrillar Muscles

The discovery of the snap action in the articulation and the demonstration of its importance in normal flight suggested to us the early experiments of Gasser and Hill (1924) on the responses of the frog muscle to quick changes in length. The theory was proposed by Boettiger and Furshpan (1950) that the opposing longitudinal and vertical indirect flight muscles were both in complete tetanus. By this it was not meant that the tension is smoothly maintained, but that the muscle is kept in the active state as described by Hill (1949). In such a tetanus or maintained active state, tension is a function of length and of the velocity of shortening. The opposing muscles cannot usually neutralize each other, since the position at which they would have the same length is unstable; because of the setting of the articulation, one muscle would be lengthened and the other shortened when flight begins. In the shortening muscle, tension would be expected to fall rapidly after the critical period. On the return stroke the antagonist would quickly lengthen the muscle. To obtain work from such a system the reappearance of tension during the lengthening must be delayed, so that at each length the muscle has greater tension while shortening than lengthening.

The first step in testing this theory was to show that fibrillar muscle could be put into typical isometric tetanus. Since we already had a body of information on flies, the first experiments were performed on one of the anterior vertical muscles of *Sarcophaga*. McEnroe (1952) reported that, with one end of the muscle detached and coupled with a recording lever, a steady tension of 250 mg. could be obtained during tethered flight. The tension increased before the start, was continued during flight, and slowly disappeared at the end of flight. Upon stimulation the muscle went into tetanus. Much larger isometric tensions were later obtained in large Tabanids (McEnroe, 1954).

The original theory was therefore substantiated to some extent. As far as the excitatory process was concerned, the muscle was in a maintained active state similar to tetanus during flight. The changes in tension necessary for the production of the wing cycle must result from the changes in length, in the speed of shortening, and in the speed of lengthening. Pringle (1954) found similar responses to stimulation in the tymbal muscles of those species of cicadas possessing fibrillar muscle to produce high-frequency tones. He accounted for the relengthening of the muscle without the usual rise in tension by assuming a deactivation by release. The deactivated state was considered to last a short time, requiring immediate

restretching of the muscle for the muscle to take advantage of the deactivation. The possibility of reactivation on stretch was also suggested. Our results with intact flies (Fig. 5E,F) show that after a fast stop the shortened muscle may remain short for the duration of several cycles without the return of tension. When the inhibition to movement is removed, the shortened muscle can be as rapidly lengthened by its antagonist as in the normal stroke. Lengthening, therefore, must be as important for the redevelopment of tension as shortening is to the fall of tension.

Since the fly preparation was technically difficult to handle because of the very small movements possible, the study was continued with large bumble bees. Upon removal of the head and abdomen, the thorax was impaled on two needles pushed into a small mounting board. A third needle, inserted into the cuticle at right angles to the other two, firmly anchored the thorax with the posterior end oriented upward. The phragma to which the longitudinal muscle is attached was exposed, cut from its connections with the articulation, and fastened with a double hook to an RCA mechanotransducer for recording muscle tension. The transducer could be raised and lowered precise amounts in order to study tension at different muscle lengths.

Fibrillar muscle of the bumble bee exhibits marked summation to a series of maximum stimuli, as does that of the fly and of the tymbal muscle. The response of the bee preparation to stimulation at 8 per second is shown in Fig. 11A. The single isometric twitch is quite small as compared to the complete tetanus attained at about 40 stimuli per second. Relaxation is very slow, taking one-half to one second for the tension to drop to zero after the cessation of stimulation. The slow relaxation explains the stops noted in Fig. 6B,C.

A typical tension-length diagram of the muscle in maximum tetanus is shown in Fig. 9. The maximum amount the muscle can shorten is only about 12% of its rest length, or 0.9 mm. With the arms of the phragma attached to the articulation, the muscle is held in the thorax at the length at which it develops maximum isometric tension, and shortening is limited to 0.1-0.2 mm. The curve of passive tension is also shown. This limited movement means that only a portion of the tension-length curve is used in flight. Since the isometric tension varies little over this range (Fig. 9), the fall in tension necessary for the work cycle must result from the rapid shortening. In these isometric contractions there was no evidence of oscillatory behavior, the muscle acting as other skeletal muscle in all regards.

The relation between isometric and isotonic contraction is shown in Fig. 10. For these experiments the mounting board with the bee thorax preparation was fastened to a thin piece of metal hinged to a support. To load the

muscle, weights were attached to the piece of metal. Changes in length were recorded by a second transducer coupled to the metal strip by a light spring. The tension recorder was connected to the oscilloscope to give vertical deflections of the beam and the length recorder to produce horizontal deflections. The tension-length relations of the muscle were therefore drawn out instantaneously on the screen.

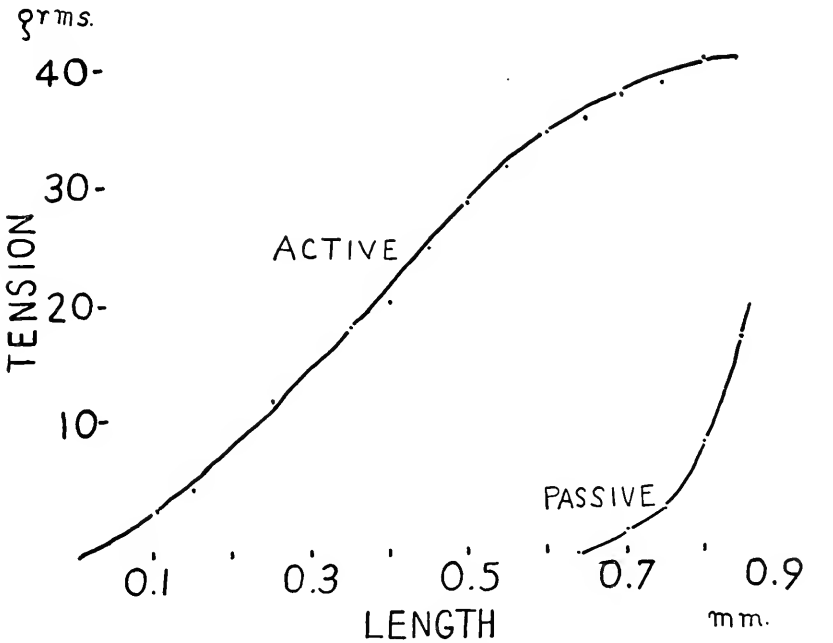

Fig. 9. Tension-length relations of stimulated (active) and unstimulated (passive) longitudinal flight muscle of bumble bee, *Bombus*. The muscle was stimulated to maximum complete tetanus. The active tension curve was obtained by allowing the muscle to shorten isotonically without a weight and then to build up tension at the shorter length isometrically. The absence of the weight explains the difference between Fig. 9 and Fig. 10. The passive curve was obtained by stretching the unstimulated muscle.

An example of an isotonic response is illustrated in Fig. 10, reconstructed from observations made on a number of preparations. The unstimulated muscle was loaded with 14 grams (*a*). On stimulation the muscle shortened to *b* isotonically, and then began to oscillate with increasing amplitude to a maximum *c-c'*. Upon the cessation of stimulation the muscle lengthened and the oscillations decreased as shown by the envelope *c-a, c'-a*. If the oscillations are prevented, the muscle shortens to *d*, a point on the active tension-length curve. By jarring the preparation the oscillations again appear, the muscle lengthening and the amplitude increasing

again to *c-c'* as indicated by the envelope *d-c, d-c'*. The direction of movement around the tension-length loop can be found by dimming the trace through the Z-axis by the output of either the length or the tension recorder. By noting whether the dimmer trace is above or below the brighter, one can immediately determine whether the tension is higher or lower on muscle shortening and so whether the system is doing work on the muscle or the muscle on the system. In the case where the muscle is vibrating the

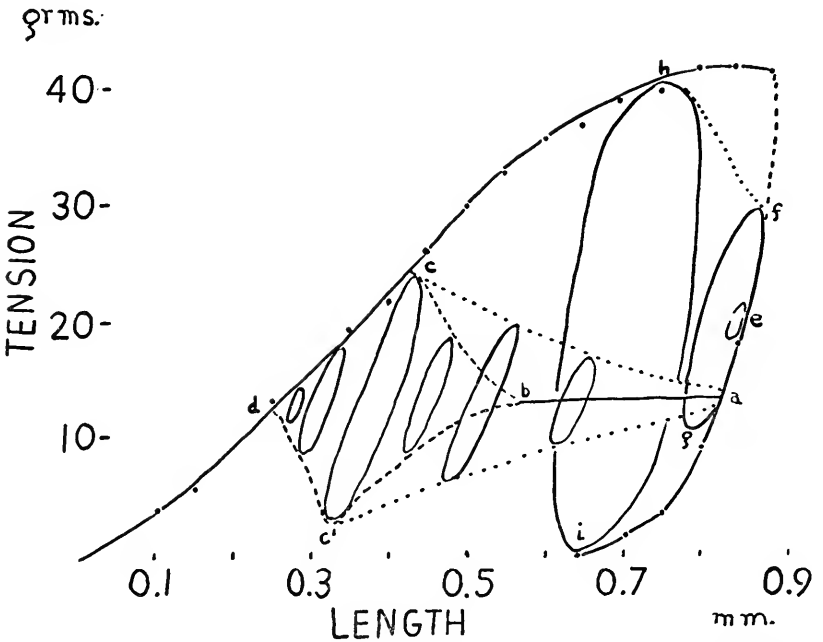

Fig. 10. Behavior of the longitudinal flight muscle of the bumble bee, *Bombus*, in isotonic contractions with a weight. The loops are not actual records but are drawn from observations on many preparations. The records were obtained with strain gages giving instantaneous measure of tension and length. Tension was applied to the horizontal plates and length to the vertical plates of a cathode-ray oscillograph to give the dynamic tension-length diagrams shown. Muscles stimulated at 60 per sec. to complete tetanus. See text for explanation of details.

platform, as in Fig. 10, the tensions are higher on shortening since the muscle is doing work. The area enclosed in the loop represents the work done by the muscle against damping forces in the mechanical system. By introducing more frictional damping, larger loops were obtained. Many different loops are possible within the area enclosed by the curves of active and passive tension, depending upon the load, the damping forces, and the characteristics of the contractile element of the muscle.

The contractile element is in series with a very stiff spring. To describe the theoretical implications of the tension-length loops in terms of these two elements, a simple model can be used. In this model the elastic element is represented by a spring and the contractile element by one's arm muscles. The muscles support the spring, to the end of which a weight is attached. If the weight is set in motion by an external force its movements will soon be damped out. One is instructed, however, to keep the weight in motion at the same amplitude and therefore must contrive to put into the system just enough energy to overcome the damping forces. One can do this most efficiently by shortening and lengthening his muscle in the same sinusoidal movement as the spring and weight. If the muscle moves in phase with the spring, no energy is transferred. But by moving in the same rhythm, and slightly out of phase with the spring, one can maintain the motion. The movements of the muscle must be slightly ahead of the movements of the spring. This is only possible because of the inertia of the weight. If the movement of the weight is more heavily damped, one's movements must be more out of phase with the spring to maintain the system in motion. The tension-length diagrams of the above model would show loops similar to those of Fig. 10. The area of the loop would be greater with greater phase shifts. Tension would be greater during shortening and the movement of the beam around the loop would be counterclockwise, the area representing work the muscle does against damping forces.

With spontaneous vibrations the loops are always counterclockwise. When, however, a mechanical system is used to make the excited muscle shorten and lengthen at different frequencies, the movement is either clockwise, the system doing work on the muscle, or counterclockwise, the muscle doing work on the system. The phase angle is a function of frequency and shifts from a minus value, length reaching its maximum before tension, to a plus value, tension reaching its maximum before length. In the former case, area represents work done by the muscle, and in the latter, work done on the muscle.

The significance of the loop can be summarized as follows: (1) the slope of the major axis of the loop is determined by the compliance of the excited muscle, (2) the area of the loop is a measure of the work done by the muscle against external damping forces and is a function of the maximum tension, the maximum length, and the phase angle between tension and length. (3) the position of the loop in the tension-length area depends upon the load and the change in the force the muscle can exert as velocity increases. When velocity is zero the muscle shortens until it attains the minimum length at which it can just sustain the load. In vibration as velocity increases, the muscle, because of the force-velocity relation, cannot exert at this short length a tension equal to the load. The muscle must

therefore lengthen, as illustrated in Fig. 10, until it can exert an average force, in the dynamic state, equal to the load.

The large loop *h-i* (Fig. 10) represents a possible tension-length loop of the muscle operating in normal flight. For sinusoidal motion the area of this loop can be calculated from the following relation

$$\text{work per cycle} = \pi \, P_o x_o \, \text{Sin} \, \theta$$

where P_0 is one-half the maximum tension change, and x_0 is one-half the length change. Using the data — $P_0 = 20$ grams, $x_0 = .0075$ cm., and $\theta = 30°$—the work is 225 ergs per cycle. If the antagonist muscle does the same work and the frequency is 100 cycles per second, the power output per second is 45,000 ergs. Since only about two-thirds of this can be converted into usable work by the wings (Hocking, 1953), only some 30,000 ergs are available to move the bee. This is probably about one-half that necessary. For the muscle operating in the insect one or more of the following must be greater than the values used above: the maximum tension, the maximum length, or the phase angle.

It has not yet been possible to load the muscle properly; consequently, loops of this size have not been experimentally obtained. The action of the articulation, the air resistance, and the wing inertia of the intact animal cannot be imitated easily. Were this possible, we have every reason to believe that the preparation would be able to do the amount of work required of it by the insect in flight.

Some additional information can be obtained from the response of the muscle to transient rapid changes in length (Fig. 11) (Boettiger and Furshpan, 1954a,b). For these experiments the platform on which the preparation was mounted was attached to a small rod running through a bearing and fastened to the center of the diaphragm of an earphone. By an on-and-off switch the current through the earphone coil could be controlled to produce small changes in length of the muscle, 0.05-0.2 mm. Tension and length were recorded as a function of time.

Two kinds of controls were used. In B the muscle was passively stretched to about 30 grams and then subjected to changes in length. The tension in this stretched unstimulated muscle followed very closely the changes in length. This result demonstrated that our experimental setup was adequate and that the unstimulated muscle behaved as a simple physical system. The same muscle stimulated to produce isometrically the same tension, and subjected to the same length changes, gave the response shown in C. Upon lengthening the muscle after a rapid shortening, the full length was attained before the full tension. The muscle produced a lower tension at each length when being stretched than it had while shortening.

A second control is shown in D, where the stimulated longitudinal micro-

fibrillar muscle of a moth was subjected to the rapid changes in length. At the completion of lengthening the tension was above the initial tension at this length, while in the bee muscle it was below. Fibrillar and microfibrillar muscle differ in their response to changes in length.

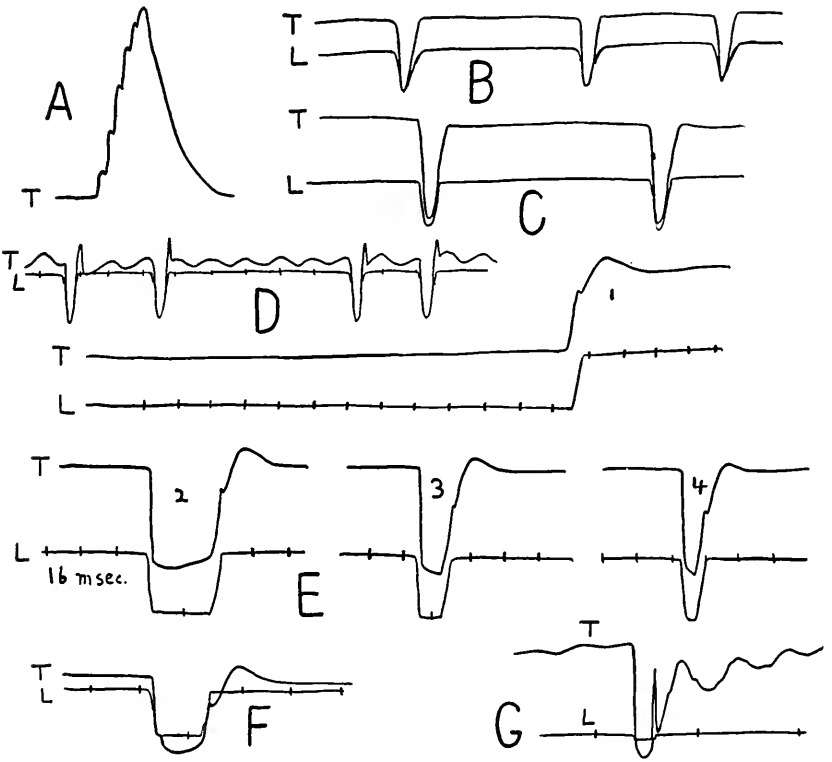

Fig. 11. The effect of rapid transient changes in length. T is muscle tension and L is muscle length. All marks on the length record show the instant of stimulation at 60 per sec. A, isometric myogram of bumble-bee muscle, stimulation at 8 per sec. to show summation of contraction; B, response of unstimulated bee muscle stretched to give about 40 gms passive tension; C, same muscle stimulated to give active tension of about 40 gm.; D, flight muscle of a moth showing behavior of nonfibrillar muscle; E, bumble bee, successive records showing effect of decreasing interval between release and stretch; F, wasp, *Sphecius*; G, bumble bee, *Bombus*.

In E is a series of records in which the lengthening of the muscle is delayed a variable time after the imposed shortening. At the completion of shortening, the tension falls slightly for about 10 msec and then rises, if it is not relengthened, to a tension characteristic of the new shorter length. This rise in tension after quick release is typical of muscle and even of the glycerinated muscle model. Relengthening the muscle during the 10 msec. before the tension starts to rise results in a somewhat greater active tension

rise after the completion of the lengthening than if relengthening is delayed. A record on a large wasp is shown in F, since it gave quite a large fall in tension following shortening. These drops in tension do not appear to result from mechanical factors in the experimental setup but are rather the result of some lengthening of the contractile elements.

If as in E (1) the muscle is rapidly lengthened after the initiation of stimulation, but before the tension has increased greatly, one may sometimes see the same effect as found when the muscle is lengthened after a rapid shortening. The tension continues to rise after the completion of the stretch.

A very rapid stretch after a shortening results in a sharp rise in tension followed by a precipitous fall after the completion of the movement, and then a rise to the isometric level, G. In the bee during flight one stroke lasts about 5 msec. In this experiment the muscle has been stretched in 1 msec. This viscous-like behavior of the muscle must set an upper limit to the speed of the system.

These transient responses of the muscle show that, following a rapid shortening, the contractile elements lengthen slightly and the tension falls. During the subsequent rapid relengthening, only a portion of the tension has returned by the end of the movement, as little as 30% in the best cases. The tension continues to rise very rapidly after the muscle has attained its initial length and may overshoot the isometric tension at which shortening began. The rise is due to the shortening of the contractile elements.

Many of the characteristics of the flight machinery found in the study of intact insects now find explanation in the physiology of fibrillar muscle. The muscle is basically an elastic system through which chemical energy is furnished by the contractile elements as necessary to overcome the damping forces tending to halt motion. Since the snap action, acting in the same manner as inertia, tends to resist movement, work must be done against it as well as against air damping at the beginning of the stroke. The elastic energy so stored maintains movement toward the end of the stroke when muscle tension is falling.

If we now place the muscle we have studied back into the insect from which it was isolated, coupling it to the mechanical system described, and to its antagonist, we can give it the proper label and file it away. At some future time, however, as we remove it to study its structure we may perhaps see something more than its external morphology, in fact, one of nature's most successful solutions of heavier-than-air flight.

Summary

Insects, making use of the special mechanical properties of an exoskeleton, have evolved two principal types of flight mechanisms. In the syn-

chronous type the usual direct correlation exists between the nerve impulses and the muscle response. In those insects possessing fibrillar flight muscle the mechanism operates in a different manner. During the active state initiated by each nerve impulse a variable number of contraction cycles may occur. Studies of this mechanism were made on the large fly *Sarcophaga bullata*. Recordings of action potentials from the muscle with external and internal leads show that processes involved in building up the active state are the same in fibrillar muscle as in many other striated muscles.

The special properties of the asynchronous mechanism result from the nature of fibrillar muscle and the mechanical system with which it functions. A snap action is demonstrated in the articulation that is shown to depend upon the relations of the first two axillary sclerites with three components of the basic thoracic structure. Records of movements of these components during flight reveal their action under a variety of conditions and features of the physiology of fibrillar muscle as well.

Using the longitudinal flight muscle of the bumble bee, *Bombus*, it was possible to study the mechanical properties. Isometric twitch is small. A smooth tetanus is obtained at 40-60 stimuli per second. Tension-length relations of maximal isometric contractions are typical but show that shortening is limited to 10% of muscle length. *In situ* the shortening is mechanically limited to 1%.

Only when the active muscle is allowed to shorten with an inertial load, or is rapidly stretched after a rapid shortening, are its special properties revealed. In the first case the dynamic tension-length relation is a loop, the area of which represents the work done in one cycle against viscous damping forces such as the movement of the wings through the air. The response to rapid stretch when the muscle has less than the tension characteristic of its length (as after rapid shortening), in contrast to that of other muscles, shows that the muscle may be stretched to full length while tension rises to only a fraction of its initial value. Following the attainment of full length the tension rises rapidly.

The presence of special mechanical properties in the mechanism moving the wings is correlated with the operation of fibrillar muscle. Together these features constitute the principle machinery to move the wings.

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NEUROMUSCULAR MECHANISMS*

C. A. G. Wiersma
California Institute of Technology

It has become well established that the control of muscular contraction by the nervous system in the typical vertebrate striated muscle is a specialized case, and that other types of control are present. From a comparative viewpoint this is a logical development. For the nervous control of muscular action must be a secondarily evolved process, since the contractile elements can be brought into action without nervous structures in protozoa, and most likely also in sponges. Therefore, it is likely that the original function of the nerves was regulation of myogenic contractions and that only subsequently the nerves obtained more complete control. It may well be that in, e.g., the smooth muscles of the digestive tract in all phyla the regulatory function of the nervous system has been maintained. However, for the body musculature, especially the parts involved in quick withdrawal reactions, nervous activation became necessary at an early stage, whereas slower and more tonic muscles may have kept a greater independence. But again, for quick reaction more than one method of activation is used. In the vertebrate striated muscle fiber it is the development of a conducted muscle action potential which spreads the excitation from the end plate to the rest of the fiber, but in many arthropods conduction along branches of the nerve fibers is certainly mainly responsible for this spread. It will be the task of future investigations to discover to what extent the possibilities roughly outlined above are adequate for the explanation of the control of the muscles in the different phyla, how far as yet unknown mechanisms have been realized, and also how far the different muscles of one animal are under a similar type of control.

Muscles with fast and with slow contraction speeds are widespread. It is interesting to note that in protozoa contractile fibrils differing vastly in this respect are present. The types of innervation of muscle fibers of one muscle are not always similar, as has been demonstrated in the frog muscles, many of which have two types of muscle fibers each with its own type of innervation. Kuffler (1955) has reviewed the work which established this. The "slow" or tonic system shows, in contrast to the conventional fast system, multiple nerve endings on the muscle fibers and an ab-

* This review will be largely limited to those newer papers, which have appeared since the author's previous reviews (Wiersma, 1952-53) which dealt partially with the same topic.

sence of twitches and of conducted muscle action potentials. Instead, only slow contractions and facilitating junctional potentials are present. The two nerve-muscular systems are here very independent. In arthropods, on the other hand, all indications are that the same muscle fibers are involved in the various contraction types (see below), and that the type of innervation is much the same. In other phyla fast and slow contractions are sometimes definitely due to two different types of muscle fibers; but it will be again necessary to obtain more information before meaningful comparisons with the better-known mechanisms can be made.

The use of intracellular electrodes may help much for the rapid increase of our knowledge about these questions. However, the results with decapod crustaceans to be described may be a warning that, even with these methods, the finer details may be so complex that conclusions as to types may be difficult to draw.

One factor influencing the effect produced by a nerve impulse arriving at the muscle fiber is undoubtedly the state of the fiber. Not only may there be previous facilitation and fatigue, but the actual state of stretch of the muscle fiber may have a considerable influence on the outcome. In vertebrate striated muscle it is well known that the heat produced during isometric and isotonic twitches is quite different. Ralston and Libet (1953) found in addition that the mere stretching of a vertebrate motor end plate may result in a conducted muscle potential when this did not occur in the relaxed muscle fiber—which brings to mind what von Uexküll (1904) long ago showed, that it is often easier to make stretched muscles contract. He found that brittle star arms would invariably contract upward when hanging down, whether or not the stimulation was nearer to the stretched side of the arm. In cases of myogenic contractions, stretch is also known to be an important factor, as in vertebrate intestine and in the heart of *Helix* (Willems, 1932). It seems likely that at least in some of these instances reflex activity is not present.

These considerations may have made it clear that a great variety of conditions exist, and that the conclusions which can be drawn from the study of any one preparation or even of the muscles of one group may have only a limited applicability to other systems. With this reservation in mind the following review of recent investigations may be undertaken.

Investigations in Arthropods

Among the invertebrates, the arthropods still remain by far the most favorable for neuromuscular studies. Their muscle fibers have lent themselves readily to the application of intracellular electrodes. The results thus obtained with insects and the intriguing studies of the relations in indirect

flight muscle of higher insects are treated elsewhere in this volume, and except for one reference will not be considered.

In decapod Crustacea the use of internal electrodes has given some remarkable results. Studying the electrical properties of the muscle membranes as such, Fatt and Katz (1953a) have shown that they differ markedly in several respects from those of vertebrate muscle fiber. These properties of the membrane may well have a considerable influence on its functional relations to contraction and inhibition. But, since at present these relations are not clear, the results of their studies will not be discussed further.

With the use of internal electrodes, Fatt and Katz (1953b) and Furshpan and Wiersma (1954) were able to show that an impaled muscle fiber of a doubly motor-innervated muscle would react on stimulation of either axon. The potentials obtained depend on the axon stimulated; in "slow-axon" stimulation considerable facilitation may be needed before the depolarization is evident, while single impulses in the "fast" axon usually give rise to a clearly observable deflection. These results were obtained with many fibers of a wide array of muscles and represent undoubtedly the normal effect. But the experiments do not necessarily prove that all muscle fibers of a doubly motor-innervated muscle receive innervation from both axons (see below). Fatt and Katz (1953b) have studied the distribution of the potentials along the fiber, impaling one at a number of loci, and have very often found insignificant differences in amplitude and time relations. These results are thus in sharp contrast to those obtained with the focal end-plate potential of mammalian muscle fibers, and make it certain that a decapod crustacean muscle fiber normally receives its excitation at the many nerve endings which each axon has along the length of the fiber.

Since there exists a good deal of variation in type of innervation and effects of stimulation in different muscles, a new terminology, with some terms already used for other preparations, has been proposed by Furshpan (1955). With a slight variation in definition these terms will be presented here. Instead of multiple innervation, an older term which covered at the same time the fact that many nerve endings are present and that more than one axon makes connection with a muscle fiber, the term *multiterminal innervation* will be used to describe the fact that one axon has a considerable number of endings on a muscle fiber. To indicate that more than one motor fiber innervates a muscle, the term *polyneuronal motor innervation* will be used (subdivisions like dineuronal motor innervation, etc. can be derived from this). But every decapod crustacean muscle fiber may have at least a dineuronal innervation, since in all known instances there is at least one inhibitor, and sometimes two, present in addition to the motor fiber or fibers (Wiersma, 1941). The types of potentials which can be ob-

tained from muscle fibers show differences that make a classification necessary. Instead of end-plate potentials the presumably purely local effects around the nerve endings will be called *junctional potentials* as was done by Kuffler and Gerard (1947) for the comparable potentials in the slow muscle fibers of the frog. The secondary response which arises from these potentials after they surpass a certain value will be called a *spike*. This will not imply that the process is conducted over the whole membrane; when the latter takes place it will be called a *conducted spike*. The conducted spike always shows, as far as known, an overshoot of the membrane potential, whereas spikes may vary from just visible enhancements of the junctional potentials to the maximum level with overshoot. Since there are strong indications that the junctional potentials (but not the spikes) of the different axons innervating the muscle fiber have different mechanical results, which do not depend on the shape or size of the potential, they must also be named with regard to the axon bringing them about. Therefore, in a double motor innervated muscle it will be necessary to distinguish between "*slow*" *junctional potential* and "*fast*" *junctional potential.*

The potentials of the contractile part of the muscle-stretch receptor organs have been studied by Kuffler (1954) and Furshpan (1955), using one or two nerve impulses only, which will presumably make the contribution of any "slow" motor axon, if present, negligible. These structures are favorable for this type of work because they constitute thin isolated strands, which we consider as single muscle fibers. They offer a clear picture of the motor innervation, the motor fiber(s) running along the length of the structure, giving off branches into it at many points (Alexandrowicz, 1951). Both Kuffler and Alexandrowicz are inclined to consider them as consisting of bundles of muscle fibers rather than single units. However, Furshpan, using two microelectrodes, failed to find any sign of high-resistance membranes between them, indicating the absence of charged membranes. The only exception found was when one electrode was in the anterior muscular segment, the other in the posterior one, separated by the intercalated region in which the sense cell has its endings.

Using the anterior muscular section, Kuffler and Furshpan have both observed spikes which were not conducted. Kuffler found that, when no spikes were present at a given time, stretching the organ by pulling it to one side would bring one about, but this spike was confined to the stretched region. Furshpan, using two internal electrodes, found that one locus might spike at a time that the other gave only a large junctional potential. When a second impulse was delivered shortly afterwards, the other locus would also spike. He could show that the speed of spread of the spike, when spiking took place all along the structure, would be the same as that of the junctional potential, and concluded that the nerve fiber plays the

primary role in the distribution of the excitation along the muscle fiber and that the spike is not the indication of conduction.

While it is thus quite possible that under normal conditions different parts of one fiber react differently to stimulation, it may be considered certain that different muscle fibers of one muscle react rather independently. In the closer muscles of crabs it was regularly observed that a single impulse in the "fast" axon would give a large spike in part of the muscle fibers, in others an abortive spike or large junctional potential, while in a different part of the muscle only small junctional potentials would be present in the fibers (Furshpan and Wiersma, 1954). Sampling in these cases has not yet been extensive enough to make certain that there is a constant gradient in this respect from the back to the front of the muscle, although this was often observed. Fatt and Katz (1953b) also report large differences between the responses of muscle fibers of one muscle.

Polyneuronal innervation is, according to the results of Furshpan (1955), also variable from muscle fiber to muscle fiber in one muscle. In many muscles with double motor innervation the indications are at present that the great majority of fibers, perhaps all, do indeed receive branches from both axons. But in the main flexor of the rock lobster, which has four motor axons supplying it, it was found that, when single penetrations of numerous muscle fibers were used, only a relatively small percentage (7%) of the muscle fibers responded definitely to all four motor axons. Response to three axons was obtained in 29%, to two in 26%, and to only one axon in 38% of the fibers. In the last case it is known that, of these, 90% responded solely to stimulation of the particular axon, which elicits the fastest contraction. Since none of the fibers tested failed to respond to at least one motor axon, these figures may represent a fair approximation of the distribution. A further significant observation of Furshpan is that it is possible to obtain spike potentials by combining the stimulation of two axons, when each gives only a junctional potential by itself. This proves that the junctional potentials caused by any of the motor axons must be considered equivalent with regard to their relation to the membrane changes.

In insects a rather similar picture has been reported by Hoyle (1955 and this volume) for locust muscles. Here only certain of the muscle fibers of a given muscle receive a polyneuronal motor innervation, the majority being supplied by a single axon.

A functional differentiation between parts of a crustacean muscle, which must be due to unequal distribution of axon types or number of their endings, has been reported (Wiersma and Ripley, 1954). It was observed that, in contrast with the great majority of leg joints, one joint in the walking legs of a hermit and of a dromid crab can rotate as well as bend at

the joint. It was found that, of the two motor fibers innervating these muscles, the "fast" axon causes rotation in one direction, the "slow" in the opposite.

Comparing muscles as a whole, differences in the process of their neuromuscular transmission can be demonstrated, not only between species and between muscles with different location, but also between homologous muscles. In *Homarus* it was observed (Wiersma, 1951) that the closer muscles of its three types of claws show a remarkable difference in their mechanical reaction to single and double nerve impulses in the "fast" axon. In a more detailed study (Wiersma, 1955) of these effects, it was found that in this species there is a strong correlation between the appearance of diphasic action potentials and twitches. In general many crustacean muscles show monophasic potentials with outside leads, even when twitches are present (Wiersma and Van Harreveld, 1938a; Wiersma and Wright, 1947). But in *Homarus* it was found that single impulses in the "fast" axon of the small claws of the second and third leg result in a clearly diphasic deflection, accompanied by a twitch. In the cutter claw of the first thoracic legs, which shows at best a slight movement of the tip on a single impulse, the deflection is largely monophasic, while in its partner, the crusher claw, which does not move at all, it is completely so. When two impulses at a short interval are given, the mechanical effect in the cutter claw is dramatic, as it closes completely and with considerable force. A nearly maximal diphasic deflection precedes this contraction. In the crusher claw no mechanical effect is obtained, and a summation of the monophasic deflections takes place. In the small claws the second deflection is only somewhat larger than the first, and the summated contraction is still not strong enough to close the claw. For this animal it seems certain that conducted spikes are responsible for the diphasic deflection. The very strong faciliation effect which a single impulse in the "fast" axon of the cutter claw exhibits, in contrast to the much weaker one of the other muscles, is a good demonstration how slight differences in properties can make crustacean muscles especially well adapted for certain special functions.

In general, the relations between junctional potentials, spikes, conducted spikes, and the resulting contractions remain uncertain. The greatest problem in this regard remains the one which has been named the paradox (Wiersma and Van Harreveld, 1938b). There can be little doubt that under given circumstances, especially on low-frequency stimulation, stimulation of the "fast" axon will result in large junctional potentials, which do not elicit a contraction, while similar stimulation of the "slow" axon will result in small junctional potentials, accompanied by a slow contraction. How this is possible when both potentials occur in the same membrane is completely unknown. This quandary is, of course, caused by our

lack of knowledge of the transfer from electrical to mechanical effects. In this connection an observation on the spread of contraction in a living muscle fiber is interesting. Matthaei and Tiegs (1955) photographed a slowly spreading contraction wave in a slightly damaged spider muscle fiber. The contraction originated from under an end-plate structure. It first spread across the muscle fiber before it went in two directions away from this region.

Further clues with regard to this problem should be obtainable from the effects of the inhibitory impulses. Fatt and Katz (1953c), studying especially the inhibition in the opener muscle of the hermit crab, have come to a number of interesting conclusions. Depending on the magnitude of the membrane potential they found that inhibitory impulses could have either no electrical effect at all (which was the condition when the membrane potential was "normal"), cause a hyperpolarization when the membrane potential was low, or give a depolarization when it was higher than normal. The time course of these inhibitory polarization effects was of the same order, but slightly longer than that of the excitatory junctional potentials.

Reductions of the excitatory junctional potential up to 90% of its value could be obtained when the inhibitory impulse preceded the excitatory one. When an inhibitory stimulus was given during the course of an excitatory junctional potential change, its decay time was speeded up. These results certainly go far in explaining the inhibition of the junctional potentials by the postulation of one inhibitor-receptor reaction, which changes the ion permeability of the muscle-fiber membrane and competes with the action of an excitatory transmitter. However, they offer no ready explanation for mechanical inhibition which occurs without even a reduction of the facilitation of the junctional potentials (Marmont and Wiersma, 1938; Wiersma and Ellis, 1942). It is difficult to believe that the membrane could change its electrical properties without at the same time influencing the facilitation process. Hence it still seems likely that the main effect of inhibitory stimulation is on a transmission process between the membrane changes and the contractile process.

It has been shown that spacing of the impulses in inhibitory stimulation can have an effect similar to that of excitatory stimulation. Ripley and Wiersma (1953) found that the same number of inhibitory impulses, when given in pairs at a short interval, gave a more pronounced inhibition in the opener muscle of the claw of the crayfish than when they were given all at equal time intervals.

That transmitters are involved seems quite certain. Concerning their nature little is known as yet, which may well be due to the way in which the nerve fibers end, sublemnally in the muscle substance. The endings may thus be well protected from the direct influence of drugs. In accord with this

concept is the fact that, when a drug is found effective in a nerve-muscle preparation, it can be shown that in most instances it has a similar effect when applied to the axon alone (Ellis, Thienes, and Wiersma, 1942). According to Florey and Florey (1954) there is evidence that, in double motor-innervated muscles of the crayfish, acetylcholine is the transmitter of the fast system, while 5-hydroxytryptamine would bring the slow systems into action since these drugs caused, on perfusion, contractions of different types. It was, however, not shown that these effects were not due to stimulations of the axon branches outside the muscle fibers.

Since it is well known that both fast and slow systems can be inhibited by the same inhibitory fibers and that the same holds true for muscles with four motor fibers, a very interesting field of research offers itself.

Florey (1954) has since withdrawn the claim that 5-hydroxytryptamine is the transmitting substance for the slow contractions. This substance causes stimulation of sensory end organs and their sensory nerves, which in turn stimulate in some way the motor nerves. The present reviewer considers it likely that this stimulation is due to an ephaptic transmission process, which takes place in a region where the slow motor axons are hyperexcitable because of an existing demarcation potential. This will be normally near their cut end.

Investigations in Molluscs

The physiology of neuromuscular transmission in molluscs still suffers from the uncertainty caused by the fact that histological methods have not yet shown conclusively where peripheral ganglion cells are present, intercalated between the motor fibers of the main nerves and their endings. A very recent preliminary publication (Bowden and Lowy, 1955) reports the presence of nerve cells in all lamellibranch muscles so far examined, among which are muscles previously believed to be free from such cells. It will have to be proven that these nerve cells and their synapses are situated in the motor pathway; but it will be necessary to consider this possibility in evaluating the physiological data, until a definite answer is obtained.

In the adductor muscles of *Anodonta*, Barnes (1955) has found evidence that the fast part of the muscles is innervated by nerve fibers which behave as typical motor axons, causing a relatively quick contraction and relaxation (it is, however, of importance to keep in mind that van Overbeek, 1931, observed that the fast part of this muscle could be brought to contraction by quick stretch after its isolation from the ganglia). Although the slow part of the muscle could be made to contract by nerve impulses, its relaxation would depend on an active process through the medium of inhibitory fibers. This view corresponds well with one held for the slow part of the adductor muscle of *Pecten* by Benson et al. (1942), who showed

that, by stimulation of certain parts of the nerve bands going to the muscle, relaxation was considerably speeded up—indicating the existence of inhibitory axons.

In the adductor muscle of *Mytilus* there is no obvious difference between fast and slow muscle fibers. In contrast to *Anodonta* and *Pecten,* in which the fast fibers are striated, here all fibers seem to be smooth (Lowy, 1955). Using the animal's naturally occurring contractions and relaxations, Lowy (1953) found action potentials present in this muscle, even during the long-lasting tonic states, in which they were infrequent. Higher-frequency bursts occurred at the onset of contraction, but also at the onset of relaxation. The latter are rather unexpected, but might come about when both excitatory and inhibitory impulses were reaching the muscle, when the inhibitory ones would inhibit the contraction but not the concurrent muscle action potentials. Lowy (1955) now reports that muscle action potentials can also be obtained from muscles, isolated from the ganglia. He proposes that these may be due either to the presence of nerve impulses in the peripheral nerve-ganglion system or be of myogenic nature; he favors the former view. With Barnes one may still have doubt whether only one type of muscle fiber is present in this muscle; for, though all fibers may be smooth, this would not be a certain demonstration that some of them cannot contract much faster than others.

Another instance in which a molluscan muscle consists of two types of muscle fibers, but this time in series with each other, has been reported by ten Cate and Verleur (1952)—the retractor muscle of the main tentacle of the snail *Helix pomatia.* The distal part is dark in color and hollow and gives much more phasic contractions than the tonic light-colored proximal part.

From these observations one may be inclined to believe that in the molluscs as in the amphibians two separate neuromuscular systems are present, of which the slower one may well be mainly under local nervous control in contrast to the situation in amphibians. Under the circumstances it would be futile to speculate about the types of innervation of the muscle fibers as such. Inhibition, for instance, may well be an effect wholly located in the peripheral nervous system, and therefore not comparable to that of the arthropods. One would like to have at hand more data about the highest forms in this phylum, such as the squid. But here the investigations of Prosser and Young (1937), which showed that a single impulse in the giant motor fibers would lead to a maximal contraction of the whole part of the mantle, whereas smaller axons seemed to give similar but more restricted twitches, are still the only indication that polyneuronal motor innervation may be a possibility in molluscs.

In the long-fibered anterior byssus retractor of *Mytilus,* whose fibers

may run the length of the whole muscle, Twarog (1954) found that applied acetylcholine depolarizes the membranes and instigates contraction. When the drug is subsequently washed out, the membranes repolarize but contraction remains. Relaxation of this tonic part of the contraction can be obtained by the application of 5-hydroxytryptamine (this is also the case when contraction is caused by other means). This drug does not cause a noticeable change in polarization and is ineffective in preventing the onset of the contraction caused by acetylcholine. In bioassays, the normal presence of these substances in this muscle was observed.

From these interesting observations it would seem that 5-hydroxytryptamine may be considered rather a relaxing than an inhibiting substance, and that active relaxation is necessary to release the "catch" mechanism, provided that the substance works directly on the muscle fibers and that only one type of muscle fiber is present.

In a very recent paper (not considered in the original manuscript) based on independently performed experiments, Hoyle and Lowy (1956) come to the conclusion that inhibitory nerves are present in the anterior byssus retractor muscle of *Mytilus edulis*. Furthermore, this muscle would have a built-in system which can fire automatically, this system consisting either in peripheral nerve cells or the spontaneous activity in muscle fibers themselves. They find again that during prolonged tonic contractions action potentials are present, although these occur only in certain areas of the muscle and not everywhere. They conclude that tonic contractions are based on the tetanic activity of such parts. They confirm that 5-hydroxytryptamine abolishes tonic responses, and that this substance does not materially affect the phasic ones. Because the muscle is unable to destroy added 5-hydroxytryptamine they consider it unlikely that it is a natural transmitter. This reviewer is less inclined than the writers to accept on this evidence the absence of a "catch" mechanism. Twarog's (1954) findings with acetylcholine, in which the fiber stays contracted even after the acetylcholine is washed out, indicates to her that such a mechanism may be present. One would want to correlate the total electrical activity of the muscle and the contraction in order to evaluate the significance of the action potentials present during rest. The presence or absence of such correlation would go far to prove or disprove the contentions of this paper. It is interesting to note that, as in crustacean muscle, the independent reactions of the muscle fibers making up the muscle are a great hindrance to this approach.

Investigations in Echinoderms

The presence of phasic and tonic muscle fibers in echinoderms was convincingly demonstrated by von Uexküll for the muscles moving the spines

of sea urchins (1900). He found that the muscles consist of two rings of radial muscle fibers, the outer ring for the relatively quick movements of the spine, for which relaxation is also fast, while the inner muscles can lock the spine so strongly into position that, when forced, it may break rather than give, or the tonic ring may be torn, so that only the phasic muscle fibers remain active. Two types of contractions have recently been reported for the pharyngeal retractor muscles of the sea cucumber, *Cucumaria*, on stimulation of the radial nerve (Pople and Ewer, 1954, 1955). The quick response did not show facilitation, but the slow one showed a marked and prolonged type of facilitation. These findings are in accord with the two types of action potentials previously obtained from the retractor muscles of the pharynx of *Thyone* (Prosser, Curtis, and Travis, 1951). Pople and Ewer argue, however, that the different contractions found are not due to two types of muscle fibers but to neuro-neural facilitation in the ganglion-cell complex, described by Smith (1950) and called the "motor complex." It seems certain that this complex must play some part in the reactions of these preparations but whether it can completely explain the different types of contraction may be doubted.

Prosser (1954) has made an electrophysiological and histological investigation of the long retractors of the body wall of the sea cucumber, *Thyone*. This short-fibered smooth muscle gives only one type of contraction, which may be considered as of the fast type (Prosser, Curtis, and Travis, 1951). On "direct" stimulation the spread of the muscle action potentials is very restricted. The reason for failure of the propagation is ascribed to the fact that many small nerve fibers pass from the radial nerve to the muscle. These behave as separate units and not as branches of one or a few axons. From these results the conclusions are drawn that the muscle is not syncytial in structure and that many unconnected motor nerve fibers are involved in the innervation of this muscle. The latter conclusion is in accord with the results of Pople and Ewer (1954) and agrees too with all previous work on echinoderm musculature.

It would seem at present that the neuromuscular systems of echinoderms may show a greater similarity to those of amphibians than do those of any other phylum, since two different neuromuscular systems controlled by many motor neurons seem to be present. However, this resemblance may still be only superficial. For it will have to be shown of what importance the peripheral ganglion cells are in the motor chain before any conclusions can be drawn.

Investigations in Annelids and Other "Worms"

With one notable exception no recent research seems to have been performed on this group. This is regrettable, since it would be of special im-

portance to compare the annelid and the arthropod neuromuscular systems. Prosser and Melton (1954) have analyzed the proboscis retractor muscle of *Phascolosoma* (Sipunculoidea) with electrophysiological and histological methods. They found both fast and slow contractions present and two types of action potentials. The muscle fibers are all smooth and short. Many parallel nerve fibers innervate the muscle and the axons can be divided into two classes, thicker ($2\,\mu$) and thinner (below 1μ). Both types of action potentials fail to be conducted after nerve degeneration. It is therefore concluded that conduction is always by nerve fibers and not by protoplasmic bridges between muscle fibers. Whether the muscle fibers also are of two types or whether dineuronic motor innervation occurs has not yet been decided. Multiterminal endings may well be present. The fast potentials have the properties of spikes and do not facilitate, the slow ones those of junctional potentials with facilitation. On fatigue the spike shows a prespike potential, but a similar phenomenon was observed for the slow potentials. It seems therefore premature to identify these phenomena with the spike and junctional potentials of the Crustacea. There is, however, a similarity in the shape of the action potentials, which are different in both cases from the conducted potentials of vertebrate muscle.

Thus in *Phascolosoma* the evidence is that relatively small muscle areas are governed by specific nerve fibers; but it should be kept in mind that in the sabellid annelids *Myxicola* (Nicol, 1948) and *Branchiomma* (Nicol, 1951) the giant fibers in the central nervous system innervate a very large part of the longitudinal musculature directly. These giant fibers consist of a conglomeration of many cells, and one may, therefore, consider that in principle the muscles would still be innervated by many axons forming a single unit. If this were the only innervation, these muscles, which form the greatest part of the whole musculature, could function only in the withdrawal reflex. For the earthworm, preparations have been described in the older literature which, with modern techniques, might yield significant results concerning this problem (e.g., Garrey and Moore, 1915).

Investigations in Coelenterates

In the "lowest" phylum in which neuromuscular transmission is present, investigation might well be very difficult. It is therefore gratifying that certain facts have gradually come to light. A most welcome contribution has been the observation of Horridge (1953, 1954) that motor-nerve impulses can be obtained from single axons, a single impulse accompanying each beat of the subumbrella in the jellyfish *Aurellia*. These results make it much more likely that the through-conducting system of Pantin (1952) in sea anemones functions indeed as he pictured it, namely by axons con-

nected by synapses with two-way conduction and with no noticeable synaptic delay, making it possible to consider these systems as simple motor-nerve fibers. As a consequence the facilitations observed have then to be located at the myoneural junctions.

From anatomical evidence of Semal-van Gansen (1952a,b) it seems somewhat doubtful whether in *Hydra* the same differentiation is responsible for the difference in reactions shown by the longitudinal and circular muscles, which are here ecto- and endodermal, respectively. The muscle fibril bundles are found to be located in the basal parts of the cells. The ectodermal bundles are in juxtaposition with each other, and more than one bundle may belong to one ectoderm cell. In the transverse fibers of the endodermal cells there is only one muscle fibril bundle per cell and these are usually well separated from each other. The nervous system consists in the ectoderm of multipolar cells, which may fuse, while in the endoderm the nerve cells are bipolar without visible connections. There may thus be a relation between these anatomical findings and the rather quick contraction of the longitudinal, ectodermal layer and the much slower stepwise contractions of the circular, endodermal fibers.

In sea anemones, Batham and Pantin (1954) and Passano and Pantin (1955) have found further confirmation of the differences in reactions of the different muscle layers. It has especially become clearer that the slow contractions and with them the slow movements of the animal as a whole may be based on spontaneous activity in the muscles.

Investigations in Tunicates

Tunicates have been investigated by Hoyle (1952) with methods similar to those used by Pantin on sea anemones, and some remarkable resemblances were shown to exist. But, as Hoyle has pointed out, until one can be more certain of the alleged underlying factors, it must necessarily remain doubtful how far the phenomena observed find their origin in the neuromuscular transmission. It is nevertheless remarkable that animals which are phylogenetically presumably so far apart seem to have many similarities in mechanisms. The sessile mode of living may be part of the explanation, but other sessile animals clearly have different mechanisms, or at least still show in part of their behavior properties which the free-living related forms possess (e.g., barnacles).

Conclusion

Some very gratifying advances have been made in the area covered by this review. In one respect, though, the recent results may be somewhat discouraging, since they have shown that slight changes in the properties of neuromuscular transmission result in great differences in readily ob-

servable reactions to stimuli and that these slight changes actually differentiate muscles and even the muscle fibers functionally. Hence a welter of phenomena is encountered which makes analysis much more difficult. Of course, this very same differentiation is in itself a highly interesting problem with many connotations, especially for such fields as transmission by synapses and integration. But it is no longer possible to think of a given special mechanism as typical for a group or phylum; instead, the whole array of variations has to be considered. We have as yet no good indication of how widely varied the mechanisms are in the arthropods nor how much of this variability is present in other phyla. It is obvious that a much more intensive study of all kinds of neuromuscular transmissions will have to be made before it will be possible to compare intelligently the different phyla with each other. It can only be hoped that the use of impaled muscle fibers will provide a way of gathering data at a much increased rate. However, the mechanical aspects of contractions should not be neglected for a comprehensive picture. There is here a field wide open for investigation, in which much more research is necessary than is at present being performed. Let us hope that this research will be done; for it will be a rewarding task, not only for its own sake but also for a better understanding of problems of wider scope.

Since the foregoing paragraphs were written, a number of papers about Coelenterates have appeared. These can be only summarily discussed. Horridge (1955 a,b,c; 1956) continued his investigations in different medusae, using neuromuscular preparations and histological investigations, but without the study of the electrical phenomena. He found that in hydromedusae *Geryonia proboscidalis* and *Aequoria forskalea* the swimming movements are governed by a circular through-conducting nervous system. In *Geryonia* this system is not influenced by the radial system, which is responsible for the movement of the manubrium by tonic contractions of its radial musculature towards a place stimulated on the bell. But in *Aequoria* contraction of the radial musculature has an inhibiting effect on the through-conducting system as in a number of other hydromedusae. In the scyphomedusa *Rhizostoma pulmo* repetitive stimulation causes a shortening of the refractory period in a part of the muscle fibers of the circular muscle of the bell. In the scyphomedusae *Cyanea* and *Cassiopea* the compensatory contractions made to keep the animals in a vertical position during swimming were studied. It was found that tonic contractions are caused by a second nervous net, the diffuse net, which acts locally and delays the relaxation time. In order to explain the all-or-none contraction of swimming due to the through-conducting nerve ring and the tonic contractions of the compensatory contractions, Horridge postulates the presence of double motor innervation of the muscle fibers. Ross (1955) has

studied the effect of temperature on the mechanical effects of single and double stimuli in the sea anemone *Calliactis*. At higher temperatures, single impulses start to give visible contractions of the longitudinal muscles, but at the same time the summation period for two impulses decreases considerably. From these results he draws further conclusions regarding the processes of excitation and facilitation.

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NEUROHORMONES OR TRANSMITTER AGENTS

JOHN H. WELSH
Harvard University

During the past two decades studies on invertebrate nervous systems have contributed greatly to our understanding of the basic mode of operation of nerve cells. Giant nerve fibers of the squid and crustacean leg nerve fibers have provided axons of conveniently large size for detailed study of the phenomena associated with conduction of the nerve impulse. Various other invertebrate preparations have furnished highly suitable systems for studies of the transmission process. Thus observations on the isolated heart of *Venus mercenaria* have told us much concerning the importance of molecular configuration in the reaction of acetylcholine with its receptor substance and revealed for the first time the role of 5-hydroxytryptamine as a neurohormone. Insect and crustacean neurosecretory systems have shown how specialized certain neurons can be, for purposes of producing quantities of transmitter agents.

The results of these studies make it increasingly obvious that the most characteristic feature of a neuron is its ability to release, at its terminations, a substance which acts on an adjacent neuron or an effector cell, or which is carried via the circulation to a distant part of the organism. This paper will summarize a small portion of the evidence for the important role of neurohormones.

It has been suggested elsewhere (Welsh, 1955) that the term neurohormone be defined as an organic compound produced by neurons and released at their endings to act as a chemical messenger or hormone, either locally or at a distance. Included among the neurohormones would be the neurohumors such as acetylcholine, nor-adrenaline, and 5-hydroxytryptamine, which often act over rather short distances, and the neurosecretory substances such as vertebrate oxytocin and vasopressin and the products of the neurosecretory systems of insects and crustaceans (see Scharrer, 1955), which normally act at some distance from the point of release.

One purpose of this discussion will be to show how much the various neurohormones have in common, especially in regard to their transport, storage, and release. A second purpose will be to review briefly our knowledge of the chemical nature and distribution of the neurohormones. One other aim will be to point out how incomplete is our understanding of the basic mechanism of action of the individual neurohormones.

Synthesis, Axonal Transport, Storage, and Release of Neurohormones

As long ago as 1914, J. F. Gaskell observed the exciter action of adrenaline on the pulsating blood vessels of the leech. Associating this effect with the presence of chromaffin cells in the nervous system of the leech, Gaskell said, "It is just possible that in the case of the leech the adrenaline passes from the cell to the periphery by way of the motor nerve itself." While this view of axonal transport of materials has since been expressed by a number of workers, there has been a rather general reluctance to accept the evidence as truly convincing. Studies of neurosecretory cells by Ernst and Berta Scharrer and others (see Scharrer and Scharrer, 1954a,b), provide evidence that synthesis of their products takes place largely in the cell body. They are then carried inside the axon to the terminals, which are often modified for storage, whence they are released on appropriate stimulation of the neurosecretory cell. Perhaps the most convincing evidence for proximodistal movement of materials in axons of invertebrate neurons comes from cutting and ligation experiments such as those of B. Scharrer (1952) on *Leucophaea* and E. Thomsen (1954) on *Calliphora*. Other workers have done somewhat similar experiments in certain crustaceans (e.g., Bliss and Welsh, 1952; Passano, 1953). When neurosecretory tracts are cut or blocked, there is an accumulation of secretory material proximal to the point of interruption. Normally, this material with characteristic staining properties is found in far greater concentration in the terminals of neurosecretory cells than elsewhere in such neurons.

Those who have been interested in neurohormones have given much thought to the question of the state in which these highly active molecules exist while yet within the neuron. Are they bound to a carrier protein or lipoprotein? Do they exist as an inactive precursor homologue requiring chemical transformation? Is the characteristic stainable material of a neurosecretory cell a carrier substance or the neurohormone? Potter (1954) has described several tinctorial types of nerve endings in the so-called sinus gland of the blue crab, *Callinectes*. Does this indicate that each type contains a different neurohormone?

There is a recent and very interesting development that helps answer some of these questions and that may prove to be one of the most important steps in our understanding of neurons. In the spring of 1954, several groups of electron microscopists (De Robertis and Bennett, Palade, Palay, Robertson) reported that axon terminals from a variety of animals, including the crayfish and earthworm, contained collections of mitochondria and small vesicles. De Robertis and Bennett (1955) de-

scribe these vesicles in the neuropile of the earthworm nerve cord as oval or spherical bodies between 200 and 400 Å in diameter, with a dense periphery and a lighter center. They find them in large numbers in presynaptic terminals and essentially lacking in postsynaptic processes. They also find them apparently lying between pre- and postsynaptic membranes and suggest that the vesicle may penetrate the presynaptic membrane and discharge its contents or possibly enter the postsynaptic cytoplasm.

There has been a growing body of evidence that acetylcholine, adrenaline, and the neurohormones of the posterior pituitary are contained in cell particles with many of the properties of mitochondria. Some of this evidence has been summarized elsewhere (Welsh, 1955). It would now appear that these mitochondria-like particles are the "vesicles" and "granules" now seen in a variety of nerve endings. It remains to be determined whether the "spheroid systems" seen by Passano (1953) in the cell body, axons, and terminals of neurosecretory cells of the marsh crab, *Sesarma reticulata*, are aggregates of smaller vesicles which are formed in the cell body and move down the axon for storage in the axon terminals.

Crustacean sinus glands, long thought to be more or less typical endocrine organs, were first recognized to be groups of nerve endings by Passano (1951) and Bliss (1951). These endings are modified for storage and release of neurosecretory substances into the circulation. Other examples of nerve endings in crustaceans that release substances directly into the circulation are the pericardial organs described by Alexandrowicz (1953) and the "sinus plates" of the prawn, *Leander*, described by Knowles (1953). The pericardial organs are neuropile-like networks of nerve fibers that spread across the openings through which blood enters the pericardial cavity. They release a heart-accelerating substance into the blood which is one of the means of regulating the heart rate (Alexandrowicz and Carlisle, 1953). Sinus plates consist of nerve endings which contain a chromatophore-activating substance (Knowles, 1953). Carlisle and Knowles (1953) suggested that groups of nerve endings that do not innervate a structure but instead store and release active materials directly into the circulation, be called "neurohaemal organs." In the insects the corpora cardiaca are regions where most of the axons from neurosecretory cells in the brain terminate and release their neurosecretory substance (Scharrer and Scharrer, 1944, 1954b). Therefore, "sinus glands," corpora cardiaca, pericardial organs, and the vertebrate posterior pituitary, all analogous structures, are examples of neurohaemal organs. Neurohumors, such as acetylcholine and nor-adrenaline, are probably stored and released in a manner similar to that described for the neurosecretory substances. Some of the evidence to support this view, gained

from observations on vertebrates, has been summarized elsewhere (Welsh, 1955).

One may picture a typical neuron, whether vertebrate or invertebrate, as an elongated cell whose cell body is its main synthetic center for the production of a specific transmitter agent or neurohormone. Lipid-coated packets of the neurohormone flow with the axoplasm to axonal endings, where they form a reserve. On the arrival of a nerve impulse, resulting in an increase in membrane permeability, a certain number of vesicles (or their contents) are released. The neurohormone may act at close range to excite an adjacent neuron or effector cell, or it may be carried in the circulation to regulate a more lengthy process, such as the activation of insect thoracic glands, which in turn produce a molting hormone.

No longer is it possible to encompass all neurons under the headings "cholinergic" and "adrenergic." Instead we must be prepared to accept a terminology that will recognize a variety of chemical transmitters or neurohormones.

The Chemical Nature and Identification of Neurohormones

In the vertebrates, acetylcholine, adrenaline, and nor-adrenaline have been isolated from the nervous system and chemically identified. There is adequate physiological evidence that these three substances act as chemical transmitters. From the vertebrate posterior pituitary gland the neurosecretory substances, oxytocin and vasopressin, have been isolated and identified as polypeptides, and their detailed structure is known.

All too often in the invertebrates the only procedure used in attempting to identify a neurohormone has been to compare the physiological effects of a nerve extract or of nerve stimulation with the effects produced by the application of a series of known candidate compounds. One has often been forced to follow such a procedure, because of the very small amounts of tissue available for chemical study. By use of extraction and bioassay, with other common pharmacological procedures, acetylcholine has been reported to be present in representatives of most of the major phyla of animals (see Prosser, 1946). Such methods do not always give the true chemical identity of a substance and should, where possible, be supplemented by other means of identification. This appears especially desirable in connection with the identification of acetylcholine, since there are other members of this class of compounds known to occur in the invertebrates (Erspamer and Benati, 1953; Whittaker and Michaelson, 1954; Augustinsson and Grahn, 1954).

Fortunately, by means of basically simple chromatographic and electrophoretic procedures, it is now possible to identify many naturally occurring organic compounds even though they are available in very small amounts.

Such methods have been applied recently in identifying choline esters and catechol and indole amines in certain invertebrates. Three examples will be cited.

Östlund (1954) has cleared up the uncertainty over the question of the occurrence of adrenaline in insects. By means of chromatographic separation, followed by elution and bioassay, he has found adrenaline, nor-adrenaline, and dopamine in a variety of insects. Adrenaline is present in least amount, while dopamine is most abundant of the three. In two lots of whole mealworms (*Tenebrio* larvae) adrenaline was present in amounts of 0.021 and 0.061 μg/gm., nor-adrenaline in amounts of 1.3 and 2.2 μg/gm., while dopamine values were between 10-15 μg/gm. in both lots. Östlund suggests that the presence of relatively large amounts of dopamine may indicate that it is the precursor of nor-adrenaline. A similar origin of nor-adrenaline has been proposed in the vertebrates.

A second example also comes from recent studies on insects. Various earlier workers (e.g., Corteggiani and Serfaty, 1939; Mikalonis and Brown, 1941) reported large amounts of acetylcholine in certain insect nervous systems. Recently some question had arisen regarding the true identity of the acetylcholine-like substance in insects. Now, however, Augustinsson and Grahn (1954), using chromatography, have found acetylcholine in the head of the honeybee. They also have evidence for the presence of one or two other unidentified esters of choline.

In another invertebrate phylum, paper chromatography has been successfully applied in the identification of a biologically active substance present in nerve tissue. Earlier observations had suggested that 5-hydroxytryptamine might be a mediator of the cardiac accelerator neurons to the heart of *Venus mercenaria* (Welsh, 1953). Chromatography revealed the presence of this indole amine in nerve ganglia of *Venus*. We now have evidence for its occurrence in nerve tissue of the gastropod, *Busycon*, the lamellibranchs, *Venus, Mactra*, and *Ensis*, and the cephalopods, *Loligo* and *Octopus* (two species).

At the present time it appears that the neurohumors of the invertebrates are, perhaps, only slightly more varied than those of the vertebrates. In addition to acetylcholine, there may be certain other choline esters or, possibly, simpler quaternary ammonium compounds acting as acetylcholine-like agents. Much of the evidence for the presence and normal action of catechol amines in the invertebrates requires confirmation. The occurrence of the indole amine, 5-hydroxytryptamine, which often has an adrenaline-like activity and which originates in chromaffin cells, as does adrenaline, makes it imperative that the identity of the biologically active amines in the invertebrates be given closer scrutiny.

We know next to nothing regarding the chemical nature of the neuro-

secretory substances of the invertebrates. Representatives of most of the invertebrate phyla, from flatworms through protochordates, have been shown to have certain specialized neurons that give histological signs of secretory activity (see E. Scharrer and B. Scharrer, 1954a,b; B. Scharrer, 1955). The products of such cells help to regulate a variety of processes, such as chromatophore activity, reproductive activity, and growth phenomena, including molting of arthropods. In fact, most physiological processes in a group like the Crustacea appear to be under primary or secondary control of substances released from the nerve endings constituting the "sinus glands." It might be added, at this point, that the discovery of organ Y in the Crustacea by Gabe (1953) and demonstration of its rôle in molt control in the green crab, *Carcinus* (Echalier, 1954, 1955), makes it appear probable that some postulated actions of neurosecretory substances in the Crustacea are actually performed by a hormone from organ Y. This gland, however, may be controlled by a neurosecretory substance. By means of electrophoresis, three different "chromactive" (chromatophore-activating) substances have been obtained from insects and Crustacea (Carlisle, Dupont-Raabe, and Knowles, 1955). In no case has the chemical identity of a neurosecretory substance in an invertebrate yet been determined, although such a step forward would appear imminent.

Modes of Action of the Neurohormones

Little is known concerning the basic mechanism of action of the neurohormones in either the vertebrates or the invertebrates. There is evidence that acetylcholine and adrenaline act at the surface of some cells to produce changes in ion permeability and in resting potential of the cell membrane, but the exact series of chemical and physical events is not known. In this section a few examples will be given of recent progress in our understanding of the modes of action of invertebrate neurohormones.

It was pointed out earlier that neurohormones appear to be of two main types, the neurohumors, which act mostly at short range and for relatively brief duration, and the neurosecretory substances, which may act at some distance from the point of release and for relatively long periods of time. Acetylcholine normally has a very short life after leaving a neuron, because of the abundance of cholinesterase waiting to hyrolyze it. The rate of destruction of the catechol and indole amines when injected or applied is often slower than the destruction of acetylcholine, but amine oxidase is of common occurrence in the invertebrates (Blaschko, 1952). Acetylcholine and certain of the amines act as transmitters of processes where rapid onset of effect and rapid recovery are required. Often they have opposing actions; nowhere is this better illustrated than in many mollusc hearts, where acetylcholine has a depressor action while 5-hydroxytryp-

tamine is excitatory. That nature seldom sticks to a set pattern, however, is seen in the exception to this rule provided by the heart of *Mytilus californianus*. Here, both acetylcholine and 5-hydroxytryptamine are excittory, as R. B. Wait (personal communication) recently observed while working at the Marine Field Laboratories of the University of Washington.

Most molluscan smooth muscle fibers are inconveniently small for use of internal electrodes in recording membrane potentials. However, the very long fibers of the anterior byssus retractor muscles of *Mytilus edulis* permit measuring of a demarcation potential in a manner similar to that long used in studying nerves. Taking advantage of this anatomical situation, Twarog (1954) finds that acetylcholine causes depolarization and a tonic contraction of the byssus retractor muscle, while 5-hydroxytryptamine relaxes tonic contractions. These observations suggest that this muscle is doubly innervated and that opposing neurohumors mediate between nerves and muscle fibers.

By a quantitative measure of the relative activities of a wide range of acetylcholine analogues, Welsh and Taub (1948, 1950, 1951, 1953) were able to show a relationship between molecular structure and biological activity on the *Venus* heart. Certain deductions could be made concerning the so-called acetylcholine receptive substance. In many respects, the patterns of pharmacological action of acetylcholine antagonists on the *Venus* heart resemble those seen in vertebrate autonomic ganglia. However, important differences provide further evidence that acetylcholine receptors which have a common basic configuration may nevertheless differ in details.

We now have considerable knowledge of the pharmacology of 5-hydroxytryptamine analogues on the *Venus* heart. The extraordinarily persistent excitatory action of certain of the ergot alkaloids and of lysergic acid diethylamide (LSD) appears due to the presence of the 5-hydroxytryptamine structure in lysergic acid and the added stable nature and stickiness of the ergot derivatives of lysergic acid (Welsh and Taub, 1948; Welsh, unpublished).

These examples of recent attempts to learn more concerning the structure-activity relations of neurohumors and their analogues, if discussed in greater detail, would show how favorable certain invertebrate preparations can be in such studies. As certain invertebrate nerve fibers have been useful in gaining further insight into the conduction process, so may other properly chosen invertebrate preparations tell us much concerning the details of the transmission process.

Since we know more concerning neurosecretory systems and the action of their products in insects and crustaceans, these groups will be used to

illustrate the type of action characterizing certain neurosecretory substances. Certain insects have groups of neurosecretory cells in the brain whose axons end largely in the corpora cardiaca. These cells produce a neurohormone that exerts a trophic influence on the thoracic glands. The thoracic glands, in turn, produce a molting hormone that has recently been isolated and crystallized, and its empirical formula determined (Butenandt and Karlson, 1954). The molting hormone influences a number of physiological processes associated with molting and subsequent growth. In this case we have a neurosecretory substance that may have to exert an influence over a considerable period of time. Also it may act at some distance from the point of release.

The neurosecretory system of decapod crustaceans is anatomically more complex than that of the insects (Bliss and Welsh, 1952; Bliss, Durand, and Welsh, 1954). Likewise its physiological role appears more involved. Products of this system are employed in bringing about color changes, retinal pigment movements, gonad development, and molting with its many attendant processes. Chromatophores and retinal pigments are directly controlled by neurosecretory substances carried in the blood. Molting in crustaceans appears to be controlled by a hormone from organ Y, the production and release of which during the intermolt period are probably prevented by a neurosecretory substance. Again we find the neurosecretory substances acting at a distance and over considerable periods of time. Until we know more concerning the chemical nature of the invertebrate neurosecretory substances we cannot hope to understand in full detail their mechanisms of action. It is possible that they act in a manner similar to that of the neurohumors but are more stable and tend to form a more lasting complex with cellular components.

In conclusion, it may be said that the invertebrates are providing useful information toward a better understanding of the neurohormones and their modes of action.

Summary

The term neurohormone (or transmitter agent) is here used to designate any organic compound that is released from neuronal endings and serves to convey a message to other cells, tissues, or organs. One type of neurohormone, which we might continue to call a neurohumor, is produced by neurons that are in close association with other neurons or with effectors. Acetylcholine, adrenaline, nor-adrenaline, and 5-hydroxytryptamine are neurohumors known to occur in certain invertebrates. A second type of neurohormone consists of the neurosecretory materials released from neurons which often end on blood spaces and which are sometimes highly modified for the production, storage, and release of transmitter agents.

The neurohumors act mainly at short range and for brief duration, while the neurosecretory materials may be carried via the circulation to distant parts of the organism where they may act over relatively long periods of time. The neurosecretory cells are sometimes organized in systems, as for example in the decapod crustaceans.

There is increasing evidence in both vertebrates and invertebrates that neurohormones are synthesized, transported, stored, and released according to a common pattern. The invertebrates provide good examples of grouped neuronal endings that are modified for storage of neurohormones. Some of these grouped endings are analogous to the vertebrate neurohypophysis.

Paper chromatography has been successfully applied in the identification of the neurohumors in certain groups of invertebrates. Examples are cited. None of the neurosecretory substances of invertebrates has yet been chemically defined although work in this direction is now going on.

We know little concerning the basic mode of action of the neurohormones in the invertebrates. Some of the doubly innervated organs, such as the molluscan heart, furnish favorable material for the study of certain phases of this problem. Here, and in some other places, there is an indication that the neurohumors may act at cell surfaces to alter permeability.

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ENDOCRINOLOGY OF INVERTEBRATES, PARTICULARLY OF CRUSTACEANS*

L. H. KLEINHOLZ
Reed College

Many of the early studies in comparative endocrinology were undertaken to seek among the invertebrates endocrine functions analogous to those known for the vertebrates; but few substantial contributions resulted, probably because the normal physiology of a particular process among invertebrates was inadequately established. The decade between 1920 and 1930 saw demonstrations of hormonal factors in physiological processes of invertebrates in which the pattern and direction of research for a number of years to come was indicated. The first of these was Kopeć's (1922) report that removal of the brain from the last instar larva of *Lymantria* resulted in a failure of pupation to occur; when the brain was reimplanted into the abdomen, pupation was initiated. Perkins (1928) and Koller (1928) almost simultaneously demonstrated that the chromatophores of crustaceans were regulated by a blood-borne substance originating in the eyestalks, instead of by nerves, as had been postulated up to that time.

The lively activity set off by these early studies has resulted in such a large and specialized body of literature in the area of invertebrate endocrinology that it would be impractical for one person to attempt a critical review and survey of this field. This subject has consequently been divided into a number of topics which can conveniently be reviewed by the speakers and participants of this symposium. I shall discuss the general endocrinology of invertebrates, particularly of crustaceans, Bodenstein will survey the endocrine basis of growth and development in insects, Welsh will analyze the function of neurohormones in invertebrates, and Scheer will discuss the metabolic aspects of molting in crustaceans.

The classical criteria in investigations of endocrine problems of vertebrates (i.e., removal of the gland suspected of endocrine function, observing the interference with a normal physiological process as a consequence of such gland removal; obtaining a restoration of the normal process by the implantation of such glands, or by the injection of extracts or separated fractions of extracts prepared from these glands; demonstration of the effective substance in the blood) can be expected to apply in

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similar studies among invertebrates. But application of these criteria has not always been feasible with the invertebrates, so that conclusive proof of endocrine function in certain physiological processes among these groups has not always been possible. For example, one of the major obstacles in the early studies dealing with endocrine regulation of chromatophores in crustaceans was the fact that the source of the chromatophore-activating hormone was unknown, other than that it occurred in the eyestalk; ablation of the eyestalks to remove the source of the hormone at the same time removed the retina, which was necessary as a photoreceptor in in the normal physiology of color change; the limitation imposed on the early investigators of this subject permitted at best the presentation of strong presumptive evidence for endocrine participation in the regulation of color change, through relatively gross deficiency and replacement experiments. An additional criticism that could be directed against those early studies which were limited to testing the effects of injected tissue extracts was that of specificity of the prepared extract, and the difficulty in distinguishing between pharmacological and physiological effects. In other words, the extent to which tissue extracts were reproducing the normal physiological process could not be readily determined.

Hanström and his collaborators (Hanström, 1934, 1935, 1937; Carlson, 1935; Sjögren, 1934) soon placed crustacean endocrinology upon a more substantial morphological basis by describing two apparently secretory structures that occurred in the eyestalks of a number of species of higher crustaceans. These structures were the X-organ and the sinus gland (first called the "blood gland" by Hanström). Experimental attempts at localizing the source of the chromatophorotropic hormones led Hanström and his colleagues to favor the view that the sinus gland was the origin, although their experiments did not conclusively exclude the X-organ from some role in color changes.

Almost simultaneously in these same years appeared a number of accounts which indicated that a number of physiological processes were regulated or influenced by hormones originating in the eyestalk. In addition to color change, which probably has been the most studied of the various endocrine-influenced functions, those of retinal pigment migration, molting and growth, general metabolism, and some phases of reproductive physiology have been the areas most closely investigated. But, despite the impressive literature that has been built up in the past two decades, our knowledge of invertebrate endocrinology is still fragmentary and incomplete compared to that of the vertebrates; basic discoveries are still being made; basic questions are still unanswered.

Instead of a detailed survey of the literature in this area, an attempt will be made here to examine the present trends in crustacean endocrin-

ology. The reader is referred to recent reviews by Brown (1952) and by Scharrer (1952). Three of the older review papers (Kleinholz, 1942; Brown, 1944; Panouse, 1947) examined the problems of crustacean endocrinology. Summaries of many of the papers given at the Symposium on Neurosecretion at Naples in 1953 are contained in the supplement to Vol. 24 of the *Pubblicazioni della Stazione Zoologica di Napoli.*

Molting Hormones

Among crustaceans, as in arthropods generally, growth is a discontinuous process; the external skeleton is shed, and a new skeleton formed; increase in size is restricted to the period between the casting of the old skeleton and the secretion and hardening of the new one. Between ecdyses may be an interval (of varying duration in different species) of lack of growth usually designated as the intermolt interval. Molting may be seasonal or continuous. As might be expected, the casting of the external skeleton in molting is only an outward superficial indication of a veritable metabolic upheaval that occurs at this time. A subsequent paper in this symposium will discuss these metabolic features in more detail. (Scheer).

The rediscovery (Abramowitz and Abramowitz, 1938, 1940; Brown and Cunningham, 1939; Smith, 1940; Kleinholz and Bourquin, 1941) of observations made earlier by Zeleny (1905) and by Megušar (1912), that eyestalk removal shortened the intermolt period in crustaceans, was the stimulus for the considerable number of recent studies inquiring into the physiology of this process and the possibility of its control by hormones originating in the eyestalk. While most of the studies cited above concerned themselves chiefly with the effects of eyestalk removal on molting, Brown and Cunningham were the first to present evidence that the accelerated molt of eyestalkless animals was due to the removal of a molt-inhibiting hormone that apparently occurred in the sinus glands; these authors found that, when the sinus gland was implanted into the body of eyestalkless crayfish, the usual accelerated molt was delayed.

Drach (1939, 1944) described a series of morphological stages that occurred in the interval between two molts of crustaceans, and in his later study investigated the effects on molting in *Leander* when the eyestalks were removed during most of these stages. Drach's observations are worth summarizing here because they established criteria for subsequent investigations of this process and indicated that it was a matter of considerable importance at what stage during the intermolt period eyestalk removal was done. Drach found that the normal molt cycle could be divided into a number of stages based on morphological characteristics, which he designated by the letters A, B, C, and D. In stage A the exoskeleton was very soft, in stage B the branchiostegites were supple; stage C could be divided

into two substages characterized by increased rigidity of the branchiostegites and the condition of the sensory hairs along the margins of the body; stage D was divided into 4 subdivisions, D_1 (divided into three subgroups), D_2, D_3, and D_4, culminating in ecdysis. In a group of 340 *Leander* measuring 25-50 mm., sampled in October, the percentage distribution of the various stages was: A and B, 2.6%; C_a, 16.7%; C_b, 21.1%; D_1', 21.7%; D_1'', 14.1%; D_1''', 6.4%; D_2 and D_3, 17.0%. Drach found that, if the eyestalks were removed in either of the C stages or in stage D_1', a significant shortening of the interval between the operation and the ensuing molt of these animals occurred as compared with unoperated control animals in comparable stages. After removal of the eyestalks in the subsequent D_1 stages or in D_2 or D_3, molting in the operated animals was not significantly accelerated over that in the unoperated controls.

The regulation of the molt cycle described above could not be clearly attributed to the sinus gland if judged by the criteria used in endocrinological analyses; it was generally agreed that eyestalk removal accelerated molting, and that implantation of sinus glands into eyestalkless animals delayed or prevented this accelerated molt, but the additional demonstration was lacking that removal of the sinus glands alone would effect the same acceleration of molting as was accomplished by removal of the eyestalks. A series of three independent studies published simultaneously (Bliss, 1951; Havel and Kleinholz, 1951; Passano, 1951b) showed that careful surgical removal of the sinus glands, leaving the rest of the eyestalk undamaged, had no accelerating effect on molting or on some of the metabolic processes associated with molting. These results were in striking contrast to the accelerated molt obtained with removal of the entire eyestalk. An explanation of these differences was offered by the studies of Passano (1953), who questioned the acceptance of the specific endocrine function of the sinus gland in accelerating molting; he postulated that, since removal of the sinus gland itself had no effect on inducing precocious molting, the delay in precocious molting seen after sinus-gland implantation might have been due to a nonspecific chemical effect rather than to hormonal action. By a series of localization experiments Passano demonstrated that removal of the X-organ induced accelerated molting in the crabs *Uca, Callinectes,* and *Sesarma,* and that implantation of the medulla terminalis, which includes the X-organ, prevented or delayed induced molting.

The role of the X-organ in crustacean endocrinology had been obscure for the twenty years following its discovery by Hanström. The investigations described above not only revealed the physiological activity of this organ in the molting process, but Bliss and Passano proposed that the

X-organ and the sinus gland constitute an anatomical complex, connected to each other by way of the so-called sinus-gland nerve (Bliss and Welsh, 1952; Passano, 1951a; but see also Enami, 1951, and Gabe, 1954). According to the hypothesis elaborated by Passano, the fibers of the sinus-gland nerve are axons arising from the neurons that make up the X-organ, and the secretory products of the X-organ cells are transmitted along the fibers of this nerve to the sinus gland; the sinus gland is considered to consist principally of the free axon endings which have been distended by the accumulation of the neurosecretory product.

While the studies cited above have resolved some of the apparent contradictions of the earlier investigations of the hormonal basis of molting in crustaceans, even more recent experiments have indicated that additional hormonal mechanisms may be involved. Carlisle and Dohrn (1953) postulated the existence within the eyestalk of a molt-accelerating hormone for which they proposed the name of growth hormone or somatotrophin. Their conclusions were drawn from experiments which consisted of the injection of a variety of extracts into *Lysmata* during the winter when the normal molting rate of this animal is low. The highest molt rates (and also mortality among injected animals) were shown by *Lysmata* injected with human chorionic gonadotrophin, mammalian posterior pituitary extract, and extract prepared from the eyestalks of female *Lysmata* collected in the summer. The effective molt-inducing properties of the remaining injected substances were, in descending order: extracts prepared from the eyestalks of female *Palaemon* collected in summer; uninjected controls; boiled extract prepared from eyestalks of female *Lysmata* collected in summer; extracts prepared from eyestalks of male *Lysmata* collected in summer; extracts from eyestalks of *Lysmata* collected in winter; and acidulated distilled water—but the differences among these last five groups are probably not significant. Because of the high mortality among their animals, Carlisle and Dohrn subjected their results to a statistical probit analysis for determining the significance of the data from their experimental and control groups. The possibility exists, however, that they may have been measuring a nonspecific chemical effect of injected substances. In subsequent studies, Carlisle (1953a) reported that the molt-accelerating effect could be obtained by feeding animals eyestalks from donor animals and by a single injection of extract (equivalent to three eyestalks). Some unanswered questions arise concerning the differences in effectiveness of extracts prepared from the eyestalks of males as against those prepared from females, and whether implants of equivalent amounts of eyestalk tissue might not have given more striking results than a single injection of eyestalk extract.

Two later reports by Carlisle (1953c and 1954) are in striking con-

trast with those of other investigators of crustacean molting. In the first of these Carlisle found that eyestalk removal in *Leander* showed no evidence of a molt-inhibiting hormone, and that the evidence pointed rather to the existence of a molt-accelerating hormone in the eyestalk. In this particular regard Carlisle seems to have overlooked Drach's (1944) study with the same species, described earlier in this section. The conclusions of these two investigators are diametrically opposed. An explanation for these striking differences is not readily apparent; Carlisle used only female *Leander,* 55-70 mm. in total length (rostrum-telson), at a temperature of 13.5 ± 1°C, in nonrunning water, the animals being fed twice weekly, and the average intermolt period being about 35 days; Drach's experimental and control animals (apparently) were of both sexes, were 25-50 mm. in total length, were maintained at an average temperature of 14° C in the month of October, were kept in running water, the animals were fed daily, and the average intermolt period was about 20 days. Apart from the possible difference of distribution between the two sexes among the animals used in the experiments of the two investigators, other differences between the two studies may have been important; Drach used smaller animals than those used by Carlisle, and thus had predictably shorter intermolt periods; Carlisle apparently paid no attention to the stage of the intermolt cycle in which the eyestalks of his animals were ablated, a factor which Drach had shown was of considerable consequence in the subsequent molt; for significant shortening of the intermolt period occurred only if the eyestalks were ablated in stage C or in the first part of stage D_1. It can be seen from Drach's data that, in a population of 340 *Leander* of the size class studied, only 60% were in these stages C and early D_1 of the intermolt cycle. An additional factor that might have contributed to these differences may be an unrecognized artifact in the experimental conditions. Scheer and Scheer (1954) observed no increase of molts in *Leander* after eyestalk removal and interpreted their results as suggesting a molt-accelerating factor in the eyestalks, similar to that proposed by Carlisle and Dohrn. Carlisle (1954) found similarly that eyestalk removal in *Carcinides* during the molting season gave no evidence of a molt-inhibiting hormone from the eyestalks, when comparisons with control animals were made. Carlisle's observations and conclusions are at variance with those of other investigators, but the resolution of these differences is not readily apparent. Scheer (this symposium) has explained the difference in results between Drach's, Carlisle's, and his studies as possibly due to distinct races of the *Leander serratus* studied (Carlisle, 1955). Most of the studies of the effects of eyestalk removal on molting have been made on crabs and crayfish; the need for a wider exploration with other species of prawns seems indicated to clarify this point.

A series of three recent short papers has presented evidence for another hormone which is concerned with molt. A paper by Gabe (1953) reported the presence in a variety of crustacean species of a structure which is named the Y-organ, located in the antennary or maxillary metamere; this structure has a highly secretory appearance during stage D of the molt cycle, and Gabe therefore postulated that it might be concerned with the molt process. Echalier (1954, 1955) undertook an experimental study of the role of the Y-organ in the molt cycle of *Carcinus*, and his first reports indicate that removal of the Y-organ results in a block in the development of the usual sequence of stages of the intermolt period: 50 young animals serving as controls had molted once within a two-months period and either had molted a second time or were close to the second molt; of 90 experimental animals from which the Y-organ had been surgically removed, 68 had not molted; of the 22 operated animals which did undergo molt, 10 had been operated in stage D_2, very close to the approaching normal molt, but were blocked in stage C of the following intermolt period. When 3-4 pairs of Y-organs were removed from donors and were implanted into *Carcinus* which had been without Y-organs, 6 survived the implantation and two of these resumed the normal intermolt cycle and molted 40 days after the implantations. While the number of experimental animals cited in this second report is small, and may represent only a preliminary study, the evidence is qualitatively such that the presence of an accelerating hormone from the Y-organ may be added to the inhibiting hormone from the X-organ-sinus gland complex to constitute the crustacean endocrine armamentarium for molting.

How such a double set of hormones for the regulation of molting is physiologically employed is still unknown. Our knowledge of the coordination of physiological mechanisms in molting, despite the wealth of morphological and physiological details of the process, is still amazingly superficial. The trigger mechanism to molt is unknown. We have only some slight indication that feeding or nutritional state, daily photoperiod (Stephens, 1955), and temperature may play a role in the process; but how these internal and external environmental conditions, along with the more complex metabolic phenomena of molt, may interplay with the hormones involved, and how the initiation and cessation of secretion of these hormones may be regulated are still largely obscure. We might be justified in predicting that secretion of the molt-inhibiting and the molt-accelerating substances must be interrelated; for it would otherwise seem physiologically uneconomical to have two opposing regulatory devices participating in so slow a physiological process as molting.

The neurosecretory complex of sinus gland-X-organ will undoubtedly be the subject of additional study, since it is apparently the basis not only

of molting but of most of the other known endocrine processes of crustaceans. The elaboration of neurosecretory droplets, and their transmission through the axoplasm of the fibers constituting the sinus-gland tract, probably could be studied histologically, but the stimulus and mechanism of release of the active material from the sinus gland may be more difficult to explain. Are the fibers of the sinus-gland tract capable of transmitting nervous impulses, and thus of participating in the release mechanism, or is release effected by a mechanism as yet unknown?

Retinal Pigment

The compound eyes of the higher crustaceans, which constitute the principal photoreceptors of these animals, are composed of a large number of ommatidial units. Each ommatidium is usually equipped with three sets of retinal pigments, which have a somewhat varied terminology in the

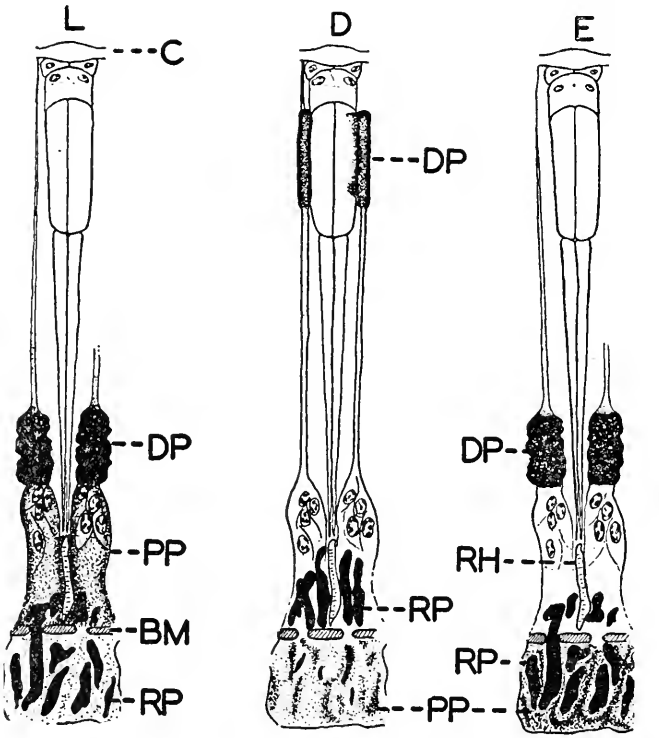

Fig. 1. Ommatidia from the retinas of *Palaemonetes vulgaris,* showing the structure and the positions of the retinal pigments in L, light-adapted eyes; D, dark-adapted eyes; E, eyes from dark-adapted animals injected with eyestalk extract; C, cornea; DP, distal pigment; PP, proximal pigment; RP, reflecting pigment; BM, basement membrane; RH, rhabdome.

older literature but have in the more recent studies been designated as the distal retinal pigment, the proximal retinal pigment, and the reflecting retinal pigment (Fig. 1).

These retinal pigments may undergo movements in response to light and to darkness, but the numbers of retinal pigments showing such movements and the extent of their movement in response to light or darkness may be a species characteristic. Thus, for example, in *Palaemonetes* all three retinal pigments undergo such movements; in *Astacus* the reflecting pigment of the retina is fixed in position above the basement membrane, while the distal and the proximal retinal pigments do undergo photomechanical movements; in *Homarus* the distal and the reflecting pigments are fixed in position and only the proximal retinal pigment moves in response to light and to darkness.

The movements of the distal and proximal pigments have been explained as functioning to screen the sensory component of the ommatidium, the rhabdome, in bright light, and to uncover this rhabdome in low light intensity and in darkness. Thus, in bright illumination the proximal pigment moves above the basement membrane of the eye and the distal pigment migrates centrally, so that the two sets of pigments form a collar around the rhabdome; in this condition light entering the ommatidium directly will stimulate the rhabdome, and light rays which enter obliquely from adjacent ommatidia will be screened out by the collar of light-absorbing black pigment, presumably melanins, which are contained in the distal and proximal cells. In darkness or in dim light the distal and the proximal pigments move away from the rhabdome so as to leave it relatively unscreened, and dim light which enters the eye may pass readily through several adjacent ommatidia to stimulate the sensory receptors of several units, and are thus more effective in stimulation than would be the case were the rhabdomes screened by the pigments. In those species where it undergoes photomechanical movements, the reflecting pigment lies above the basement membrane in darkness, and below the basement membrane of the retina in light. These white pigment granules, which appear to be a mixture of purines and pterins (Kleinholz and Henwood, 1953; Kleinholz, 1955), are believed to reflect dim light that enters the eye over several adjacent receptors, thus increasing the effectiveness of the dim light as a stimulus.

The first experimental evidence that the movement of these retinal pigments might be under hormonal regulation was shown by Kleinholz (1934, 1936), who found that injection of extracts prepared from the eyestalks of a variety of crustaceans into dark-adapted *Palaemonetes* used as the test animals brought about light adaptation of the distal and the reflecting retinal pigments (see Fig. 1); the proximal pigment was apparently un-

affected by these extracts. These observations were confirmed by Welsh (1939), who investigated retinal pigment movement in *Cambarus*, and found, not only that injected extract was effective on the distal pigment of *Cambarus*, but that use of more concentrated extracts also brought about light adaptation of the proximal pigment as well; the reflecting pigment does not move in *Cambarus*. The refractoriness of the proximal pigment of *Palaemonetes* to injected eyestalk extract (Kleinholz, 1936) may have been due to inadequate concentration of the eyestalk extract, as compared with Welsh's observation on the proximal pigment of the crayfish retina, but the possibility should not be overlooked that the differences in response of the proximal pigments of *Palaemonetes* and of the crayfish may be due to different physiological mechanisms of regulation (see below). A subsequent study by Welsh (1941) pointed to the sinus gland of the eyestalk as the presumptive source of the effective substance in *Cambarus*. Additional supporting evidence for the participation of an endocrine factor in retinal pigment migration was presented by Kleinholz and Knowles (1938), Kleinholz (1938), and Sandeen and Brown (1952). In the first study it was found that movement of the distal retinal pigment in *Leander* was not an all-or-nothing response to illumination, but that the movement of this pigment could be graded between the extremes of light and dark adaptation by varying the intensity of illumination. Sandeen and Brown reported a similar situation for *Palaemonetes*. Kleinholz (1938) found further that the amount of migration of the distal retinal pigment in *Leander* was proportional to the concentration of the injected extract, and that the graded responses observed by Kleinholz and Knowles might therefore be mediated by the amount of hormone released into the circulation. Smith (1948) extended the possibility of humoral activation of the retinal pigment to brachyuran crustaceans when he found that injection of extracts of sinus glands, optic ganglia, and other portions of the central nervous system brought about varying degrees of reduction of the glow observable at night in dark-adapted crabs; which particular retinal pigments were affected by these injections were, however, not investigated.

Critical proof of endocrine regulation of crustacean retinal pigments was incomplete. The evidence described above came predominantly from injection experiments, and could be subject to the reservations raised against evidence exclusively of this nature. In an attempt at localizing the source of the retinal pigment hormone, Welsh (1941) found in *Cambarus* that extracts of the sinus gland and extracts of medulla terminalis (seat of the X-organ) were active but those of cerebral ganglia were not. He concluded that, although some activity was shown by extracts of medulla terminalis, this might be due to residual tissue from the sinus gland or to

material which had escaped from the sinus gland during removal, and that the sinus gland might therefore be the source of this principle.

Following Welsh's study came a series of reports which, while failing to settle entirely the question of hormonal regulation with experiments involving removal of the sinus glands, at the same time gave indication that the regulation of the retinal pigments was probably more complex than hitherto believed. Kleinholz (1948a,b) reported that sinus-gland removal from one eye of the crayfish, followed by ablation of the other eyestalk, had no effect on the ability of the proximal retinal pigment to adapt to light and to darkness when such operated animals were placed under appropriate conditions of lighting; of 5 such animals placed in darkness, the proximal pigment of all was in the dark-adapted position, while, of 21 similarly operated animals kept in an illuminated environment, the proximal pigment of 20 was in the typical light-adapted position and that of one animal was irregularly light adapted. No reliable observations of the effects on the distal pigment of the crayfish were available from Kleinholz's study because of the damage caused to the retinal cones in the process of histological sectioning. Similar effects of sinus-gland removal were found by Smith (1948) in his study of glow in the retinas of the crabs *Hemigrapsus* and *Pachygrapsus*; glow, which is generally associated with the dark-adapted retina, did not appear during daylight as a result of this operation, while it was generally present at night, but was variable in degree. Kleinholz (1949) found that in isolated eyestalks of the crayfish *Astacus* the proximal retinal pigment could become light- or dark-adapted when placed in moist chambers under appropriate conditions of illumination; under the conditions of these experiments the eyestalks were isolated from the central nervous system (except for the optic ganglia contained within the eyestalk) and from the circulatory system. The result of these three studies do not support the view that a hormone from the sinus gland is solely responsible for causing the proximal pigment of the crayfish (and of the crab?) to move into the light-adapted position and indicate the possibility that the proximal retinal pigment cells of the crayfish retina may respond to light and to darkness as independent effectors. Knowles (1950) found that the proximal pigment cells of *Leander* from which the sinus glands had been removed respond in normal fashion to light and to darkness; he, too, inclined toward the interpretation that these proximal retinal pigment cells were behaving as independent effectors. In none of the studies cited has the independent-effector hypothesis been critically demonstrated to the exclusion of other possibilities. One such possibility which has been mentioned (Kleinholz, 1948a), and which may be indicated in the experiment of Knowles, is a source of retinal pigment hormone outside of the eyestalk. Conclusive evidence for such sources, beyond that resulting only

from injection experiments, would present serious limitations to the classical methodology of demonstrating endocrine regulation of these effectors.

The evidence for the sinus gland as the source of a hormone causing light adaptation of the distal retinal pigment in the Natantia seems more favorable. In addition to the evidence from the injection experiments of Kleinholz (1936, 1938), Knowles (1950) found that the distal retinal pigment of *Leander,* from which sinus glands had been removed, attained maximal dark adaptation and was not affected by changes of illumination; these results, however, were not unequivocal, for in a few such operated individuals the distal retinal pigment underwent a slight proximal migration (toward light adaptation). The latter responses might have been due to slight injury to the optic ganglia, caused during removal of the sinus glands, a condition which Smith (1948) found to result in varying degrees of light adaptation in the retina of crabs, or they might have been due to some of the other physiological possibilities mentioned above.

In recent years it has been proposed (Brown, Fingerman, and Hines, 1952; Brown, Hines, and Fingerman, 1952; Brown, Webb, and Sandeen, 1953) that, in addition to a hormone which brings about light adaptation of the distal retinal pigment of *Palaemonetes,* an antagonistic hormone, which causes dark adaptation of the distal retinal pigment, may be present. The basis for this hypothesis lies in two kinds of observations; *Palaemonetes* from which one eyestalk has been removed show less light adaptation than normal animals, while similar one-eyed animals show the same dark adaptation as normal animals; the other kind of observation resulted from studies of the kinetics of light and dark adaptation of the distal retinal pigment of animals which had been successively dark-adapted for varying periods, given light stimuli of different durations, and then returned to darkness. It was found that the ensuing variations in the rate of readaptation to darkness could be explained in terms of a hormone that causes dark adaptation of the distal retinal pigment cells. By way of experimental test of this hypothesis, these authors studied the effects on the kinetics of dark adaptation of injecting—into previously dark-adapted animals given a light stimulus and then returned to darkness—extracts of eyestalks, of central nervous system, of tritocerebral commissures, and of sea water. It was found that the rate of subsequent readaptation to darkness was greater after injection of the eyestalk extract and of the central-nervous-system extract than with the sea-water control, and that *Palaemonetes* injected with extracts of tritocerebral commissure showed less light adaptation than the controls. These authors had no success in inducing dark adaptation of the distal retinal pigment in light-adapted animals, and point out that the only condition under which it was possible in

their study to demonstrate the presence of a dark-adapting hormone was under the environmental condition of complete darkness.

Chromatophores

The first systematic study of a physiological process in crustaceans which gave evidence of endocrine regulation was that of color change, the effectors in this case being the pigment cells located within the hypodermis and on the deeper-lying organs of the body. The readiness with which these chromatophores may be discerned depends, among various crustacean species, on the degree of transparency of the overlying tissues and exoskeleton. The chromatophoral systems of different species may range through a complexity of colors, morphology, and distribution over the body surface; the physiological responses are effected by centrifugal or centripetal streaming of the chromatophoral cytoplasm in which the pigment granules are carried, resulting in the dark or colored phase of the animal when the pigment is dispersed through the interlacing processes of the chromatophores, and in the light phase of the animal's color change when the pigment granules are withdrawn from the chromatophoral processes and are concentrated near the center of the cell body; intermediate conditions between the extremes of response are also possible. The colored appearance of an animal may be determined by the abundance and distribution of a particular type of chromatophore; where there are physiologically responsive chromatophores containing an assortment of pigments in the system of an animal, the animal may be able to assume a variety of colors, depending on which chromatophore components have their pigment granules dispersed and which concentrated. In some species color changes may occur in adaptation to the color of the background, in others to change in light intensity. In investigations of color change, the most obvious component of the variegated chromatophore system has been the one usually studied, while those chromatophores which are less abundant have been relatively slighted.

The studies establishing an endocrine basis for color change in crustaceans, the subsequent observations on the diversity of response of different chromatophore systems in a variety of crustacean species, and some of the attendant problems that arose from these studies have been amply reviewed by the authors mentioned in the introductory section of this paper. In recent years a large proportion of the studies of color change has been concerned with the demonstration by extraction and injection methods of the presence of different active principles from the central nervous system of crustaceans. Many of these studies were prompted by an effort to resolve one of the early problems in this field; i.e., whether the regulation of the various types of chromatophores in a color-changing

crustacean could be explained on the basis of one hormone or was due to a number of hormones. In brief summary, Brown and a number of collaborators (see Brown, 1952, for specific citations) have reported three chromatophorotropic principles from the sinus gland of crustaceans, the distinction being based to some extent on different solubilities in ethanol and on the response of particular chromatophore types in different crustacean species: (1) a principle which causes concentration of the red chromatophores of *Palaemonetes*, (2) a principle which causes dispersion of the black chromatophores of *Uca*, and (3) a principle which causes the black pigment in the chromatophores of the telson and uropod of *Crago* to become concentrated. From the central nervous system of a variety of crustaceans two additional principles were adduced: (4) one which disperses the melanophore pigment of *Crago* both in the body and in the "tail," and (5) one which concentrates the melanophore pigment of the body of *Crago* but not of the "tail" of *Crago*.

Most of the evidence presented in attempting to settle the problem of localization of the chromatophorotropic hormones of the eyestalk and of the central nervous system has consisted of histological demonstration of apparent neurosecretory structures in the central nervous system and of injection experiments involving extracts prepared from the central nervous system. The recent explanations of the part played in molting and its associated metabolic processes by the X-organ were facilitated by deficiency experiments involving removal of the sinus gland; the advisability of similar deficiency experiments in studies of color change would seem apparent.

As long ago as 1940 Brown reported that most of the chromatophorotropic effect of whole eyestalks of *Palaemonetes* and *Uca* was contained in the sinus glands. A study by Brown, Ederstrom, and Scudamore (1939) neatly complemented the early injection experiments by examining the effects of surgical removal of the sinus glands from the eyestalk in *Palaemonetes* on the animal's subsequent color behavior. These authors found that the glands could be removed without apparent serious disturbance to the rest of the eyestalk. In such *Palaemonetes* it is to be expected that the erythrophores will become dispersed and the animal will remain in the dark phase of its color range if the sinus gland is the chief source of a chromatophore-concentrating principle; in addition the animal will be unable to adapt its erythrophores to an illuminated white background. When 16 such operated animals were tested on an illuminated white background, the erythrophore responses were such that the animals fell into three groups: 5 showed no concentration of the red pigment, remaining permanently dark; 4 showed a weak concentration of the red pigment; and 7 underwent strong concentration of the erythrophores. Thus, only

about one-third of the operated animals showed the expected chromatophore behavior. But, to test further the completeness of sinus-gland removal, eyestalks from each of these three groups were extracted and the chromatophorotropic activity determined quantitatively by injection into *Palaemonetes* and *Uca* employed as test animals. A direct relationship was found between the activity of these eyestalk extracts on the test animals and the degree of response to white background shown by the erythrophores of the operated animals whose eyestalks were used in making the extracts. Such physiological testing of the efficacy of complete sinus-gland removal is highly desirable as a critical approach to the question of localization of the origin of the chromatophorotropic hormone and indicates that there is no erythrophore response to illuminated white background on total removal of the sinus gland.

Somewhat similar results were obtained by Panouse (1946) with surgical removal of the sinus gland in *Leander*, although the proportion of successfully operated animals was slightly higher than that obtained by Brown and his co-workers; in Panouse's study 10 of 20 sinus-glandless prawns failed to show any white-background response. Here the success of the surgery was checked by inspection after the subsequent molt, when the scar at the site of the operation became transparent like the rest of the body; if a fragment of the sinus gland had escaped removal, it was readily visible and its volume could be estimated. Knowles (1950) in his study of the control of retinal pigment migration in *Leander* also used the immobility of the dark chromatophores of animals kept for 10 days under various conditions of illumination and background as an indication of successful sinus-gland removal; about one-third of the operations undertaken were successful.

The observations cited above complement the evidence from injection experiments in pointing to the sinus gland as the source of chromatophorotropic hormones. Evidence of a like nature for the origin of such hormones in the central nervous system would be technically much more difficult, if not impossible, to obtain. But even the existing evidence presents us with certain anomalies, at least with regard to the question as to whether secretion of chromatophorotropic hormone from the central nervous system can occur as a normal physiological process in color change. If surgical removal of the sinus glands can be accomplished without damage to the photoreceptor apparatus of the eyestalk, and if the pathways for visual reflexes remain intact after such surgery, physiological secretion by the central nervous system should enable such animals to adapt to an illuminated white background; the above investigators do not report such observations except those white-background responses which they explain as due to incomplete removal of the sinus glands. Thus, while there is some

evidence, predominantly from injection experiments but also, to a lesser extent, from observations of chromatophoral behavior under other experimental conditions, for sources of hormone outside the eyestalk, there is insufficient evidence for the participation of these extra-eyestalk sources of hormone in the normal physiology of color change. Panouse (1946, 1947) has expressed some reservations as to the specificity of extracts of the central nervous system in the activation of chromatophores.

Carlisle, Dupont-Raabe, and Knowles (1955), using the methods of paper electrophoresis, have undertaken separation of substances which affect various components of the chromatophore system from extracts of sinus glands and postcommissural organs of *Leander*. From extracts of sinus glands and of postcommissural organs they obtained a substance A which on injection caused concentration of the red and yellow pigments of the large and the small chromatophores. Substance B, having different migration properties and separable only from postcommissural organs, upon injection, concentrated the large red chromatophores of the body but expanded the red pigment of the small chromatophores of the body and of the uropods. Other substances showing more marked electrophoretic mobilities than A or B, but acting only on a single pigment, were separable from postcommissural extracts when the latter were allowed to remain a certain time at laboratory temperature (time and temperatures not stated); the concentration of A and B in these extracts was diminished. It is also reported that fresh extracts of the X-organ have no effect on the chromatophores, but that the same extracts after boiling cause concentration of the dark pigments. These experiments indicate the possibility of substances which by various treatments can be altered or broken down into chromatophorotropically active components.

Reproductive Systems

The question of the existence of sex hormones in crustaceans which maintain secondary sex characters has been under discussion by zoologists for many years. The basis for this discussion has been the observable change in the secondary sex characters of a number of species, either associated with parasitic castration or induced experimentally by x-ray or radium irradiation which destroyed the gonads. This subject has been reviewed by a number of the authors mentioned in the introductory section; there seems to be general agreement that evidence for the secretion of hormones from the gonads to maintain secondary sex characters is not completely satisfactory.

An attempt at experimental approach to this problem along the lines of conventional endocrine surgery by Takewaki and Nakamura (1944) has not been particularly fruitful in presenting evidence in favor of secretion

of hormones from the gonad. These authors were able to perform surgical castration upon male and female isopods, and found no consequent modification of the permanent sex characters. Oostegites, which constitute a brood pouch for developing young, formed in more than 90% of castrated females and are thus independent of the gonad. Since earlier investigators had reported that radiation castration was followed by failure of the oostegites to develop (hence the argument for a hormone from the ovary to maintain the oostegites), it seems unjustifiable from the results of Takewaki and Nakamura to explain such changes in terms of destruction of the gonad.

Reinhard (1950) proposed that differentiation of the inner rami of the pleopods of *Callinectes*, similarly associated with care of the developing young, was under the influence of an ovarian hormone. It may be unwise to extend the conclusions from one order of crustaceans, the isopods, to another, the decapods; but, in the absence of any more positive evidence for ovarian hormones, the results of the Japanese workers remain without substantial challenge.

The cement glands in the female crayfish are a secondary sex character for which the possibility of nonovarian endocrine control factors have been indicated by Stephens (1953). This author reports a factor in the eyestalks which inhibits development of the cement glands in the mature female; a possible rôle of neurosecretion from the central nervous system in regulating these structures is suggested by implantation experiments, but Stephens believes further experimental verification is desirable.

Studies reporting endocrine effects upon the gonads themselves seem to be based on more substantial evidence. Panouse (1946) was the first to report a marked effect of eyestalk removal in *Leander serratus* on the ovaries. He found that by 45 days after eyestalk removal the wet weight of the ovary was 13 times that of unoperated control animals, and the dry weight more than 30 times that of controls. These results occurred in experiments executed well before the normal breeding season; the egg laying which follows such ablation experiments appeared normal. To characterize these results more adequately as a possible endocrine effect, surgical removal of the sinus glands was done on one group of animals and was found to give similar results, but not as marked as those obtained with eyestalk removal, in explanation of which Panouse suggested the possibility of incomplete sinus-gland removal. Reciprocal experiments involving weekly implantations of 2 sinus glands into the abdomens of animals without eyestalks not only prevented the rapid growth of the ovary, but actually depressed ovarian weight below that of normal unoperated animals. The evidence from these experiments suggests an inhibitory hormone from the sinus gland, but the question arises as to whether the inhibitory effect

is on the ovary itself or on some other organ which normally secretes an accelerating or gonadotropic hormone. Experimental procedures to answer this question faced technical difficulties; but, in the light of some reports of neurosecretory structures associated with the cerebral ganglia, Panouse undertook implantations of such tissues into a small number of animals. No significant effect on ovarian growth was detectable, but Panouse himself recognized that the small number of animals (26) involved and the possibility of insufficient implantations make repetition of this experiment desirable.

Panouse's results were confirmed on a number of other crustaceans by Brown and Jones (1947, 1948) and by Carlisle (1953b). A study of the reproductive cycle in the female crayfish by Stephens (1952) postulates the participation of a number of hormones, two from the sinus gland and two from the cerebral ganglia, but these proposals are still speculative and require experimental verification.

On the other hand, results reported by Arvy, Echalier, and Gabe (1954) present more adequate evidence for an additional source of an endocrine substance which is gonadotropic in function. These authors find that bilateral removal of the Y-organ (described above in the section on molting) in sexually immature *Carcinides* results in ovaries in which oogonia and mitoses are rare, follicle cells and vitellogenesis of the oocytes are reduced, and cytolysis of oocytes has occurred. Comparable anomalies appear in similarly operated males. Removal of the Y-organ in sexually mature males and females has no observable effect, such operated animals not being different from normal controls given blank operations. At this stage the authors are unable to decide whether the effect on the gonads is a general metabolic one, because of the arrest of the molting rhythm, or whether it is a specific gonadotropic effect limited to gametogenesis. The Y-organ may be a structure which is physiologically in balance with the sinus gland in regulating ovarian growth, a relationship that would be worth closer examination.

Other Activities and Other Phyla

In very few animal phyla other than the arthropods have endocrine processes been demonstrated in much detail. For the most part, studies among the other phyla consist of scattered observations, which have been summarized in some of the early reviews, particularly that of Hanström (1939). On the other hand, there have been a number of studies of neurohumoral activity among invertebrates, many of which are reviewed in this symposium by Welsh. Recent studies of the distribution of catechol amines in invertebrates (Östlund, 1954), reviews of the pharmacology of indolealkylamines, particularly of 5-hydroxytryptamine (Erspamer, 1954),

and studies of activity of crustacean pericardial organs on the crustacean heart (Alexandrowicz and Carlisle, 1953) all give indications of endocrine characteristics and the possibility of function as a neurohumor in normal physiological processes. Further studies will be able to distinguish between physiological and pharmacological effects of these substances.

Laviolette (1950) has verified reports by earlier investigators of hormonal influence of the gonad on secondary sexual structures of the genital tract in gastropod mollusks. Fragments of the hermaphroditic gonad of *Mesarion*, from which all spermatozoa were absent and containing only fully grown oocytes, were implanted into young *Arion* or into individuals with infantile genital tracts. The hosts were sacrificed one month later and the genital tract accessories (albumen gland, ovospermiduct, copulatory pouch, and the calcareous glands of the neck of the genital atrium) were found considerably modified in comparison with the controls: while the host had not increased appreciably in size, the genital tract had trebled; the genital tract in controls of the same age remained infantile. When a fragment of ovospermiduct from a young *Arion* was recovered from the general cavity of an adult *Kobeltia* in which it had been implanted for five weeks, the fragment had appreciably increased in size and had differentiated histologically toward the type of structure found in the adult. Similar results were obtained on homotransplantation of the albumen gland between two individuals of *Limax* of different ages. Castration was successfully performed on adult *Limax* at an age of 10 months, when the genital tract is fully developed. Three months afterward, appreciable regression of different parts of the genital tract, particularly of the albumen gland, had occurred; regression of the penis was less marked.

One additional area of possible endocrine function should be mentioned here, that of the tunicates among which Carlisle has made some observations. An old report of Hogg (1937) on the occurrence of a gonadotropin in the neural gland complex of tunicates when tested against the mouse had not been widely accepted, probably because of the small number of test animals involved; Carlisle (1950) repeated these and additional tests on male toads, also with small numbers of test animals, with results indicating gonadotropic activity. More appropriately related to tunicate physiology is an hypothesis proposed by Carlisle (1951) that the gonadotropic hormone from the tunicate neural gland constitutes the afferent portion of a gametokinetic reflex. The basis of this hypothesis is that injection of human chorionic gonadotropin into *Ciona* and into *Phallusia* caused release of gametes from the gonads; injection of extract prepared from the neural complex of 1,000 *Ciona* into 9 *Phallusia* provoked a similar response in 6 out of 9 individuals. From the results of additional experiments involving the nervous system of tunicates Carlisle proposed that a hormone from

the neural gland passes to the central nervous system (the neural ganglion) by a nonvascular route, and that the efferent pathway of the gametokinetic reflex consists of a nervous discharge along nerves to the gonads, effecting release of gametes. The high concentration of the *Ciona* extract (1,000 glands) injected into 9 animals may raise some question as to whether this is a true physiological hormone effect; the nervous efferent portion of the proposed gametokinetic reflex could be tested by electrical stimulation of the effector nerve from the ganglion to the region of the gonad.

Summary

Hormonal factors have been indicated in the regulation of some physiological processes among crustaceans: molting and associated metabolic phenomena; retinal pigment migration; chromatophoral behavior in color change; and some aspects of reproductive physiology. The application of critical standards of experimental methodology reveals that, despite an abundant literature in the areas mentioned, conclusive proof of endocrine intervention in some of these processes is lacking.

Molting among crabs and crayfish seems to be influenced by a molt-inhibiting hormone originating in the sinus gland-X-organ complex, although some investigators, studying Mediterranean prawns, fail to reveal evidence for a molt-inhibiting hormone and postulate instead a molt-accelerating hormone. On the other hand, preliminary evidence for the origin of a molt-accelerating hormone in a newly described structure, the Y-organ, has been presented for the crab, *Carcinus*. It is possible that these two structures may be interrelated in controlling the molt cycle of crustaceans.

Most of the studies of endocrine factors in the regulation of the retinal pigments of crustaceans have been made on crayfish and prawns. For the distal retinal pigment, there is direct evidence, although not unequivocal, that movement of this pigment into the light-adapted position in prawns is mediated by a hormone from the sinus gland; studies of the kinetics of light and of dark adaptation in *Palaemonetes* point to the possibility of a hormone that causes dark adaptation of the distal retinal pigment. Hormonal regulation of the proximal and the reflecting retinal pigments has been less certainly demonstrated.

Deficiency experiments and injection experiments point to the sinus gland as the source of chromatophorotropins. The evidence for a source of chromatophore-activating hormone outside the crustacean eyestalk is less satisfactory, being adduced predominantly from histological studies and the injection of extracts of central-nervous-system tissues; technical difficulties make deficiency experiments less feasible than with the sharply circumscribed sinus gland.

No convincing evidence has been presented for the maintenance in crustaceans of secondary sex characters by hormones from the gonads, although it has been indicated that the cement glands of the female crayfish may be regulated by nonovarian hormones. Two apparent sources of gonadotropic hormones have been described: one originating in the sinus gland is inhibitory, since removal of the sinus glands leads to hypertrophy of the ovary; the other, whose specific function is less certain, originates in the Y-organ; removal of the Y-organ in sexually immature *Carcinides* results in degenerative changes in the ovaries and testes, but removal in mature animals has no observable effect.

A few studies are described where endocrine factors have been proposed for secondary sex characters in gastropod mollusks, and as part of a gametokinetic reflex in tunicates.

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HUMORAL DEPENDENCE OF GROWTH AND DIFFERENTIATION IN INSECTS

DIETRICH BODENSTEIN
Medical Laboratories, Army Chemical Center, Maryland

The postembryonic life of insects presents a series of developmental steps interrupted by molts, by means of which the immature organism gradually attains its adult form. The insect undergoes a larval or nymphal molt when it retains its juvenile characteristics. It metamorphoses when adult structures occur after the molt. Molting is usually accompanied by growth, but it also always involves differentiation. Whenever the animal passes from one stage to the next, morphogenetic events of great complexity take place. These lead in the case of the skin, for instance, to the deposition of an entirely new cuticle with all its often very complicated structural elements. These processes of growth and differentiation are under the control of hormones. The humoral situation prevailing at any given stage regulates and guides the expression of the developmental characters. Obviously the manifestation of these developmental events depends not only on hormones but also on the target organs that respond to these humoral stimuli. In the present account, special emphasis will be given to a discussion of the responsive behavior of the reacting tissue. Those interested in other aspects and further details of insect endocrinology may consult the recent reviews by Wigglesworth (1954) and by Bodenstein (1953b and 1954).

THE HUMORAL CYCLE

Although this paper will concern itself mainly with an analysis of the target material, it is necessary for background information to give a short account of the humoral cycle that triggers and controls the target responses. The humoral mechanism which causes the insect to undergo a larval molt or to metamorphose can be briefly outlined as follows. Prior to each molt, a humoral cycle is set into motion by impulses which in certain cases are known to be nervous in nature. They provoke the secretion of a hormone from special cells in the brain, the so-called neurosecretory cells. Under the influence of this brain hormone, the prothoracic glands become activated and produce hormone. This hormone apparently acts directly on the target organs. Its initial activity causes a wave of proliferation in the epidermal cells and thus sets the stage for molting. Because of this growth-promoting ability, the prothoracic-gland hormone has been

called a growth hormone. As the titer of the hormone gradually rises, under the influence of this hormone alone, the target organs respond by differentiation of imaginal structures. For example, the epidermis will lay down the complicated imaginal cuticular pattern under the influence of a sufficient hormone titer. Since it inaugurates imaginal differentiation, the prothoracic-gland hormone has also been called a differentiation hormone. Thus growth and imaginal differentiation are caused by the same hormone.

The sequence of events is different when the production of the prothoracic-gland hormone is followed by the release of a second hormone from the corpus allatum. In the presence of the allatum hormone, the response of the targets is modified. After the mitotic wave induced by the prothoracic-gland hormone has run its course, the allatum hormone causes the targets to form larval structures; thus the allatum hormone is responsible for larval differentiation and in its presence a larval molt ensues. Since the juvenile, or larval, characters are maintained by the presence of the allatum hormone, the latter has been named the juvenile hormone. During the entire larval life of the insect, the allatum hormone is present in sufficient titer to cause larval molts. Only in the last larval stage is the allatum hormone titer too low in relation to that of the prothoracic-gland hormone to make its effects felt; under these conditions, the animal metamorphoses, that is, the targets differentiate into imaginal structures. One will notice that the development of larval features is controlled by the combined action of these two hormones. As a matter of fact, the allatum hormone can make its effects felt only in the presence of the prothoracic-gland hormone, for it is the latter that sets the molting process into motion. The special humoral balance prevailing at any time and the state of responsiveness of the target organ to this balanced hormone system together determine the features of the insect characteristic of any stage.

Growth

Growth in insects is often cyclic. At definite time intervals the immature insect molts. Molting can be regarded as the visible expression of growth. By the term "growth" we mean cell multiplication, unless otherwise indicated. Now it has been known for a long time that the first perceptible indication of molting is the occurrence of a mitotic wave in the epidermal target, which is followed by differentiation. At each molt, therefore, the target exhibits two main developmental reactions, namely growth and differentiation. Only for didactic reasons will these two developmental events be treated separately. Actually, both are closely related and often occur simultaneously. We assume that cell multiplication can only occur in a hormone-conditioned environment, which at the molting time reaches its

effective threshhold; during the nonmitotic intermolt periods there is apparently too little growth hormone in the system to be effective. The experimental evidence for the above assertions is as follows.

Cyclic Growth

The necessity of the prothoracic-gland hormone for growth is well illustrated in experiments on *Drosophila* (Bodenstein, 1943). Imaginal discs transplanted into the abdominal cavity of adult male flies do not grow. But growth in these organs can be induced by the simultaneous transplantation of ring glands, which in these animals produce the prothoracic-gland hormone. Thus only in the presence of the hormone is mitotic activity possible. Similarly, in tissue culture experiments (Schmidt and Williams, 1953) one finds that spermatogonia of *Platysamia cecropia,* isolated in a hanging drop of caterpillar blood, divide only when blood containing prothoracic-gland hormone is used; no growth occurs in spermatogonia isolated in blood containing no prothoracic-gland hormone. The humoral dependence of the cyclic mitotic wave in the cells of the epidermis is also well documented. Mitosis in the epidermis takes place only when prothoracic-gland hormone titer has reached a certain threshold shortly before molting (Wigglesworth, 1934; Kühn and Piepho, 1938). Mitosis never occurs when the hormone concentration is prevented experimentally from reaching the effective level. In the other hand, whenever a molt is induced experimentally, mitotic activity in the epidermis is also induced. No mitosis is seen in the epidermis during the intermolt period, when the prothoracic-gland hormone titer is expected to be very low.

The cyclic rise of the hormone level and the associated induction of mitosis are also evident in wound healing. If small wounds (needle pricks) are made in the ventral coxal skin of the cockroach leg, they are closed by a migration of the epidermis cells over the wound surface. This depletes the area surrounding the wound of cells; thus it lacks a normal cell density. This situation is regulated during the next molt when, under the influence of the normal prothoracic-gland hormone level, the mitotic wave starts in anticipation of this molt. At this time, increased cell division at the depleted areas brings back the normal cell density (Bodenstein, unpublished). In similar experiments on the cuticle of *P. cecropia,* much the same observations were made, for here too it is at the time of the occurrence of the mitotic wave and not before that the deficient cell number is restored (Smith and Schneiderman, 1954).

Continuous Growth

Quite different is the course of events in the growth behavior of the imaginal discs of *Drosophila* larvae. During the entire larval period these

discs grow at a rather constant rate; at least, there seems to be no peak of growth associated with the molting times (Enzmann and Haskins, 1938). Growth of these discs seems independent of the cyclic increase of hormone. Indeed, much growth occurs in these organs during the intermolt periods when the hormone level must be subthreshold. Does this indicate that the discs do not depend on the prothoracic-gland hormone for their growth? It does not, for, as mentioned before, these discs transplanted into an adult male host only grow in the presence of hormone supplied by the ring gland. Growth of these discs is therefore only possible in an environment properly conditioned by prothoracic-gland hormone. The fact that these discs do grow during the larval intermolt period indicates the presence of the hormone in the larval system in a titer sufficient for the growth of the discs but inadequate for the induction of molting. The same is apparently true for the imaginal discs of lepidopterous larvae, where the mitotic activity of the epidermis is definitely cyclic but where the discs seem to grow continuously (Eassa, 1953). From all this one must conclude that the ability of the different tissues to respond with growth to a given titer prothoracic-gland hormone varies. Some tissues are able to grow in a low, others only in a higher titer.

In this connection, one will recall the wound-healing experiments, where the reduced density of the epidermis, brought about by migration of the cells towards the wound, was regulated during the following mitotic cycle. In another insect, *Rhodnius*, the regulation of the cell density does not await the next mitotic event, but mitotic activity occurs in the depleted areas a short time after cell migration (Wigglesworth, 1937). Thus depletion is cause enough to inaugurate cell division. Mitosis in this insect can even be induced without wounding, that is to say without disturbing the normal cell density, just by placing denatured protein material on the outside of the cuticle (Wigglesworth, 1937). Factors emanating from the applied material apparently pass through the cuticle, altering the epidermis cells in such a way as to make them capable of mitosis. These facts do not indicate that *Rhodnius* epidermis cells can divide in the absence of prothoracic-gland hormone. They imply that changes in cell density, as well as the application of certain substances, are able to alter the responsive capacity of the cells. The altered cells are now able to respond to the low hormone titer prevailing in the animal during the intermolt period. The epidermal cells of *Rhodnius* are apparently easier to alter experimentally than those of the roach. In the latter, density changes do not make the cells capable of immediate mitosis. But density changes have altered the roach epidermis cells also; this is evident from their reaction during the mitotic wave period, where the cells in the regions of low density divide more frequently than those in normal density regions. Of course, the ob-

served differences in reaction between these two species might be based on differences in the prothoracic-gland hormone concentration during the intermolt periods, for which we have no accurate measurement. If the hormone titer in *Rhodnius* is relatively high, a rather slight change in the reaction threshold of the *Rhodnius* epidermis cells could bring about mitosis.

Hormone Concentration and Growth Rate

The above considerations bring up the question of whether there is a definite relation between hormone concentration and growth rate. If so, one would expect that the growth rate would be a function of the hormone concentration, within the limits of the growth capacity of an organ. Information on this is provided by experiments in *Drosophila* (Bodenstein, 1943). Larval organ discs were transplanted into the abdominal cavity of adult male flies, together with a varied number of ring glands. It was found that the growth of a disc was more pronounced in proportion to the number of ring glands which were transplanted together with the disc. In other words, the growth velocity of the discs increased in proportion to the amount of prothoracic-gland hormone present in the system. The hormone produced by four ring glands was sufficient for the expression of the disc's maximal growth capacity, since eight ring glands did not cause more rapid growth than four ring glands. The importance of the hormone concentration as a significant factor in the regulation of growth velocities is thus evident. The dependence of the growth velocity on the concentration of prothoracic-gland hormone is well illustrated in another set of experiments. There is good evidence to suggest that the concentration of the hormone is greater in late larval stages than in younger ones. If this is correct, one would suppose that organs taken from younger donors and transplanted into older hosts would grow better in their new environment. This experiment has been performed on *Drosophila*, where organ discs of younger larvae were transplanted into the body cavity of older larval hosts (Bodenstein, 1940). As was anticipated, the young discs grew better in the older hosts than in those of their own age group. And vice versa, older discs transplanted into younger hosts were retarded in their growth.

Growth Regulation

These experiments not only illuminate the specific subject under discussion; they also shed some light on the complicated problem of growth regulation. In their new surroundings, the young transplants grow faster than they would have if left in their own environment; they also cease their rapid growth once they have caught up in size with the corresponding

organ discs in their new hosts. Likewise, the older discs in younger hosts are retarded only until the equivalent host discs have reached the same size. Once host and transplanted organs are in size harmony, their growth continues alike; this phenomenon is known as growth regulation. Now the question arises: Why do the young discs ever catch up with the size of the host discs, for the latter are, after all, growing in the same high hormone titer? The answer may be found in the physiological age of the discs. The younger discs must be more responsive than the older ones to the same hormone concentration. As the younger disc grows larger and becomes older, it must gradually lose its higher responsive capacity, until host and graft tissue have the same reaction threshold. For the combination of older discs in younger hosts, the same holds true. The younger host has a low titer of prothoracic-gland hormone—too low to support much growth in the older disc, but still high enough to allow for considerable growth of the responsive young organ disc. For the first time, then, we have here an indication that the responsive capacity to humoral stimuli changes with the age of an organ. At any time in development, the prevailing hormone concentration and the responsive state of the target together determine the growth velocity of an organ. This relationship underlies the characteristic growth achievements which we call growth regulation.

Additional information relating to responsive differences between young and old issues is provided by the following observation. *Drosophila* has three larval instars. When eye discs of young third-instar larvae are transplanted into late third-instar individuals, the transplant metamorphoses prematurely in synchrony with the host, and gives rise to a small but otherwise normal eye. Yet eyes from second-instar larvae transplanted in the same manner are unable to metamorphose synchronously with the host tissue. They continue to grow until they have reached the age at which they can respond with differentiation to the humoral factors (Bodenstein, 1939). The essential point here is that very young organ discs can respond to a certain prothoracic-gland hormone titer only with growth, while older discs respond to the same titer with differentiation. The evidence thus suggests that the young organ's response is a growth response, and that the differentiation response is acquired later in development. As the organ discs become older, they respond with differentiation to a hormone titer which earlier elicited growth only. However, it must be understood that a rather young organ, which in a low hormone titer responds with growth, can be made to differentiate when the titer is raised. This is the case in the above-mentioned experiment, where young third-instar discs transplanted into older hosts differentiated prematurely. Other experiments with similar implications will be discussed later in relation to differentiation phenomena.

Regeneration and Growth

Attention must now be given to another aspect of development—namely, regeneration, in which growth plays so important a rôle. If the prothoracic-gland hormone is as vital for the control of mitotic activity as the foregoing considerations seem to indicate, its effects should certainly be demonstrable in the processes of regeneration. Recent evidence provides proof for this contention (Bodenstein, 1955). Although most insects regenerate their legs readily in the immature stages, they lose this power in the adult stage. Legs of adult cockroaches amputated at the femur-trochanter level are never replaced by regeneration. However, the transplantation of prothoracic glands into such adult individuals restores the power of regeneration. Under the influence of the hormone, the adult animals are caused to molt. When this occurs, the amputated leg is replaced by a well-formed regenerate. Since growth is an integral part of the process of regeneration, the importance of a hormone-conditioned environment for the expression of growth is again evident.

Now the roach is an insect in which cell multiplication is a cyclic affair. One may therefore assume that during the intermolt periods there prevails a hormone titer too low for the support of growth. Yet, as far as regeneration is concerned, this titer is adequate for the growth of the regenerating tissues, for regeneration starts and continues after amputation of the leg at any time during the nymphal intermolt period. This implies that the regenerating cells respond with growth to a hormone titer which is too low to bring about the same reaction in nonregenerating cells. One will recall that young cells exhibit the same high sensitivity to the hormone. In this respect, the behavior of the regenerating cells resembles that of young cells. Since regeneration recapitulates in many aspects early developmental processes, the characteristic growth response of the regenerating cells is not astonishing. As in young cells, the growth rate of the regenerating cells should fall off with age. Since, concomitantly with the decrease in the responsiveness of the regeneration cells, the hormone titer increases as the animal gets closer and closer to the molting stage, the overall growth velocity of the regenerate is the expression of these two interacting forces. From this point of view, regeneration might be looked on as a special case of growth regulation.

Growth of Adult Target Organs

The adult insect has no prothoracic gland and is therefore unable to molt and hence to grow. Yet, if the adult is experimentally supplied with prothoracic-gland hormone, mitotic activity can be induced and the insect made to molt again. The adult tissue has retained its powers of growth.

Perhaps a higher titer of hormone is needed to induce growth in the adult than in the larval insect. Experiments on this point are not clear. It is certain that regeneration is more easily induced in larval than in adult animals (Bodenstein, 1955). However, mitosis in the epidermis of adult *Rhodnius* can be induced simply by wounding (Wigglesworth, 1937). In this case, mitotic activity was brought about in the apparent absence of prothoracic-gland hormone. This is evidence that under certain conditions, mitotic activity may occur even in the absence of this hormone; there are other instances with like implication. These facts cannot be denied and we have no intention of minimizing their importance. Yet the rôle of the prothoracic-gland hormone for growth is a vital one, and it is this aspect of the problem of growth which we have emphasized in the above discussion.

Differentiation

Age and Imaginal Differentiation

It is a curious fact that growth and imaginal differentiation in insects are controlled by the same hormone—the prothoracic-gland hormone. Whether an organ responds with growth, or with differentiation, depends on the age of the organ and on the hormone concentration to which the organ is exposed. For instance, in the humoral environment of a late last-stage *Drosophila* larva, an eye disc taken from a young last-stage larva will begin its imaginal differentiation at the same time as do the host tissues—that is, prematurely. But if this eye disc had been left in its own young donor, it would have continued to grow. Still younger discs transplanted into mature larvae are unable to differentiate synchronously with the host (Bodenstein, 1939). Similarly, the genital *anlage* of a fourth-stage cockroach nymph transplanted into a last-stage nymph is unable to differentiate its imaginal structures at the metamorphosis of the host (Bodenstein, 1953a). The same is true for the wing buds of a first-stage *Rhodnius* nymph transplanted in the same manner (Wigglesworth, 1934). These young *Rhodnius* can be made to metamorphose prematurely into miniature adults, but their wing buds fail to differentiate to imaginal completion.

On the other hand, other structures, as for example the integument, appear ready at all times during the postembryonic period to differentiate adult structures if supplied with the appropriate hormone stimulus. One is able to make the skin of newly hatched caterpillars form imaginal skin, bearing scales, by transplanting it into a mature caterpillar (Piepho, 1938). Thus, in synchrony with the differentiation of the host, the transplanted young skin will differentiate prematurely into adult skin. The

above-mentioned prematurely metamorphosed *Rhodnius* nymph which gave rise to a dwarf imago with adult integumental structures is another example. Here again, we witness the same phenomenon which we already encountered in the growth response—namely, that age is an important factor in differentiation, and that different tissues possess different thresholds of responsiveness.

Differentiation and Hormone Titer

Not all tissues respond alike to the same hormone titer. If in a last-stage lepidopterous larva the production of the prothoracic-gland hormone is cut short by the application of a ligature before much hormone has been given off into the blood, the skin will respond with pupal differentiation only at certain body regions. More pupal cuticle will be formed when the ligature is applied later in development. Finally, if the hormone concentration is high enough, the entire cuticle of the caterpillar will pupate (Kühn and Piepho, 1938). Or, when in *Drosophila* a leg and genital disc are implanted together into the abdominal cavity of an adult host, and the differentiation of these transplants is then initiated by the transplantation of the ring glands, only the leg disc completes its imaginal differentiation, while the genital disc remains pupal. Even the various regions of an organ may differ in their competence to respond with differentiation to the prothoracic-gland hormone. In the presence of a rather low hormone titer, only the tarsal segments of a *Drosophila* leg disc differentiate to imaginal completion, while the rest of the leg structures remain pupal (Bodenstein, 1943). More examples could be added, all suggesting that certain cells respond more easily with differentiation than others to the same hormone concentration.

Differentiation of Adult Structures

The amazing plasticity of the target material is best illustrated in the redifferentiation of adult structures. As before mentioned, adult insects can be made to molt again by supplying them with prothoracic-gland hormone. Under these conditions, the adult individual is able to develop almost all its imaginal characters anew. Not only is the entire imaginal skin redifferentiated, but also the differentiation of such complicated structures as the external genital apparatus, scales, sense organs, etc. is completed to perfection. Not once, but several times is the adult skin able to accomplish this task. Although the adult tissues still possess great developmental capacities, there are certain restrictions, and again the extent of the restrictions seems to vary with the type of species and tissues involved. For example, the mature cockroach wing is a structure which the adult organism is unable to rebuild (Bodenstein, unpublished); and yet

the imaginal scale-bearing cuticle of the adult wax moth can be made to form scales again (Wiedbrauck, 1953). The scales of the new imaginal skin develop from the original scale-forming cells. These secondary scales are normal in size but simpler in form. Apparently, the formative cells have gradually lost their ability to form normally shaped scales. On the contrary, this adult cockroach integument loses none of its complicated architecture even at successive extra-imaginal molts (Bodenstein, 1953a). Even a small feature characteristic of the adult skin, namely the loss of the molting sutures in the head and thoracic skin, is not restored in the molting of the adult. The adult is thus unable to get out of its exuviae.

Larval Versus Imaginal Differentiation

The reader must recall an earlier statement of the utmost importance for the understanding of the following comments. It has been said that differentiation takes place not only in the development of the adult organism, but that complicated processes of differentiation also occur at each larval molt, when, from instar to instar, larval characteristics of great specificity are laid down anew. Larval differentiation is thus distinct from imaginal differentiation. The presence not only of prothoracic-gland hormone but also of corpus allatum (juvenile) hormone is necessary for larval differentiation. The latter apparently alters the course of differentiation, leading it toward larval characteristics. Imaginal differentiation ensues at metamorphosis, because at the last larval stage the allatum hormone titer in the organism is too low to support larval differentiation effectively. The latter occurs at the larval molts, because at these stages the allatum hormone titer is higher in relation to the prothoracic-gland hormone titer than it is at the last larval stage. It is possible experimentally to prevent the developmental system of the last larval stage from metamorphosis, i.e., from imaginal differentiation, by supplying the organism anew with allatum hormone through transplantation of larval corpora allata. Under these conditions the reacting tissues are forced to respond with larval differentiation and an extra larval molt occurs. Many experiments of this kind, by which supernumerary larval molts have been induced, attest to the validity of this concept. They also prove that the tissues of the final larval stage are still able to respond to the allatum hormone with larval differentiation.

The question now arises: Is the target material of all stages equally responsive to the allatum hormone or is its responsive threshold to this hormone gradually lowered as the tissues become older? Transplantation of adult cockroach skin into younger nymphal stages demonstrates that the capacity of this tissue to respond to the allatum hormone has apparently been lost, or considerably reduced. Although the grafted pieces of

skin may molt as many as three times with their nymphal host, they retain their adult characteristics regardless of the presence of allatum hormone in the nymphal host (Bodenstein, 1953a). Adult structures of most insects tested in this manner show a similar behavior. In order to cause adult skin to revert into larval skin, a much higher titer of allatum hormone is apparently necessary than that prevailing in normal stages. Such a high titer was provided for the adult tissues in an experiment on *Rhodnius* (Wigglesworth, 1940). When the adult insect was supplied with a great number of corpora allata, the adult skin could at least partially revert to nymphal skin.

However, the skin of not all adult insects has this low responsive threshold. The skin of the adult wax moth was exposed to humoral systems containing different amounts of allatum hormone (Weidbrauck, 1953). When exposed to a larval system with its high allatum hormone titer, adult skin occasionally reverted to larval skin. In a lower allatum hormone titer, pupal cuticle was often formed, while the adult structures were repeated most frequently in an even lower allatum hormone titer. Since larval skin always responds with larval differentiation in a larval humoral environment, while in the same environment adult skin shows this response only occasionally, it follows that the target gradually loses its responsive capacity to the allatum hormone with age. But adult tissues may not entirely lose their capacity to respond with larval differentiation. They merely need a higher allatum hormone titer than the younger ones to elicit this response. We have seen that the responsive capacity of the targets to the prothoracic-gland hormone increases with age. This implies that the target systems change their responsive competence in the course of their development. Younger tissues respond with greater ease to the allatum hormone, but older tissues with greater ease to the prothoracic-gland hormone.

Furthermore, it must be noted that, even within the same individual, different body regions vary in their reaction thresholds to the allatum hormone. This point may be illustrated by some recent experiments on moth larvae (Piepho and Heims, 1952). The removal of the corpora allata from young caterpillars at the beginning of the intermolt period results at the subsequent molt in the formation of pupal skin. Obviously this operation has not left enough allatum hormone in the organism to support larval differentiation. If the allata are removed somewhat later in the intermolt period, when the allatum hormone titer is expected to be higher, then the postoperative molt is a partially pupal molt. Only certain regions of the cuticle show pupal characters, while the greater part of the skin has differentiated larval structures. Thus not all regions of the cuticle exhibit the same response to a given allatum hormone concentration. Essen-

tially the same result is obtained when corpora allata in varied number are transplanted into last-stage caterpillars, where the allatum hormone titer is normally a low one (Piepho and Heims, 1952). Under the impact of the increased allatum hormone titer, the cuticle responds at the next molt with a varied degree of larval differentiation, instead of with pupation. The more allata are implanted, i.e., the higher the hormone titer is raised, the wider spread is the larval differentiation of the cuticle. A complete larval molt can be induced if the number of allata implanted is large enough. By means of two quite different procedures, namely by the reduction of the allatum hormone titer in the young organisms or by the increase of this hormone titer in the last-stage caterpillar, it is possible to change the normal sequence of differentiation.

Changes in Target Organ Differentiation Thresholds

The ability of epidermal cells after wounding to restore their normal cell density by increased cell division and the induction of division by the application of certain materials to the surface of the skin have been cited to illustrate how special conditions not affecting the humoral situation can alter the responsive capacity of target cells. An interesting phenomenon with like implication was found in experiments on wound healing in relation to larval differentiation (Piepho, 1950). The experiments are as follows: Several small wounds were made in the cuticle of last-stage wax-moth caterpillars. A few corpora allata were then implanted into one of these wounds. Before these animals molted, the wounds healed by the formation of a thin wound epithelium which was protected on the outside by a cover of coagulated blood. These individuals then molted into pupae which were normal, except that at the wound regions the epidermis, instead of laying down pupal cuticle, responded with the formation of larval cuticle. The implication is clear. The threshold of the epidermal cells for larval differentiation has been lowered by regeneration. The epidermis, we may say, has been rejuvenated, for it responded with larval differentiation to an allatum hormone titer that was too low to elicit a larval response in the nonregenerated epidermis. The high sensitivity of the regenerated epidermis can also be maintained beyond the first postoperative molt, as the following observation demonstrates. Again, a wound was made in the cuticle of the last-stage larva, but this time ventrolaterally in the region of an abdominal proleg. Then six corpora allata were transplanted. This caterpillar molted into a pupa which showed at the wound region a rather large area made up of larval cuticle. From within this larval region, a complete larval proleg protruded. The pupa finally molted again, giving rise to the imago, where, surrounded by the scaled imaginal skin, the larval area containing the larval proleg was still present. Therefore,

the regenerated epidermis had maintained its high sensitivity to the allatum hormone through the period of two successive molts. This case strikingly illustrates the importance of the target's response in determining the final developmental achievement.

As indicated above, regeneration seems to rejuvenate last-stage epidermis, making the wound epidermis more responsive to allatum hormone. The validity of this contention gains support by one further ingenious observation and experiment by Piepho and Heims (1952). These investigators found that the skin of wax-moth caterpillars is composed of two types of cuticle. The skin of the larval head, pronotum, and anal plates is made up of a brown, hard cuticle. The skin of the rest of the body consists of a soft yellowish cuticle. Now the hard, brown cuticle, of which the entire pupa is made, very much resembles the larval head, pronotum, and anal plate cuticle in its morphological as well as its histological structure. The cuticular pattern of the caterpillar is a mixture of larval and pupal cuticle. Thus, in normal development, the larval epidermis reacts to a normal larval allatum hormone titer with the formation of two cuticular types—a larval and a pupal type. One will recall that the pupal type of cuticle is much less sensitive to the allatum hormone, but that by regeneration it can be sensitized. If the hard, dark larval cuticle is actually the physiological equivalent of the pupal cuticle, it too should be sensitized by regeneration. To test this, a small window was cut in the hard, dark cuticle of the pronotum and the pre-anal plate of young third-stage caterpillars. It was observed during the following molts that the wound epidermis in some of the cases actualiy formed soft, yellowish cuticle, which stood out in striking contrast to the surrounding dark cuticle. The epidermis of the larval pronotum and pre-anal plate can indeed be sensitized by regeneration. Without the addition of extra allatum hormone, but under the influence of a normal larval allatum hormone titer, the dark larval cuticle can be made to form cuticle of the soft yellowish type. It is possible, the authors believe, that the other larval sclerites also possess a slightly lower sensitivity to the allatum hormone than the nonsclerotized region of the body. The typical larval cuticular pattern is thus basically a sensitivity pattern. In normal development, the epidermis reacts according to its inherent sensitivity pattern to the prevailing allatum hormone titer, which results in the differentiation of a cuticular pattern, typical for each stage.

Regeneration and Differentiation

The knowledge gained from experiments on wound healing has been supplemented by studies on regeneration. The role of hormones in the growth phase of regeneration has been discussed. As far as the differentiation of the regenerate is concerned, the evidence suggests that it too

depends on humoral factors. By the transplantation of prothoracic glands, it is possible to induce leg regeneration in adult cockroaches (Bodenstein, 1955). Since the adult insect is normally unable to regenerate its legs, regeneration is induced through the agency of the experimentally supplied prothoracic-gland hormone. A certain titer of the hormone is needed not only for the initiation of regeneration but also for sustaining its progression. The responsiveness of the organs to the hormone seems to decline somewhat with age, for the adult tissues respond with less ease than the nymphal tissues to the same hormone titer. Nevertheless, the leg of an adult individual can be induced several times to differentiate anew by regeneration, attesting to the amazing plasticity of the formative adult materials. In order to respond with differentiation to the prothoracic-gland hormone, an immature organ has to reach a certain degree of development. The same is observed in regeneration (Bodenstein, 1941). A regenerating moth wing disc has to acquire a definite state of organization before it becomes competent to respond with imaginal differentiation to the prothoracic-gland hormone. The close relationship between the processes of ontogeny and regeneration is again evident.

Before ending this section, it must be mentioned that, in the walking-stick *Dixippus*, regeneration seems to be controlled by the corpus allatum (Pflugfelder, 1939). Molting and leg regeneration can be induced in the imago by the transplantation of nymphal corpora allata. Since there is little doubt that molting in all insects is under the control of the prothoracic-gland hormone and since the prothoracic gland has degenerated in the adult insect, it is difficult to understand how molting in *Dixippus* is brought about by the implantation of corpora allata. Further experiments are needed to clarify this issue.

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THE HORMONAL CONTROL OF METABOLISM IN DECAPOD CRUSTACEANS

Bradley T. Scheer
University of Oregon

During the last seven years, I have been concerned with the problem of the mechanism of action of eyestalk hormones in decapod crustaceans. It is widely assumed, on the basis of the fact that hormones are active at extremely low concentrations, that these substances act by some catalytic mechanism. When I began to work on crustaceans, it seemed that the metabolic actions of crustacean hormones were few and simple, and hence that a study of these actions might lead immediately to the discovery of specific effects on enzyme systems. The state of blissful ignorance which made such an opinion possible did not last very long. By now, I am convinced that the metabolic actions of crustacean hormones are just as complex and numerous as are those of vertebrate hormones, and that crustacean metabolism is no simpler than is vertebrate metabolism.

In the course of this work, I have become increasingly aware of the fact that we really know very little about metabolism in the crustaceans, and consequently we know even less about its control by hormones. It is my purpose here to review briefly some of the available information about oxygen consumption and the metabolism of organic substances in the decapods, and to summarize what we know about the action of hormones on this metabolism.

Oxygen Consumption

We may begin with oxygen consumption as an index of general metabolic activity. Spallanzani, in his memoir on respiration published in 1803, was the first to demonstrate oxygen consumption in crustaceans. The information accumulated since that time has been brought together by Zeuthen (1947); it may be summarized by saying that the oxygen consumption of crustaceans is not notably different from that of other cold-blooded animals of the same size.

The most interesting aspect of oxygen consumption in decapods is its variation during the intermolt cycle. I shall use the phrase "intermolt cycle" in the sense of Drach (1939) to indicate a series of morphological and physiological changes between one molt and the next, such that the animal at the end of the cycle is in the same condition as, though older and usually larger than, when the cycle began. I shall also use Drach's sub-

division of the cycle into four major phases: A, the period of absorption of water and increase in size; B, the period of hardening of the integument; C, the period of completion of the integument, tissue formation, and storage of reserves; D, the period of preparation for the molt.

Scudamore (1947) was first to observe that oxygen consumption increases prior to a molt in the crayfish *Cambarus propinquus*. Edwards (1950) also found, in *Uca pugilator* and *U. pugnax*, a diurnal cycle of oxygen consumption, with a maximum at night; the latter cycle corresponds with the cycle of motor activity. Brown, Bennett, and Webb (1954) observed this diurnal rhythm, and in addition tidal, semilunar, and lunar rhythms. Scheer and Scheer (1954b) have recently examined the premolt increase in oxygen consumption in *Leander serratus*, and have found that it is not simple. For animals in the C_1 to C_3 stages of Drach (1944), we find an oxygen consumption of 14.47 μl per animal per hour after correction to mean body weight. For an animal of the same size in stage D_1, in which the resorption of the inner layers of the old integument is beginning, the oxygen consumption rises sharply to 18.52 μl/hr. The rise is transient, however, and in stage D_2, marked by formation of the principal layer of the new integument, the value falls again to 13.29 μl/hr. Finally, in stage D_3, immediately preceding the molt, when the new integument is fully formed and absorption of the inner layers of the old integument is completed, there is a second rise to 17.64 μl/hr. Whether this complexity is typical of other decapods is uncertain; it is clear that the hormonal factors operative in the Neapolitan race of *Leander serratus* are qualitatively different from those observed in many other decapods, even in other races of the same species.

Evidence for the hormonal control of molting was obtained by Megusar (1912), but was not interpreted as such. Brown and Cunningham (1939) showed that removal of the eyestalks from crayfish (*Cambarus immunis*) is followed by molting at an earlier date than in normal controls, and that implantation of sinus glands delays molting in eyestalkless animals. At that time, there was evidence that the sinus glands in the eyestalks are endocrine in function, and a molt-inhibiting hormone, formed in the sinus glands, was postulated to explain the observations of Brown and Cunningham (1939). The accelerating effect of eyestalk removal on molting has been reviewed by Passano (1953), who has also provided evidence that the molt-inhibiting factor is formed by neurosecretory cells in the X-organ of the eyestalk, and liberated from the sinus glands. The same conclusion was reached independently by others, and their results are reviewed by Passano (1953). Drach (1944) has established for the Roscoff race of *Leander serratus* that eyestalk removal is only effective in shortening the intermolt cycle when the operation is performed before stage D_1; this

suggests that the molt-inhibiting hormone is formed or active only before this stage.

Scudamore (1947) showed that eyestalk removal not only results in precocious molting, but also increases oxygen consumption. Bauchau (1948a) observed a similar increase in the crab *Eriocheir sinensis*, as did Edwards (1950) in *Uca pugilator* and *U. pugnax*. Frost, Saloum, and Kleinholz (1951), using *Astacus trowbridgi*, and Bliss (1951, 1953), with *Gecarcinus lateralis*, showed that sinus-gland removal has little or no effect on oxygen consumption, whereas eyestalk removal is followed by an increase. This result suggests that the factor active on oxygen consumption, like that responsible for molt inhibition, is formed outside the sinus gland, presumably in the X-organ, and merely stored in and liberated from the sinus gland. Edwards (1950) reported that the diurnal cycle of oxygen consumption in fiddler crabs (*Uca* spp.) is eliminated by eyestalk removal. Brown, Bennett, and Webb (1954) found that the tidal rhythm, but not the diurnal rhythm, is eliminated by this operation.

It is tempting to consider that the molt-inhibiting factor and the factor which inhibits oxygen consumption are identical. In this connection, it is of interest that the Naples race of *Leander serratus* gives no evidence of a molt-inhibiting hormone. We removed eyestalks from 114 animals in all stages of the cycle, and observed no acceleration of molting or of any steps leading to molting (Scheer and Scheer, 1954a). In these same animals, eyestalk removal is without effect on oxygen consumption. The mean value, corrected for body weight, for 25 normal animals was 14.50 μl/hr.; that for 25 eyestalkless animals was 12.45 μl/hr.; the difference was not statistically significant (Scheer and Scheer, 1954b). The Naples race differs in its response to eyestalk removal from that at Roscoff, where Drach (1944) found an increased frequency of molting following eyestalk removal. The Plymouth race of *Leander serratus*, which is morphologically distinguishable from that at Roscoff, behaves like the Naples race with respect to the effect of eyestalk extirpation on molting (Carlisle, personal communications). There is also the possibility that the effect of eyestalk removal in increasing oxygen consumption is indirect, and results from an increase in general motor activity.

Growth and Nitrogen Metabolism

The intermolt cycle is closely connected with processes of growth. Many observers, beginning with Baumberger and Olmsted (1928), have noted a distinct increase in size immediately after ecdysis. This increase results primarily from the intake of water through the digestive tract (Baumberger and Olmsted, 1928; Drach, 1939). Removal of the eyestalks, in those species in which this operation results in acceleration of molting,

generally causes an increase in size beyond that of normal animals (Smith, 1940; Abramowitz and Abramowitz, 1940). The increase is not merely the consequence of increased frequency of molting, but results also from a greater than normal increase in size following the first ecdysis (Scudamore, 1947; Bauchau, 1948b). Koch (1952) has raised the question whether this increase in size, which he confirms, is true tissue growth, in the sense of formation of new tissue. He observed in *Eriocheir sinensis*, no difference in the nitrogen content of the exuvia or of the entire body between normal and eyestalkless animals of the same size before molting. After molting, however, the eyestalkless animals had much less nitrogen than normal animals of the same size. Koch (1952) concluded, therefore, that the increased size of eyestalkless animals is not the result of increased synthesis of tissue protein.

This conclusion is less firmly based than might appear. Drach (1939) showed that, in normal crabs (*Cancer pagurus, Maia squinado*), the synthesis of new tissue does not begin until the C stage, some days after ecdysis; the volume increase, on the other hand, is complete within a few hours. Renaud (1949) finds, for *Cancer pagurus*, that the total amount of nitrogen in the digestive gland decreases after ecdysis until stage C, after the carapace is completely calcified. Then the nitrogen increases, to a maximum in stage D_1, the beginning of preparation for a new molt. Since Koch's animals were analyzed soon after ecdysis, he may have missed this later increase. Neiland and Scheer (1953) examined the effect of fasting and removal of the sinus gland on the body composition of *Hemigrapsus nudus* in the C stages of the intermolt cycle. Female crabs had a higher protein nitrogen content, on a body-weight basis, than males. Fasting for 23 days resulted in a decrease in the protein nitrogen content of females to the level of the males, with no change in the latter. Removal of the sinus glands, superimposed on fasting, resulted in a decrease in protein nitrogen in both sexes. We concluded that removal of sinus glands induces an increased tissue catabolism in fasted animals. It would be of interest to know the effects of eyestalk removal from fed animals. Kincaid and Scheer (1952) found evidence of an increased organic-matter content in fed crabs following extirpation of the sinus glands. This work was done before we were aware of the source of the eyestalk hormones in the X-organ, and requires repetition with eyestalk removal. We concluded tentatively that the eyestalk principle involved directs tissue metabolism towards tissue growth and away from processes concerned in preparation for ecdysis (Neiland and Scheer, 1953). This conclusion requires further experimental test.

Carbohydrate and Lipid Metabolism

In mammals and many insects, glycogen is the most readily available

energy store; when it is exhausted, lipid reserves are drawn upon, while tissue protein is utilized last of all. This picture is less well established for invertebrates, but some insects appear to use protein quite as readily as they use carbohydrate or lipid in fasting (Chauvin, 1949). The presence of glycogen in the digestive gland of crustaceans was first noted by Bernard (1879); reserves of lipid in this organ were demonstrated by Cuenot (1893). In 1922, Morgulis found reducing substances in the blood of *Panulirus argus*, and in 1923 he showed that added glucose disappears from the blood. Hemmingsen (1925) performed similar experiments with fresh-water crayfish and followed the time course of glucose disappearance. He concluded that glucose is removed from the blood more rapidly then it can be oxidized, and hence that regulatory mechanisms must exist. In 1924 Verne showed that glycogen accumulates in the integumentary tissues immediately before a molt, but is lacking from these tissues at other times; as chitin is formed, glycogen disappears. He therefore concluded that the glycogen serves as a precursor of chitin. Hoet and Kerridge (1926) observed a decrease in muscle glycogen immediately before a molt, paralleling an increase in the reducing value of the blood. Krishnan (1954) has found fructose in the muscles of *Carcinus maenas*; the content is minimal in the C stages, and rises as the molt approaches.

Renaud (1949) made a very thorough study of the carbohydrate content of the tissues of *Cancer pagurus* in relation to the intermolt cycle. In the digestive gland, the level is low following ecdysis, and continues to decline immediately afterwards, reaching a steady low level after water absorption is completed (stage B_1). The carbohydrate content begins to increase sharply after tissue growth is completed, reaching a maximum in stage D_2, when formation of the new integument begins, after which it declines. The glycogen content of the hypodermis follows a similar course, but reaches its maximum earlier, at the beginning of stage D_1 (resorption of inner layers of old integument). The reducing value of the blood (blood sugar) rises steadily from a minimum at 24 mg. per 100 ml. immediately after ecdysis to a maximum of 48 in stage D_1. This increase in reducing value is the result of two factors—first, the decrease in water content of the animal and hence of blood volume during the intermolt cycle, and, second, the increase in nonfermentable reducing substances in the later stages of the cycle. When the absolute amount of fermentable sugar in the blood is calculated for an animal weighing 100 gm. immediately after molting, it is found to decrease steadily from a value of 7.7 mg. in stage A_2 to one of 2.7 mg. in D_2. The nonfermentable reducing substances increase from a minimum of about 2 mg. glucose equivalents in stage C_3 to a maximum of 11 mg. in stage D_2. Both the fermentable and nonfermentable constituents reach a maximum at the actual time of ecdysis. The glucosamine content,

and the absolute amount of this substance in the blood, increase steadly from C_2 until the molt, and then fall off very sharply. The glucosamine content of the hypodermis increases during the same period, but less sharply, while the glucosamine content of the digestive gland varies inversely with that of the hypodermis, reaching its maximum in stage C_3. These changes are considered to be consistent with the transformation of glycogen through glucose and glucosamine to chitin. Malaczynska-Suchcitz (1949) and Travis (1955), using histochemical methods, confirmed these observations qualitatively with respect to glycogen, and concluded that glycogen is the precursor of chitin.

This view was also supported by the work of Scheer and Scheer (1951) with *Panulirus japonicus* and *P. penicillatus*. Glucose labelled with radioactive carbon was administered to these lobsters by intrapericardial injection, and the tissues were analyzed subsequently for radioactivity. In experiments lasting up to 18 hours, the bulk of the radioactivity was recovered in the water- and alcohol-soluble fractions of the tissues. Relatively high counts were, however, found in the alcohol precipitate from deproteinized extracts of digestive gland, which would contain the glycogen, and, after 18 hours, a substantial part of the radioactivity was found in the chitin of the integument.

A more startling result of this experiment was the observation that little or none of the radioactivity could be recovered in the respiratory carbon dioxide, even after 18 hours. This is conclusive evidence that glucose is not used primarily as a substrate for oxidative metabolism. Further evidence is provided by the observation that glucose has no stimulating effect on the oxygen consumption of surviving fragments of digestive gland or muscle of *Panulirus japonicus* (Scheer and Scheer, 1951) or muscle of *Hemigrapsus nudus* (Hu, unpublished). Moreover, glucose is apparently not removed from solution by these tissues. Further supporting evidence comes from the observation that, in 23 days of fasting, *Hemigrapsus nudus* shows no change in glycogen content (Neiland and Scheer, 1953). We may therefore conclude that the primary role of glucose in decapod metabolism is in the formation of chitin, with glycogen as a reserve or intermediate. We should note, however, the curious results of Krishnan (1954); he finds a definite stimulation of oxygen consumption by glucose and fructose in muscle extracts from *Carcinus maenas* in the presence of large concentrations of potassium cyanide.

Recent results of Hu (unpublished) from this laboratory indicate that glucose metabolism in *Hemigrapsus nudus* may differ significantly from that reported for *Panulirus*. Radioactive glucose was injected into C stage *Hemigrapsus*, and the rediocticity recovered from the respiratory CO_2, glycogen, and a new polysaccharide containing glucose, galactose,

and fucose; only the glucose of this polysaccharide was labeled, and no label was found in the chitin.

Evidence that carbohydrate metabolism is under control of an endocrine factor in the eyestalks was obtained first by Abramowitz, Hisaw, and Papandrea (1944). They found that eyestalk extract, or extracts of the sinus glands, of *Uca pugilator* or of *Callinectes sapidus* had the effect of increasing the reducing value of the blood in these crabs. Kleinholz and Little (1949) performed similar experiments with *Libinia emarginata* and obtained similar results. Moreover, they were able to provide evidence that the increased reducing value is primarily an increase in the fermentable fraction. Neither Abramowitz and coworkers (1944) nor Kleinholz and Little (1949) observed any decrease in blood reducing value following eyestalk removal; no determinations of fermentable reducing substances were made on eyestalkless animals. Scheer and Scheer (1951) removed eyestalks from *Panulirus japonicus* and *P. penicillatus*, and found a significant decrease in blood reducing values following the operation. The values for eyestalkless animals were approximately 53% of those for unoperated controls. Injections of eyestalk extracts caused a marked increase in reducing value in normal or eyestalkless specimens, and the increase was entirely in the fermentable fraction. Indeed, in two of three experiments, the increase in the fermentable fraction was considerably greater than the increase in total reducing substances.

Kleinholz and Little (1949) and Kleinholz, Havel, and Reichart (1950) repeated earlier observations concerning the effects of anoxia, anaesthesia, and adrenaline on the blood reducing values in *Libinia emarginata*, *Astacus trowbridgi*, and *Callinectes sapidus*. They found that the hyperglycemia following asphyxia is mediated by the sinus gland in all three species, since the hyperglycemia failed to occur in animals from which this gland had been removed. In *A. trowbridgi* the hyperglycemia following chloroform anaesthesia or adrenaline injections likewise depends on the sinus gland, but in *C. sapidus* adrenaline produces hyperglycemia even in eyestalkless animals. Kleinholz et al. (1950) concluded that the diabetogenic hormone of the eyestalk is responsible for most of the hyperglycemic effects; the action of adrenaline in *C. sapidus* may result from activation of a source of hormone outside the eyestalks, or from a direct action of adrenaline on carbohydrate metabolism.

Scheer and Scheer (1951) attempted to learn something of the mechanism of action of the diabetogenic factor. Glucose tolerance studies showed that the rate of removal of injected glucose from the blood is much greater in eyestalkless than in normal lobsters. In view of the evidence already discussed, that the principal function of glucose is in the formation of chitin, we concluded that the diabetogenic hormone acts to restrain the conversion

of glucose to chitin. Moreover, since the latter process is of importance in preparation for the molt, it is conceivable that the diabetogenic hormone and the molt-inhibiting hormone are identical. This view is further supported by the observation of Schwabe, Scheer, and Scheer (1952) that eyestalk removal is followed by an increase in the glycogen content of the epidermis in stage C in *Panulirus japonicus*.

The metabolism of lipids has been much less well studied in decapods than the metabolism of carbohydrates. Smith (1915) observed decreases in the lipids of blood and hepatopancreas immediately following a molt. Paul and Sharpe (1919) found that the fat content of the digestive gland increases as the molt approaches, while the content of phosphatides decreases. Renaud (1949) followed the content of total lipids and various lipid fractions through the intermolt cycle of *Cancer pagurus*. Lipid content of the digestive gland falls after a molt until feeding begins in stage C_1. It then rises continuously until feeding stops in stage D_1, when it begins to fall again. This suggests clearly that lipids constitute a primary store of energy in the normal periods of inanition in the intermolt cycle. However, Neiland and Scheer (1953) found no decrease in lipid content of whole stage C specimens of *Hemigrapsus nudus* during 23 days of fasting. Removal of the sinus gland provoked a marked decrease in lipid content of fasted animals. This suggests a hormonal control of lipid metabolism; but further studies are needed.

Renaud (1949) found that the fatty-acid content of the hepatopancreas of *Cancer pagurus* follows the same pattern of variation in the intermolt cycle as does the total lipid. Phosphatides, cholesterol, and unsaponifiable lipid follow a similar pattern, but the amount of change is much less. As a result, the ratio of phosphatides to total lipids is maximal at the beginning of feeding (C_1), minimal at the end of the feeding period (D_1). Histochemical studies with *Panulirus argus* (Travis, 1955) generally agree with the quantitative studies, indicating an accumulation of lipid in the hepatopancreas during stage C_1 and its utilization in stage D. In stage D, fatty acids and cholesterol appear in the integumentary tissues as well, and Travis (1955) suggests that they function in the formation of the integument.

It is remarkable that, in view of the many studies of the hormonal control of color change, so little attention has been paid to metabolism of the pigments, and particularly of carotenoids. Schwabe, Scheer, and Scheer (1952) found a steady deposition of lipochromes, presumably carotenoids, in the digestive gland throughout stage C in *Panulirus japonicus*, paralleling the pattern observed in other species for other lipids. Eyestalk removal had no effect on this deposition, or on the utilization in stages B or D in males. In females, on the other hand, eyestalk removal resulted in a dis-

tinct decrease in carotenoid content of the gland in all stages except late C, when there was a very marked increase. This suggests that sex is a factor in carotenoid metabolism, which is not surprising in view of the extensive deposition of carotenoids in the eggs.

Lenel (Lenel and Veillet, 1951; Lenel 1953a,b, 1955) has observed that removal of eyestalks from *Carcinus maenas* in stage D results in a sudden change in the color of the pigmented layer of the new integument from dark to bright red, with no change in the total carotenoid content. This suggests that an eyestalk factor, presumably not the molt-inhibiting hormone, is concerned in the conjugation of carotenoids with protein which occurs during formation of the integument.

Tissue Metabolism and Enzymes

The changes in oxygen consumption and the metabolism of protein, carbohydrate, and lipid which we have discussed are presumably reflections of changes in enzymic processes occurring at the cellular level. Ultimate explanations of the way in which hormonal factors bring about or influence metabolic changes will presumably have to be made in terms of the actions of hormones on cellular processes of metabolism. Little progress has been made in this direction. We may assume that the basic pattern of tissue metabolism in crustaceans is similar to that in mammals. Wherever careful studies of enzyme systems have been made, in microorganisms, in plants, in invertebrates, or in vertebrates, the same fundamental patterns of oxidative enzymes, glycolytic enzymes, and enzyme sequences have been found, with only minor variations in initial substrates and products and in the relative importance of alternative pathways. However, very few such studies have been made with crustaceans.

Ball and Meyerhof (1940) found succinic dehydrogenase in the heart muscle of *Homarus americanus*, but could not demonstrate the enzyme in skeletal muscle. Scheer, Schwabe, and Scheer (1952) found no evidence for the operation of this enzyme, or of other oxidizing enzymes of the citrate cycle, in muscle or digestive gland of *Panulirus japonicus*. Kermack, Lees, and Wood (1954) were likewise unable to find any utilization of substrates for the citrate cycle in muscle or digestive gland of *Homarus vulgaris*. The oxygen consumption of fragments of digestive gland of *Panulirus japonicus* was not inhibited by fluoride or malonate; we did find evidence of an active phenol oxidase system in this tissue (Scheer, Schwabe, and Scheer, 1952). Krishnan (1954) confirms earlier results demonstrating a tyrosinase in the blood of *Carcinus maenas*. The activity of the enzyme varies in the intermolt cycle, reaching a maximum in the late D stages. This variation in activity is attributed to changes in the oxidation-reduction potential of the blood. There is also a cyanide-insensitive

system in muscle which takes up oxygen in the presence of fructose or glucose. This system is most active in the C stages, and least active immediately before molting. The activity of the system appears to decrease in captivity, and this decrease is accelerated by eyestalk removal. The available evidence, then, suggests that the oxidative metabolism of crustacean tissues may deviate from the usual pattern. There is clear need for a careful investigation of the problem with modern biochemical techniques.

The first studies of the effects of hormonal factors on tissue oxidations in vitro were those of Kuntz (1946, 1947, 1948), which have unfortunately been published only in abstract form. She first reported (1946) that the dehyrogenase activity of tissue extracts is increased by addition of eyestalk or sinus-gland extracts. We were able to confirm this in experiments with *Carcinus maenas* and *Panulirus japonicus*. However, the facts (1) that eyestalk removal does not alter the dehydrogenase activity of lobster tissues, and (2) that eyestalk extracts will increase oxygen consumption of tissue homogenates, but not of surviving tissue fragments, led us to doubt the significance of this observation. No consistent variation in tissue respiration with the intermolt cycle could be established in *P. japonicus*, and the only effect of eyestalk removal was that of decreasing oxygen consumption of tissues from males to the lower levels normally characteristic of females (Scheer, Schwabe, and Scheer 1952). Kuntz (1947, 1948) has also reported a stimulating effect of sinus-gland extract on the phosphorylation of arginine by tissue extracts; this work has not been repeated or extended.

From these very scanty and unsatisfactory results, two possible alternatives emerge: (1) The well-known effect of eyestalk principles on oxygen consumption may not be a direct effect on tissue metabolism, but rather an effect on motor activity, which in turn alters oxygen consumption. (2) The effect may actually be exerted on tissue metabolism, but more refined techniques are required to demonstrate this.

Conclusions

It appears from this brief survey that the best-established effect of hormones on metabolism in crustaceans is the effect of an eyestalk principle on the conversion of glucose to chitin. The intermediate steps in this conversion are not known; presumably glucose is aminated to form glucosamine, the amino sugar is acetylated, and the acetylglucosamine is then polymerized to form chitin. The recent discovery of the compound uridine-diphosphate-acetylglucosamine suggests the possibility that this compound is an intermediate in chitin synthesis. If the sequence of reactions could be established, the way would be open for a test of the action of eyestalk principles or of fractions isolated from eyestalk extracts on individual reac-

tions in the sequence. Such a test would be the sort of thing I had in mind seven years ago when I started this work. Recent studies by Hu (unpublished) indicate that there are no uridine-diphosphate compounds in the crab *Hemigrapsus nudus* in stage C; there is, however, no evidence that chitin synthesis is occurring during this stage.

In the meantime, I have developed a great interest in the whole problem of crustacean metabolism and its relation to the life of these animals. Fascinating problems have emerged concerning the relation of tissue growth to the molt cycle, the regulation of nitrogen metabolism and growth, the use of stored materials as energy sources in fasting and in tissue formation in the intermolt cycle, the question of sex differences in metabolism, the nature of tissue oxidations, and many others.

In closing, I should like to mention just two of these problems. Through all of our work, whenever we have distinguished between the sexes, we have found quantitative differences in metabolism—in tissue oxidations, carbohydrate metabolism, protein metabolism, and metabolism of lipids—between the sexes. Moreover, these aspects of metabolism were usually influenced differently by eyestalk extirpation, most often in such a way that sexual differences were eliminated by the operation. There is evidence that eyestalk principles are concerned in sexual matters, and it would be most interesting to learn more of the metabolic actions of the sex hormones of crustaceans.

Second, the intermolt cycle involves complex and interrelated physiological events; the life of a crustacean is not simply a matter of molts separated by long intermolt periods, but each molt is followed by a sequence of profound changes in all aspects of metabolism leading to the next molt. The molt-inhibiting hormone, for the existence of which there is now abundant evidence, is only one of many crustacean hormones. Carlisle and Dohrn (1953) have demonstrated a molt-accelerating hormone in the prawn *Lysmata seticaudata*, and we have recently provided evidence that the chromatophorotrophic hormones of *Leander serratus*, some five or six in number, are specifically involved in individual stages of the intermolt cycle (Scheer and Scheer, 1954a). It is reasonable to conclude, therefore, that many of the metabolic events concerned in the intermolt cycle are under hormonal control, and that the orderly progression of the animal through the cycle results from a complex sequential action of numerous endocrine factors. The unravelling of some of these actions should prove a most fascinating and enlightening task.

Summary

The oxygen consumption of crustaceans is not different from that of other poikilotherms of comparable size. The rate of oxygen consumption

increases prior to a molt, and varies in accordance with diurnal, tidal, and lunar cycles as well. The diurnal cycle, at least, corresponds with a cycle of bodily activity. The variation of oxygen consumption in the intermolt cycle, and in some of the shorter cycles as well, is under control of endocrine factors in the eyestalks. One of these may also be a molt-inhibiting factor. Eyestalk removal is followed by an increased frequency of molts, resulting from a decrease in the length of the intermolt period, in most but not all crustaceans. Eyestalkless animals also increase in size following a molt to a greater extent than do normal animals; the increase results from increased uptake of water, and it is not known whether increased tissue formation also occurs later in the intermolt cycle.

A principal function of glucose in some crustaceans appears to be as a precursor of chitin, rather than as a substrate for oxidative metabolism or glycolysis. Glycogen acts either as a direct precursor of chitin or as a storage form for glucose. The conversion of glucose to chitin is influenced by an endocrine factor in the eyestalks which may also be the molt-inhibiting factor. This factor is also involved in the regulation of the level of reducing substances, and especially glucose, in the blood. Lipids may serve as energy stores during fasting, and there is some evidence that their metabolism is also under endocrine control. The processes of cellular metabolism in crustaceans are poorly understood, and evidence concerning their endocrine control is scanty and conflicting.

The variations in metabolism with sex, the control of such variations by hormones, and the complexity of the hormonal control of events in the intermolt cycle all provide fruitful fields for future investigation. The hormonal control of glucose metabolism offers the most promising opportunity for test of a postulated mechanism of hormone action. Better knowledge of the details of intermediary metabolism in crustaceans is essential to our understanding of any of these problems.

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OSMOTIC AND IONIC REGULATION IN AQUATIC INVERTEBRATES

JAMES D. ROBERTSON
University of Glasgow

The study of osmotic and ionic regulation in aquatic animals was furthered by Krogh's (1939) discussion of the literature up to 1938. In recent years, work in this general field has centered principally on problems of active transport of ions in tissues; various reviews are available (e.g., Brown and Danielli, 1954). This paper reviews recent work on ionic regulation in marine invertebrates, and osmoregulation in invertebrates living in fresh and brakish water. A short account is given of some problems of salt and water balance in crustacean nerve and muscle.

MARINE INVERTEBRATES

Most marine invertebrates have body fluids in osmotic equilibrium with sea water, the principal exceptions being some prawns and the grapsoid crabs. All show to a varying extent ionic regulation, which may be defined as the maintenance in a body fluid of concentrations of ions differing from those of a passive equilibrium with the external medium. Many marine invertebrates have considerable quantities of protein in their blood plasma, a feature which complicates the interpretation of concentration differences between plasma and sea water, owing to the Donnan effects produced by the indiffusibility of the proteins and the formation of calcium-protein complexes. Although protein concentrations may reach 80 gm./liter in the decapod Crustacea and exceed 100 gm./liter in the cephalopod Mollusca (Robertson, 1949, 1953), the Donnan ratio does not seem to exceed 1.03 and only 10-20% of the calcium is in the form of a calcium proteinate.

By comparing the composition of plasma or coelomic fluid as drawn from an animal with the composition of a sample dialyzed against sea water across a collodion membrane, a true idea is obtained of the extent by which it differs from that resulting from a passive equilibrium.

Table 1 gives a selection from data on nearly 40 invertebrates (Robertson, 1949, 1953, 1954). In general, ionic regulation of body fluids is slight in the lower, less highly organized groups such as echinoderms, tunicates, polychaetes, lamellibranchs, and gastropods, and in the mesogloea of the jellyfish *Aurelia*. With the exception of the prosobranch gastropods, these animals have very low protein concentrations in their body fluids, usually below 1 gm./liter. In the more highly organized decapod crustaceans and

TABLE 1. IONIC REGULATION IN SOME MARINE INVERTEBRATES

	Concentrations in plasma or coelomic fluid as percentage of concentration in body fluid dialyzed against sea water					
	Na	K	Ca	Mg	Cl	SO_4
COELENTERATA						
Aurelia aurita	99	106	96	97	104	47
ECHINODERMATA						
Marthasterias glacialis	100	111	101	98	101	100
TUNICATA						
Salpa maxima	100	113	96	95	102	65
ANNELIDA						
Arenicola marina	100	104	100	100	100	92
SIPUNCULOIDEA						
Phascolosoma vulgare	104	110	104	69	99	91
ARTHROPODA						
Maia squinado	100	125	122	81	102	66
Dromia vulgaris	97	120	84	99	103	53
*Carcinus maenas**	110	118	108	34	104	61
Pachygrapsus marmoratus†	94	95	92	24	87	46
Nephrops norvegicus	113	77	124	17	99	69
MOLLUSCA						
Pecten maximus	100	130	103	97	100	97
Neptunea antiqua	101	114	102	101	101	98
Sepia officinials	93	205	91	98	105	22

* Webb (1940).

† This grapsoid crab is the only animal in the table which is hypo-osmotic (ionic concentration 86% that of sea water).

in the cephalopods, regulation may extend to every ion. High concentrations of protein, chiefly hemocyanin, are characteristic of these groups, and values between 105 and 150 gm./liter are found in *Eledone, Sepia,* and *Loligo,* with rather lower values (29-80 gm./liter) in the crustaceans.

Regarding individual ions, calcium is usually higher than the equilibrium values, except in some of the animals lacking calcareous skeletons, such as tunicates, jellyfishes, polychaetes, and *Sepia.* Magnesium remains high in all the groups except the sipunculids and some of the crustaceans. An interesting feature about the decapods is the correlation between the magnesium level in the plasma and the ability to move quickly (Robertson, 1953). Active crabs such as the portunids *Carcinus* and *Portunus,* and the grapsid *Pachygrapsus,* as well as the lobsters *Nephrops* and *Homarus* which are capable of quick movements, all have low magnesium values ranging from 14-48% of those in sea water. *Pachygrapsus crassipes* and the lobster *Panulirus interruptus* from the California coast also have low magnesium values (Schlatter, 1941). On the other hand, slow-moving crabs such as *Dromia* and the spider crabs *Hyas* and *Maia* have high magnesium values ranging from 84-101%. It may be rather fanciful

to suggest that these three genera are living in a state of partial magnesium anaesthesia, with depressed neuromuscular transmission. One might object that other ions and hormones influence activity, and point to the general anatomy of a spider crab as being unsuitable for swift movement. Yet there seems to be some support for a causal relationship in Crustacea between reduction of magnesium and increased activity, at least according to studies on the neuromuscular system of the legs (Katz, 1936; Waterman, 1941; Boardman and Collier, 1946).

Sodium and chloride are the least variable ions in different specimens of a species, as one might expect, since they form such a large proportion of the total ionic concentration, which is kept at the same level as that of sea water, within 1-2%. A reduction of magnesium as in *Nephrops* and *Phascolosoma* is accompanied by a rise in sodium to maintain osmotic and cation balance.

Sulphate in body fluids is usually less than the equilibrium value with sea water, and falls below 30% in *Loligo* and *Sepia*. Reduction in this anion is compensated by increase in chloride to maintain cation-anion balance and osmotic equilibrium.

In body fluids potassium tends to be the most variable ion in specimens of a species, but the mean values are fairly characteristic of species and groups. In all so far examined except the Homaridae (*Homarus* and *Nephrops*), potassium is higher than the equilibrium value with sea water, and highest of all in cephalopods, the mean values in the octopus *Eledone*, the cuttlefish *Sepia*, and the squid *Loligo* being 152%, 205%, and 219%, respectively. The meaning of the lower potassium in lobsters is not clear, but it may have some physiological significance in relation to the balance between the various ions, since these animals have very low magnesium concentrations. In cephalopods, calcium and magnesium ions remain near equilibrium levels with sea water, and perhaps the high values of potassium contribute to the activity and powers of active movement of members of this class, since moderate increases in potassium ions are known to have a stimulatory action on the neuromuscular system (e.g., Wells, 1928; Ross and Pantin, 1940).

The mechanism of ionic regulation in many groups is unknown, although one may attribute it to the activity of the cells in contact with the external medium in such groups as coelenterates and tunicates. In the Arthropoda and Mollusca rather more is known, at least in the decapod crustacean and cephalopod groups.

In these animals an output of fluid through the excretory organs is balanced by the uptake of a similar quantity of fluid elsewhere, so that the weight remains constant. If the excretory apertures are closed, the weight of the animal increases and estimates can be made of the amount of fluid

produced. Analyses of the fluid from the antennal glands and molluscan excretory tubules show that differential excretion of ions is taking place (Table 2), tending to eliminate magnesium in the crustaceans and sulphate in all the animals, while conserving the other ions. In general, these differences in the excretory fluids from pure ultrafiltrates of the blood are tending to alter the levels of ions in the plasma, in relation to sea water, towards those actually found.

TABLE 2. ANTENNAL GLAND SECRETION AND RENAL SAC FLUID COMPARED WITH BLOOD PLASMA

	Concentrations as percentages of plasma values (on water-content basis)					
	Na	K	Ca	Mg	Cl	SO_4
CRUSTACEA						
(Antennal gland secretion)						
Maia squinado	100	98	99	109	101	214
Cancer pagurus	97	81	90	125	96	134
*Carcinus maenas**	95	78	94	390	98	224
Palinurus vulgaris	98	65	86	137	101	98
Homarus vulgaris	99	91	64	180	101	159
Nephrops norvegicus	98	83	81	130	101	106
Palaemon serratus†	82	86	95	670	106	380
CEPHALOPODA						
(Renal sac fluid)						
Eledone cirrosa	102	90	87	89	97	136
Sepia officinalis	79	50	70	68	100	215

* Webb (1940).

† Parry (1954), on ml. basis. *Palaemon* shows hypo-osmotic regulation, but "urine" is isosmotic with blood.

Remainder of analyses from Robertson (1939, 1949, 1953).

A deficiency of cations in the renal fluid of *Sepia* is apparent from Table 2, since they are only 50-79% of those in the plasma. The missing cation is ammonium, NH_4^+, which may be excreted in amounts as much as 146 m.equiv., forming 24% of the total cation equivalents. The production of ammonium in such amounts, presumably shortly after feeding and protein breakdown, presents a problem in osmotic regulation which is solved in *Sepia* by reduction of the other cations, so that the "urine" remains isosmotic with the plasma.

Loss of water and salts through excretory tubules must be made good by absorption of a similar quantity of fluid through permeable portions of the integument, usually the gills. Absorption of pure water would alter the osmotic pressure of the blood, and ions must be absorbed with the water in concentrations sufficient to make it isosmotic with the fluid it is replacing. Consequently Robertson (1939) and Webb (1940) deduced that sodium, potassium, calcium, and chloride must be taken up against concentration gradients in many invertebrates, although magnesium and

sulphate may penetrate in accordance with the diffusion gradient in those animals in which the plasma levels of these ions are low.

I have recently made careful analyses of sea water in which *Carcinus maenas* has been kept in order to obtain the net uptake or output of water and ions. In this crab the secretion from the antennal glands amounting to about 5% of the body weight per day is balanced by the absorption of an

TABLE 3. IONIC BALANCE IN INTERMOLT PHASE OF CRABS *(CARCINUS MAENAS)*

The net uptake or output (*actual*) and the output of urine (*antennal gland secretion*) over two 7-day periods are given for two crabs. Crabs were kept individually in 500 ml. sea water containing in mg. Na 5022, K 182, Ca 192, Mg 606, Cl 9050, SO_4 1263. Uptake or output is not significant [ ] unless it exceeds the following values: Na 24.1, K 3.8, Ca 2.1, Mg 7.5, Cl 11.5, SO_4 15.2.

Weight	Period in days		Uptake (+) or Output (—) in mg. Na	K	Ca	Mg	Cl	So_4
♂ 107.9-106.9	1- 7	Actual	[—18]	—10.0	—10.0	[+ 4.0]	[— 5]	— 40
		Antennal gland secretion*	—363	—11.5	—15.5	—58.8	—612	—111
106.9-106.4	8-14	Actual	[+22]	—11.0	—12.0	[— 6.0]	— 35	— 24
		A. g. sec.*	—360	—11.4	—15.4	—58.4	—608	—111
♂ 114.7-112.9	1- 7	Actual	[— 3]	—13.0	—16.0	[— 5.0]	— 35	— 29
		A. g. sec.*	—384	—12.1	—16.4	—62.3	—649	—118
112.9-113.5	8-14	Actual	[— 8]	— 8.0	[— 1.0]	+ 8.0	[— 5]	— 18
		A. g. sec.*	—382	—12.1	—16.3	—62.0	—645	—117

* Calculated figures based on average composition of this secretion, and average uptake of fluid, 4.7% of body weight per 24 hr. (Webb, 1940).

equivalent volume of fluid through the gills (Webb, 1940). The results of balance experiments in two crabs are given in Table 3. With respect to sodium, chloride, and magnesium, the crabs are essentially in balance within the accuracy of the analytical methods, the output through the antennal glands and other possible sources such as the gut being offset by uptake of similar quantities of ions, the sodium and chloride against a concentration gradient. Potassium balance over a weekly period is negative, and most of the loss would seem to be in the secretion from the glands. Calcium is also lost from the crabs in small quantities, which are usually less than the amounts calculated as being excreted, so a slight uptake may have taken place. Results with sulphate show a net loss of this anion, but from the amounts excreted by the antennal glands it is clear that considerable uptake may take place, amounting to more than half of that excreted.

It seems, therefore, that the incoming fluid contains all ions except perhaps potassium, these ions with the exception of magnesium and sulphate being taken up against concentration gradients.

Following up the work of Baumberger and Olmsted (1928) who found increases at molt in the freezing point of the blood and tissues of *Pachygrapsus crassipes*, a crab showing hypo-osmotic regulation in sea water, I have found that the total ionic concentration of *Carcinus* increases in the premolt phase and falls again after molt. Expressed as percentage of the sea-water concentrations, the total mg. ions in the plasma rise from 100.9 in the intermolt crabs to 107.5 in the premolt phase, falling to 102.9 within 24 hours of molting owing to the absorption of sea water by the gut. Thereafter, a further fall to 98.2 takes place within the next fortnight. Baumberger and Olmsted did not investigate the mechanism of the increase in osmotic pressure. In *Carcinus* there is an increase in all the ions except sulphate, but how this takes place is not clear.

Characteristic changes in the calcium content of the plasma are found in *Carcinus* at different stages of the molt. Taking sea-water values as 100, intermolt crabs have a mean calcium content of 130 (range 113-160). In the premolt phase the mean is 163 (range 140-189), the increase being correlated with resorption of salts from the old skeleton. A fall to 120 (range 103-134) takes place immediately after molt, presumably owing to dilution of the blood with sea water. After two days the mean figure falls to 88 (range 66-105). At this stage and for several weeks after molt, calcium is being withdrawn from sea water, and it is possible that a good part of this takes place by inward diffusion according to the concentration gradient. In this postmolt phase calcium is probably being withdrawn by the epidermis from the plasma to be deposited as calcium carbonate and, to a small extent, calcium phosphate in the integument. This continuous withdrawal reduces the calcium content of the plasma, enabling continuous absorption from the external medium to take place.

Invertebrates in Brackish and Fresh Water, and in Semiterrestrial Habitats

Active uptake of salts in crustaceans is considered to take place through the gills, but it is often difficult in experiments to eliminate the complications of simultaneous output of salts from antennal glands and the functioning of the gut.

An advance has been made by the recent finding that the isolated gills of the grapsoid crab *Eriocheir sinensis* are still able to absorb sodium chloride from a solution of 8 mM/liter, about 1/30-1/40 of the concentration in the blood (Koch et al., 1954; Koch, 1954). The absorption stops in the absence of oxygen, and is reversibly inhibited by small concentrations of carbon dioxide, potassium cyanide, sodium azide, and sulphide, and irreversibly inhibited by silver, lead, and mercury salts. These gills, which are of relatively homogeneous histological composition, are

obviously promising material for the investigation of ion uptake. Koch (1954) has found that many basic dyes, including methylene blue and pyocyanine, are reversible inhibitors of salt absorption. These dyes contain a quaternary NH_4 group, and tetramethyl ammonium chloride is also an inhibitor. Koch believes that the inhibitory action of basic dyes is due to their anticholinesterase activity. Cholinesterase is present in the blood of *Eriocheir* and very probably in the gill epithelium. The classical inhibitor of cholinesterase, eserine (physostigmine), reversibly inhibits the salt absorption in *Eriocheir* gills, and also the absorption of ^{22}Na by the anal papillae of the midge *Chironomus plumosus*.

Invertebrates which live and breed in fresh water have a wide range in blood concentration. The lamellibranch *Anodonta* has a concentration equivalent to about 4-5% sea water, Δ 0.08° C (Potts, 1954a), whereas the crayfish *Astacus* has a concentration ten times as much, Δ 0.81° C (Scholles, 1933). In *Astacus* and *Cambarus* the urine produced by the antennal glands has only about one-tenth the concentration of the blood (Scholles,1933; Lienemann, 1938), but in *Anodonta* and the gastropod *Lymnaea* the urine is more concentrated, about 60% and 70% of the blood concentrations, respectively (Picken, 1937).

Eriocheir, on the other hand, produces urine isosmotic with the blood at all dilutions of the external medium, and this is also true of the freshwater river crab *Potamon edule*. While *Eriocheir* is perhaps predominantly a brackish-water crab, young crabs penetrate hundreds of miles up rivers, and the mature animals emigrate to the sea to breed (Krogh, 1939). By its very active uptake of ions, this crab maintains a much higher concentration of salts in its blood than *Astacus,* sustaining a Δ of 1.18° C in fresh water, although freshly molted animals have a mean of 0.86° C, 27% lower. *Potamon* likewise has a high osmotic concentration in the blood, Δ 1.17° C (Schlieper and Herrmann, 1930), but it is better adapted to fresh water since it shows direct development, the young individuals hatching from the eggs with all their appendages and becoming perfect miniature crabs after one molt.

The breeding of *Eriocheir* in the sea, and the fact that mature egg-bearing females transferred to fresh water cannot survive more than a few days (Scholles, 1933) emphasizes the fact that all stages must be capable of living in fresh water for complete adaptation to this environment. In *Carcinus,* the eggs from ovigerous females will develop normally only within a salinity of 28-40‰, although adults are found within 4-31‰ in the Zuiderzee and Den Helder areas (Broekhuysen, 1936), and there must be other cases where the powers of osmoregulation of the adults are not matched by those of their larvae. Another example is *Mytilus californianus,* which seems to show no regulation in diluted sea water. Gametes

and larvae die in diluted sea water in which the adults can survive indefinitely in the laboratory (Fox, 1941).

Potts (1954c) has recently made some interesting calculations of the thermodynamic work performed in osmotic regulation in brackish- and fresh-water animals. He shows that the lowering of the blood concentration of animals in brackish water is the chief means of easing the strain on osmoregulatory mechanisms, the production of a hypo-osmotic urine giving little advantage to the animals until the medium falls well below 50% sea water. In fresh-water animals, however, hypo-osmotic urine can reduce osmotic work by 80-90%, and be of significant value even when many times more concentrated than the external medium, since most of the benefit is secured in the early stages of reducing the urine concentration, not when the concentration is approaching that of the medium.

Applying his equations to data for *Eriocheir* in fresh water, Potts calculates that the osmotic work is 0.176 cal./hr. for a crab of 60 gm., about 1.3% of the total metabolic energy calculated from the oxygen consumption (14 cal./hr.). If the crab had been able to produce urine as hypo-osmotic as the external medium (instead of being actually isosmotic with the blood), the osmotic work would have been 0.0375 cal./hr. If the crab had maintained in fresh water the high concentration found in its blood when in sea water, the value would have been 0.725 cal./hr.

Similar calculations for *Anodonta* suggest 0.0145 cal./hr. for the total osmotic work, of which 0.0131 is done at the body surface and 0.0014 (about 10% of the total) at the excretory organ. The total work constitutes 1.2% of the total metabolism (1.2 cal./hr. for a 60-gm. mussel).

Well-marked powers of osmotic regulation are shown by crabs with a semiterrestrial habitat, and Jones (1941) found that the most homoiosmotic of a series he studied was the burrowing fiddler crab, *Uca crenulata.* This crab maintained a relatively low blood concentration when exposed to air for 12 hours, although the water of the branchial chamber had concentrated and was about 20% above the concentration in the blood. In *Pachygrapsus crassipes,* Gross (1955) found that in air salts were absorbed from the branchial tissues, but that the contribution they made to the increase in salinity of the blood was relatively small. The mechanism by which a crab like *Pachygrapsus* can maintain hypo-osmoticity in ocean water or more concentrated water is obscure. The activity of the antennal glands requires the absorption of water against a gradient to replace that lost in the antennal secretion, and the latter is, according to Jones (1941), isosmotic with the blood at all dilutions of the external medium. Such active absorption has been shown by Gross (1955) by measuring the con-

ductivity of the external medium, when crabs with blood concentrated by evaporation in air were replaced in the medium. But what part, if any, is played by drinking has still to be discovered.

Prosser et al. (1955) find that the antennal secretion of *P. crassipes* is slightly hypo-osmotic to the blood in 50% sea water in which the animal maintains hyperosmotic regulation. In 170% sea water the crabs show hypo-osmotic regulation and the urine is isosmotic with the blood. At the lower salinity the antennal gland secretes magnesium at a concentration four times that of the blood, but in 170% sea water the secretory activity becomes so great that a concentration of ten times is attained. Although the blood concentration of sodium increases with increasing salinity of the medium, the level of sodium in the secretion actually decreases concomitantly. The authors therefore conclude that increased active outward transport of magnesium by the antennal glands in some way reduces the excretion of sodium. This reduction in sodium is probably necessary for the maintenance of cation-anion balance.

Ocypode albicans burrows in sandy beaches near or above the high tide level. It seems to be very homoiosmotic, judging from the chloride content of the blood (Flemister and Flemister, 1951). In this species the authors found very different chloride values in the antennal gland secretion, which follow those of the environment but at a higher level, and seem to suggest that the secretion may be hyperosmotic in concentrated sea water. The urine-blood chloride differences would not be as large if the concentrations were compared on a water-content basis, and if plasma were used instead of whole blood.

The coconut crab *Birgus latro*, an anomuran like the hermit crabs, is even better adapted to terrestrial conditions (Gross, 1955). If given the choice of both fresh and sea water, the concentration of its blood is about 85-92% of that of the sea water, with extremes of 64% and 123% when fresh water or sea water is available alone. Water can be drunk from small puddles by being passed by chelipeds and maxillipeds to the other mouth parts, and the branchial chamber can also be kept moist by means of water passed to it by the appendages.

The isopod *Ligia oceanica* studied by Parry (1953) lives in crevices about high tide level, and feeds mainly on seaweed detritus. It survives in aerated sea water, the freezing point of the blood being kept fairly constant in 50-100% sea water. The blood of animals kept in atmospheres of lowered humidity becomes concentrated, the integument being apparently fairly permeable to water. Parry considers it to be a marine animal which is able to tolerate internal osmotic changes, without any special mechanisms which would enable it to become a truly terrestrial animal. Considerable ionic regulation is found in the blood, where concentrations of magnesium

and sulphate are much below those of sea water, and sodium, potassium, calcium, and chloride higher.

TABLE 4. IONIC COMPOSITION OF THE BODY FLUIDS OF SOME FRESHWATER INVERTEBRATES AND PARASITES

	*Anodonta cygnea** (lamellibranch) mE/kg.H_2O	*Sialis lutaria*† (neuropteran) mE/liter	*Gastrophilus intestinalis*‡ (dipteran) mE/kg.H_2O	*Ascaris lumbricoides*§ (nematode) mE/liter
Na	15.6	109	206	129
K	0.49	5	13	25
Ca	16.8	15	7	12
Mg	0.38	38	38	10
Cl	11.7	31	17	53
SO_4	1.5	....	6	....
HCO_3	13.6	15	17	....
$HPO_4+H_2PO_4$	0.4	....	40	26
Other anions	....	....	75	....
Total cations	33.3	167	264	176
Total anions	27.2	46	155	79
Total ionic concentration in mg. ions	50.9	187	*ca* 347	234
Δ °C of body fluid	0.078	*ca* 0.63	0.872	0.655
Δ °C of environment	*ca* 0.02	*ca* 0.01	? 0.8	0.869

* Potts (1954a).
† Shaw (1955a).
‡ Levenbook (1950).
§ Hobson et al. (1952).

Table 4 gives some indication of the range in composition of body fluids in fresh-water and parasitic animals, and the relatively incomplete data available for the anions in three of the animals. In *Anodonta* the concentration of calcium exceeds that of sodium, but about 5 mE are believed to be present in some nondiffusible, nonionized form. The high calcium value is related to the fact that in this shelled mollusc the blood is saturated in respect to calcium carbonate (Potts, 1954*a*). *Sialis* larvae like other insects maintain a fairly high osmotic concentration, but the inorganic ions account for little more than half. Presumably free amino acids make up the deficit, and Shaw (1955*a*) suggests that dicarboxylic amino acids may make up the anion deficit.

Similar discrepancies occur in the horse-bot larvae *Gastrophilus* and *Ascaris* from the pig. In the former organic acids and unidentified phosphorus compounds form a large fraction of the anions, and free amino acids come to about 73 mM/kg. water, or about 15% of the total osmotic concentration. Although *Ascaris* body fluid is markedly hypo-osmotic to the contents of the small intestine of the pig, the electrolyte composition of the two fluids is very similar (Hobson et al., 1952).

From the data available concerning osmoregulation in fresh-water animals, it is apparent that the mechanisms of the regulation vary in different animals or groups. Lamellibranchs like *Anodonta* with large surfaces in contact with the external medium and low metabolism are unable to maintain a high osmotic gradient between blood and water. Despite the low gradient, the water flux of these animals, as measured by Potts (1954b) from the rate of urine production, varies from about 5% of the weight per day at 0° C to 24% at 18° C. If the animal is narcotized with ether or barbiturates, the production of urine stops, but the animal increases in weight, owing to the osmotic uptake of water (Florkin and Duchâteau, 1948). In an Australian fresh-water mussel *Hyridella australis*, closure of the shell forms an effective seal from the external medium, eliminating weight changes in short-term experiments in different media, and allowing the lamellibranch to survive periods of desiccation up to three months (Hiscock, 1953).

In the larvae of the mosquito *Aedes aegypti*, the body wall is relatively impermeable to water except across the anal gills or papillae, which also actively absorb chloride from the environment. Using ingenious technical methods for the small quantities of fluid available, Ramsay (1950, 1951, 1953) has shown that the fluid eliminated by the rectum is markedly hypo-osmotic to the haemolymph, and contains less sodium and more potassium. These ions enter the body through the anal papillae and are excreted by the Malpighian tubules into the intestine in amounts which vary directly with those in the external medium, but the sodium of this intestinal fluid is always less and the potassium more than the values of these ions in the blood.

Sialis larvae, however, have no special ion-absorbing organs, and their tracheal gills are in fact, as well as in name, respiratory organs. The excretory fluid in the rectum, presumably produced by the Malpighian tubules, is not strongly hypo-osmotic, since its conductivity is as much as 65% that of the blood (Shaw, 1955a). It contains large quantities of ammonium and bicarbonate ions, is chloride-free, and has only small quantities of sodium and potassium. Sodium, potassium, and chloride of the blood increase slowly when the larvae are kept in dilute solutions of sodium or potassium chlorides. At the same time the concentrations of these ions in the rectal fluid increase, but only potassium exceeds the value in the blood, reaching a concentration of three to six times.

In these experiments most of the uptake of ions in *Sialis* takes place from fluid absorbed into the gut. The osmotic intake of water through the cuticle is much less than in *Anodonta*, about 4% of the weight per day at 20° C.

The gut of aquatic animals is much more permeable to water and ions

than the outer integument, and more attention needs to be paid to the possibility of exchanges in this way, particularly since Fox (1952) records anal and oral intake of water in many transparent crustaceans. By keeping *Carcinus maenas* in sea water colored with phenol red, I have found little absorbed in the first few days in intermolt specimens, but much is absorbed during the molt, the phenol red concentrating several times in the foregut and fluid in the digestive glands.

Some aquatic animals such as coelenterates manage without excretory organs. In fresh water an animal like *Hydra*, with its large surface in contact with the external medium, must regulate its water and ionic content, but the mechanism responsible is practically unknown. Lilly (1955) finds that the ectoderm and endoderm cells of isolated tentacles or whole animals shrink in sucrose solutions stronger than 0.04 M, and believes the internal osmotic pressure is equivalent to that of 0.04-0.05 M sucrose. Using radioactive isotopes of sodium (^{22}Na) and potassium (^{42}K), she finds that these are concentrated in *Pelmatohydra*, a steady state being reached after 12 hours. Accumulation of potassium was more pronounced than accumulation of sodium, but the exact localization of the ions in cells or mesogloea was not determined.

Ionic and Osmotic Aspects of the Composition of Tissues

An osmotic steady state probably exists between cells and the internal medium, but few direct measurements have been made. In isolated muscle fibers of the bivalve *Mytilus*, Potts (1952) found the freezing point to be within 1.5% of that of the blood, confirming Krogh's (1939) vapor pressure determinations on muscle press juice in the same species. Potts also found osmotic equilibrium between the eggs of the sea urchin *Psammechinus* and sea water. Organic substances form a large part of the osmotic concentration of cells, and some recent analyses illustrate their importance.

In leg nerves of *Carcinus* the amino acids aspartic, glutamic, taurine, and alanine total 271 mM/kg. fresh weight or 313 mM/kg. water (Lewis, 1952), forming 28-29% of the total osmotic concentration, assuming it to be that of the crab Ringer solution in which the nerves were dissected out (1096 mg. ions or mM/kg. water). Inorganic ions, acid-soluble phosphorus compounds, and small amounts of bicarbonate, lactate, and keto acids accounted for another 65% of the concentration, leaving only a 6% deficit unexplained. Lewis has suggested that aspartic and glutamic acids are important in the cation-anion balance of crab nerve, forming 39% of the total anions.

The amino acids of crustacean muscle may be present in amounts even greater than those in nerve. Camien et al. (1951) found about 272 mM/kg.

in *Homarus* muscle, and Kermack et al. (1955) rather more since they included taurine, not determined by the former workers. Proline, glycine, and taurine are the most abundant, followed by glutamine, valine, arginine, and alanine. Trimethylamine oxide and betaine were found by Kermack et al. in considerable quantities, and also a small amount of volatile base. All these substances must contribute to the osmotic concentration of lobster muscle. Approximate concentrations in mM/kg. were: amino-acids 221-285, mean 256, trimethylamine oxide 75, betaine 67, volatile base 8, total about 406.

TABLE 5. COMPOSITION OF *NEPHROPS* MUSCLE

	mg. ions (mM)/kg. H_2O	mE/kg. H_2O
Sodium	83.2	83.2
Potassium	166.6	166.6
Calcium	5.2	10.4
Magnesium	19.1	38.2
Chloride	109.9	109.9
Sulphate	3.1	6.1
Bicarbonate	2.2	2.2
Inorganic phosphate	19.1	30.8*–21.5
Arginine phosphate	74.2	76.4*–74.2
Adenosine triphosphate	12.1	44.8*–38.7
Remaining acid-soluble P, mostly hexose monophosphate	15.1	29.3*–21.7
Lactate†	7.8	7.8
Total cations	274.1	298.4
Total anions	243.5	307.3*–282.1
Amino acids	496.0	....
Trimethylamine oxide	72.0	....
Total mg. ions‡	1,085.6	....
Plasma	1,100.0	....
Sea water	1,094.0	....

* Assuming intracellular pH of 7.0; second figure pH 6.0.

† Boyland (1928) for *Homarus*.

‡ If concentration of betaine is as high as 90 mM, found by Kermack et al. (1955) in *Homarus*, total concentration would be 1,176 mg. ions/kg. water.

An analysis of the muscle of *Nephrops norvegicus*, the Norway lobster, is shown in Table 5 (Robertson, unpublished). The organic phosphate compounds make up half the total anions and over 10% of the osmotic concentration. Amino acids account for nearly half the concentration, and trimethylamine oxide for about 7%. If betaine is present in the concentrations found by Kermack et al. in *Homarus*, about 90 mM per kg. water, the total concentration would exceed that of the plasma by 7%. It would be premature to discuss the inference that some of the base and perhaps some of the phosphate must be bound until the concentration of betaine is determined. It is almost certain, however, that most of the intracellular calcium and magnesium is in the form of unionized complexes.

Calculations of intracellular ions have often been made in the past by assuming that all the chloride of the muscle is extracellular. Thus on this basis, Hayes and Pelluet (1947) considered that in lamellibranchs and in cephalopods the cells contained only potassium—no sodium, calcium, or magnesium. Steinbach (1940) likewise thought that no sodium was present in the muscles of the sipunculid *Phascolosoma*. The basic assumption that there is no chloride inside cells seems to be false, as is shown by the data of Table 6. The *Carcinus* analysis was made directly on isolated muscle fibers, those of Krogh and myself on muscle press juice and whole muscle respectively, corrected for extracellular fluid. In estimations of the latter, Krogh used the sodium thiosulphate and ammonium thiocyanate "spaces," that is, the space into which thiosulphate or thiocyanate injected into the blood distributed itself in the muscle, after allowing several hours for even distribution. I used inulin for an estimate of extracellular space in the *Nephrops* muscle, and found that 11.5% of the muscle water was probably extracellular. All these "spaces" are lower than the chloride space.

TABLE 6. INTRACELLULAR COMPOSITION OF MUSCLE OF SOME MARINE INVERTEBRATES

	mE/kg.H_2O					
	Na	K	Ca	Mg	Cl	SO_4
Mytilus (Krogh, 1939)	121	137	....	....	284	84(?)
Eriocheir (Krogh, 1939)	33	136	....	....	139	....
Carcinus (Shaw, 1955b)	54	112	10.4	33.8	53	....
Nephrops (Robertson)	27	177	7.6	40.5	56	2

The data show over 50 mE intracellular chloride in these muscles, and considerable quantities of sodium and magnesium, with rather less calcium. The chloride content of *Mytilus* muscle is half that of the blood, and the sum of the anions greatly exceeds the figures for cations, even allowing an extra 50 mE for calcium and magnesium. Sulphate may have been overestimated, but the chloride figure is supported by that of Fox (1941) for *Mytilus californianus*, 203 mE/kg. fresh weight in the muscular foot. Krogh's figure on the same basis is about 240 mE/kg. for *Mytilus edulis*, and the salinity of sea water in his case was slightly greater. Such high salt contents are not universal in lamellibranchs, since Hayes and Pelluet (1947) find only 68.5 mE Cl/kg. in the whole muscle of *Mactra*.

A comparison of the ratios of intracellular to extracellular ions is given in Table 7. Relative to the blood, *Mytilus* has more sodium, chloride, and sulphate and less potassium inside its cells than any of the crustaceans. The accumulation of potassium in *Nephrops* and *Eriocheir* is twice that of *Carcinus*, and the only other ion accumulated in the crustaceans is mag-

nesium in *Nephrops*. It may be noted that the plasma magnesium in this animal is only half that of *Carcinus*.

TABLE 7. RATIOS OF CONCENTRATIONS INSIDE MUSCLE CELLS TO THOSE OF PLASMA (ON WATER CONTENT BASIS)

	$\frac{Na_i}{Na_o}$	$\frac{K_i}{K_o}$	$\frac{Ca_i}{Ca_o}$	$\frac{Mg_i}{Mg_o}$	$\frac{Cl_i}{Cl_o}$	$\frac{SO_{4i}}{SO_{4o}}$
Mytilus	0.238	6.7	....	....	0.513	1.600
Eriocheir	0.071	18.4	....	....	0.312	....
*Carcinus**	0.109	8.8	0.281	0.68	0.096	....
Nephrops	0.052	20.6	0.235	1.97	0.106	0.055

* Recalculated from Shaw's (1955b) figures, assuming a water content of 0.945 mg./ml. in the plasma.

Present-day views on the ionic composition of cells (Ussing, 1949; Hodgkin, 1951) suggest that most of the potassium is held electrostatically by nondiffusible organic anions, organic phosphates, and protein, and that the low value for sodium is maintained by a process of active extrusion of sodium ions. On this hypothesis cellular potassium stands in Donnan equilibrium with the outside potassium, and

$$\frac{[K_i]}{[K_o]} = \frac{[Cl_o]}{[Cl_i]} = r$$

In *Eriocheir* these ratios are respectively 18.4 and 3.21, in *Carcinus* 8.8 and 10.5, and in *Nephrops* 20.6 and 9.39. Only in *Carcinus* are the theoretical requirements approximately satisfied. It seems that in *Eriocheir* and *Nephrops* active uptake of potassium by a metabolic process must be postulated, since the Donnan equilibrium is inadequate to explain the ratios, and the binding of large amounts of internal potassium in unionized complexes is very improbable.

The composition of *Mytilus* muscle (Table 6) as determined by Krogh is inconsistent with the presence of any large amount of organic anions, and the ratios of 6.7 and 2.0, respectively, do not suggest a Donnan equilibrium.

Intracellular cations may not be evenly dispersed but localized in relation to intracellular structures (Steinbach, 1947; Dubuisson, 1954). In *Nephrops* the juice pressed from the muscle has the same chloride content as whole muscle, on a water-content basis, but only 74-80% of the sodium, potassium, and acid-soluble phosphorus, and less than 10% of the calcium and magnesium. This suggests that most of the calcium and magnesium is bound to structural proteins not present in the soluble proteins of the juice.

Summary

Characteristic patterns of ionic regulation are found in the marine invertebrates, although most of these animals show no osmotic regulation. These patterns vary from slight regulation in the more simply organized groups like echinoderms, polychaetes, and lamellibranchs to pronounced regulation in the more highly organized Crustacea and Cephalopoda. In the two latter classes, selective excretion of ions in the excretory fluids and controlled uptake of ions by the more permeable surfaces are essential features in their ionic regulation. A correlation between level of magnesium in the blood plasma and the activity of the animals is evident in the decapod crustaceans, the more active ones having low values of magnesium.

Calculations by Potts (1954c) of the osmotic work done at the body surface and excretory organs of brackish- and fresh-water animals show that in *Anodonta* and *Eriocheir* it amounts to less than 2% of the total metabolic energy.

In aquatic insect larvae the Malpighian tubules can regulate the sodium and potassium concentrations in the blood.

The osmotic concentration of the blood of insect larvae as found by freezing-point measurements cannot yet be accounted for by the sum of analyzed constituents, and serious discrepancies exist in the matter of cation-anion balance. However, fairly satisfactory agreement is found in these respects between crustacean nerve and bathing fluid, and between crustacean muscle and plasma.

In two out of three crustaceans the intracellular potassium concentrations in muscle and extracellular potassium are not in Donnan equilibrium with extracellular and intracellular chloride.

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RECENT ADVANCES IN KNOWLEDGE OF INVERTEBRATE RENAL FUNCTION*

ARTHUR W. MARTIN
University of Washington

From the position of advantage which we now occupy because of the accomplishments of earlier workers, we can look back over a long accumulation of facts about the excretory organs of invertebrate animals. Detailed descriptions of the gross and microscopic anatomy are available for many species. Intelligent inferences as to function are possible from a knowledge of the structure, and many of these inferences have been confirmed by the cytological picture following special methods of treatment of the tissues, and more recently by quantitative analysis. Adequate reviews devoted primarily to this type of work have been prepared, as for example the review of the renal histology of molluscs by Turchini (1928), those of Bruntz (1904) and Lison (1942) on the arthropods, and that of Willem (1910) on flatworms, annelids, molluscs, and several minor phyla.

A special case of the histological approach has been called for some time the method of "physiological injection." Nonfatal doses of vital dyes were injected into living animals, some time was allowed to pass, and the animals were then fixed and sectioned for the identification of the cells and organs which responded specifically by the accumulation of the injected material. Such studies appear to have identified organs which are excretory by virtue of the accumulation within themselves of specific compounds, the removal from the circulating blood being essentially complete, and these structures have been called the kidneys of accumulation by some authors. The distinction of these from kidneys of elimination has been broken down to some extent by the realization that, at a more propitious moment from the standpoint of the availability of water, the cells of accumulation may lose the accumulated wastes and revert to ordinary status. For examples of recent studies of this kind attention is called to a paper of Palm (1952) on insects and one by Husson (1951) on an amphipod.

As an appropriate immediate descendant of the morphological studies, biochemical methods have been applied to the problem of excretion in invertebrates. The very important generalizations that have been established regarding the form of excretion of nitrogenous wastes may be mentioned here as a magnificent accomplishment of this method of study.

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The inorganic composition of the urine is related to that of the other body fluids and to that of the external environment. Its study has become an integral part of the subject of osmoregulation which, by its importance, has been accorded a separate treatment in this symposium (Robertson).

The subject to which this review is limited may properly be called the quantitative physiological approach to renal function. It may be described as the method of controlling the chemical composition of the blood in one or more of its constituents and following the performance of the kidney by analysis of serial samples of urine collected quantitatively. As has been emphasized by Smith (1951), the animal must be in normal physiological state during this time, having recovered from anaesthesia or shock. How well these criteria can be extended to invertebrate animals may be judged from the results presented below. This approach should enable the investigator to form a critical judgment as to the rôle of three important mechanisms in urine formation. These mechanisms are filtration, the active transport of materials from the filtrate back to the blood, and the active transport of materials from blood to urine. The method should also provide a wealth of detail about the normal rates and maximum excretory capacities for a variety of normal and test substances.

The terminology of the field under consideration is not in a satisfactory state, nor is the terminology adopted here one carefully planned to take care of future needs. Suggestions for a terminology would be welcome and vigorous discussion might lead to more consistency for the future than now prevails. Secretion, to at least one cytologist (Bowen, 1929), meant the synthetic steps within the cell resulting in formation of a new substance, and the release of that substance from the cell was to him "excretion," no matter what important function the substance might then have. This limitation of the term secretion is unacceptable to physiologists, and indeed would appear to be generally so because of the meaning of the root: to place apart, to separate, or to sever. A more recent author (DeRobertis, 1948) says of secretion that it " . . . is the process by which cells absorb substances, transform them chemically or in concentration, and expel them. The products of secretion may be utilized by other cells, may stimulate or inhibit other cells, may act chemically on other substances or may be eliminated from the organism. These transformations imply work done by the cells, since, in chemical transformation or in fluid transfer against a concentration gradient, a certain quantity of energy is always consumed. In excretion nonmodified substances are expelled along favorable concentration gradients without expenditure of energy by the cell. Nevertheless both processes frequently are more or less intermingled and it is difficult to separate them clearly." It seems to the writer that we must try to distinguish them clearly.

A terminology has been developed by the vertebrate renal physiologists which suffices for their needs but which is not helpful in dealing with animals which do not possess the same structures, even though the analogues are there. At the beginning of his monographic work Smith (1951) clearly identifies filtration with the glomeruli. Further terms are linked inseparably with tubules, for example: "The tubules then 1. might absorb substances from the glomerular filtrate and return them to the peritubular fluid (tubular reabsorption of glucose, etc.), 2. they might remove substances from the peritubular fluid and discharge them into the tubular urine (tubular excretion of phenol red, diodrast, etc.), 3. they might elaborate new substances that could be discharged into the tubular fluid (tubular secretion)."

If we cannot use these terms it appears wise to define the terms to be used hereinafter. The meaning assigned to *filtration* will usually be quite clear. Any process assigned this name will be understood to mean transport under some kind of pressure head through a semipermeable cell or membrane from blood, hemolymph, coelomic fluid, or intracellular fluid into a structure leading out of the body. Diffusion processes will, of course, accompany this and following processes. In most animals such a filtrate might well carry away organic and inorganic compounds of use to the animal. These useful compounds, having been filtered, may be reabsorbed into the blood or hemolymph. The term *reabsorption* will be used consistently in this sense. The material traverses cells and it is not intended that the pump be thought to be different in principle from the pumps that work in the other direction. Cells of the kidney may actively transport material from the blood, etc. to the lumen of a structure leading to the outside of the body. Active transport in this direction will hereafter be called *secretion* without any consideration of chemical transformations being required or not. These definitions leave the term *excretion* as a general, nondefinitive, term to encompass the sum of all these activities. The nonmorphological term *kidney* will be applied to those specialized excretory tissues, exclusive of cells of the digestive tract, which discharge wastes to the outside of the body, since we are not yet ready to distinguish on a functional basis the different kinds of nephridia which have been described. Where an author has applied a specific term to a tissue being described, his term will be repeated without morphological implications. The term *blood* will be used merely to indicate a circulating fluid unless there is a well-recognized blood system independent of a coelomic fluid or circulating extracellular fluid.

Instead of using a phylogenetic approach, I wish to discuss each of the three major mechanisms in turn, drawing on the material which is available from any invertebrate phylum in which forms have been studied in

such a way as to contribute to an understanding of mechanism. The final product will lack the grace of form which has been given to the same story told phylogenetically by Carl Schlieper in the *Fortschritte der Zoologie,* Vol. 9 (1952), and by G. S. Carter in *A General Zoology of the Invertebrates* (1951). The reader is referred to those works for considerable detail that must be omitted here. The result of this approach, it is hoped, will be critical comparison of the part played by these mechanisms in urine formation.

Filtration

It is most profitable to begin our discussion with a consideration of filtration; for where filtration of the blood occurs in the process of urine formation it will be an initial process, producing a fluid which will probably be modified by other processes before the discharge of the urine from the body. When these other processes are inhibited, the composition of the urine will revert more and more nearly to that of a filtrate and in this relationship we have ready at hand a test for the presence of filtration. Of the invertebrate animals as a whole it might be well to choose to start with a phylum for which a considerable body of evidence has been accumulated and to pass from well-established facts to a consideration of the groups for which only inferences can be made. Three different molluscan classes have yielded evidence of one kind or another for filtration.

Molluscs

In a general way it would appear essential that fluid accumulating in a coelomic cavity be in some sort of steady-state relationship to blood, and that filtration through the lining of the coelom would participate to a greater or lesser degree in maintaining this steady state. Whenever we deal with a coelomic fluid, consequently, we are already dealing in part with a filtrate. With the elaboration of a part of the coelom for excretory functions, this primitive filtration might be refined or suppressed. Molluscs possess a reduced coelom of which one part becomes a pericardial chamber, another the cavity of the renal structures (Goodrich, 1945). These chambers commonly remain in contact one with the other through a renopericardial canal and may participate together in the excretory function. The renopericardial canal is frequently ciliated, the beat being in the direction of the kidney lumen. Movement of fluid through the lumen of the glandular portion of the kidney may then be accomplished either by the contraction of muscles within the kidney walls or by pressure set up by general body movements.

In the lamellibranch molluscs the atria are very thin-walled structures in which the pressure is rhythmically elevated above the general tissue

TABLE 1. A COMPARISON OF THE RATES OF URINE FORMATION OF MARINE, FRESH-WATER AND TERRESTRIAL INVERTEBRATES—FROM THE LITERATURE

Phylum Habitat and Species	Environ-mental Temperature (centigrade)	Average Body Weight (grams)	Data from Measure-ment of Filtration Rate (ml./gram/day)	Data from Measure-ment of Urine Flow (ml./gram/day)	Urine per 24 Hours as % of Body Weight	Remarks	Observer	
MOLLUSCA								
MARINE								
Octopus vulgaris	....	4,000	....	0.02	2.0	Sacs tied	von Fürth	1900
Octopus hongkongensis	10°	10,000	0.1	....	10.0	Sacs catheterized	Harrison	1954
Octopus hongkongensis	10°	20,500	....	0.062	6.2	Sacs tied	Martin (unpublished)	1955
Sepia officinalis	....	895	....	0.042	4.2	By sucrose excretion	Robertson	1953
FRESH-WATER (shell weight omitted)								
Anodonta cygnea	17°	50	4.7	....	470.0	Pericardium open	Picken	1937
Anodonta cygnea	15°	50	....	0.48	48.0	Dilution of inulin	Potts	1954b
TERRESTRIAL (shell weight omitted)								
Achatina fulica	25°	50	1.2	....	120.0	Kidney duct catheterized	Martin, Harrison, and Stewart	1953
ARTHROPODA								
MARINE								
Carcinus maenas	15°	42.7	....	0.10	10.0	Pores closed by wax	Nagel	1934
Carcinus maenas	....	....	....	....	5.0	.....	Webb	1940
Cancer sp.	....	....	....	....	3 –10.0	.....	Robertson	1939
Homarus americanus	....	500	....	0.48–0.192	4.8–19.2	Higher rates when injected with fluid	Burger	1953
Maia squinado	....	2,190	....	0.03	3.0	Bladders may not have been completely emptied	Bialaszewicz	1932
BRACKISH-WATER								
Palaemonetes varians	....	....		....	3.6	In 50% sea water	Parry	1955
Palaemonetes varians	....	....	....	....	10.0	In 100% sea water	Parry	1955
Palaemonetes varians	....	....	....	....	39.1	In 5% sea water	Parry	1955
FRESH-WATER								
Cambarus clarkii	....	12.8	....	0.052	5.2	Pores cemented for 4 to 8 hours	Lieneman	1938
Cambarus clarkii	....	32.2	....	0.049	4.9	Pores cemented, ⅓ of cases	Maluf	1941b
Potamobius astacus	13–14°	e.g. 61.5	....	0.037	3.7	.....	Herrman	1931
ANNELIDA TERRESTRIAL								
Lumbricus terrestris	....	4.0	....	0.60	60.0	.....	Wolf	1940
Pheretima posthuma	....	1.44	....	0.47	47.0	Mass sampling	Bahl	1945

pressure even when the animal is enclosed within a tight shell. Filtration in such circumstances has been sought and found by Picken (1937). Measurements proved that the hydrostatic pressure of the blood exceeded the colloid osmotic pressure by a substantial margin. By exposing and opening the pericardial sac of *Anodonta cygnea*, a fresh-water lamellibranch, he was able to demonstrate a copious flow of a fluid judged to be an ultrafiltrate of the blood, though the exact location of the filtering membranes was not identified. The fluid was nearly free of protein, hence was not blood, but otherwise corresponded to blood in its composition. Florkin and Duchateau (1949) confirm the filtration in *Anodonta*, identifying the ventricle as the site of filtration and finding the same amount of Ca, Cl, and PO_4 in pericardial fluid and in blood.

The evidence for filtration as an initial process in urine formation in *Anodonta* has been further strengthened by the studies of Potts (1954b), who sought to determine the filtration rate by measuring the quantity of inulin lost from the blood stream into the surrounding water per unit of time. It will be noted that Potts made the implicit assumption that inulin is neither secreted nor reabsorbed. The independent active transport of water would not affect the validity of his computations as long as these primary assumptions are correct. From his data Potts computed inulin "clearances" and found that the rate of filtration measured in this way compared favorably with an independent estimate of the rate of urine formation by the technique of placing the animals in an isotonic medium and following the rate of weight loss. It is assumed that salt and water gain or loss will be negligible by any other route than urine formation. The measurements made in this way showed a rate of filtration only about one-fourth that obtained by Picken but, as may be seen from Table 1, a rate still much higher than that for most invertebrate animals. The conditions of Potts' experiments were more physiological than those of Picken's since back pressure due to the presence of an uninjured pericardial sac and a nephrostome of small dimensions would limit filtration.

Martin, Stewart, and Harrison (1954) have followed still a different technical procedure in studying filtration in the gastropod, the giant African snail *Achatina fulica*. It is possible to chip away the shell of this terrestrial form, exposing the heart and the kidney without blood loss. Fig. 1 shows the relationship of these structures and the points of insertion of small, plastic catheters into pericardial sac, kidney, and kidney ducts. There is the usual renopericardial canal communicating between the pericardial sac and the kidney lumen, but repeated attempts to show a significant amount of filtration into the pericardial sac failed. That filtration was nevertheless taking place, probably through the cell layers of the kidney itself, seems quite clear from the results of the quantitative physio-

logical experiments performed. It is suggested that the source of the pressure is the arterial blood pressure, the kidney of the gastropod receiving afferent renal blood vessels directly from the ventricle of the heart.

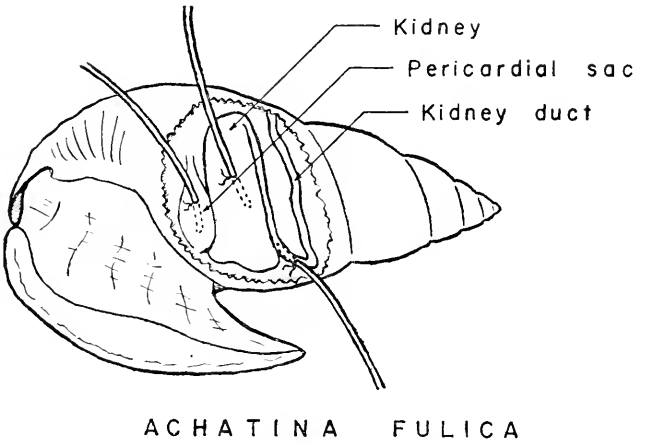

Fig. 1. Appearance of a specimen of *Achatina fulica* prepared for sampling from pericardial sac, kidney lumen, and kidney duct.

In addition to a catheter in the kidney duct which permitted quantitative collection of urine, a soft plastic catheter was tied into the perivisceral space, entry being made under ether anaesthesia through the foot. Inulin was injected at regular intervals and blood and urine samples collected alternately for long periods of time. In those experiments in which the urine was allowed to flow freely from the catheter, the urine/blood inulin ratio was usually one and remained nearly so at high rates of urine flow. Continuous reabsorptive and secretory processes were taking place during this time, as was shown by the reabsorption of glucose and the active concentration of injected dyes. The poisoning of reabsorptive or secretory processes brought the U/B ratios of such substances close to that obtained simultaneously for inulin. These results are most satisfactorily understood on the assumption of a process of filtration.

A free flow of urine from the kidney of the snail is probably a rare occurrence in nature. The presence of a tortuous duct and muscular sphincters will allow for much water reabsorption under conditions where water conservation is important. It is well known that hibernating snails may produce no urine whatsoever. The effects of such back pressure may be illustrated in the following experiment. A tightly fitting catheter was tied into the kidney duct and the preparation tested for leaks by the injection of faintly colored water. The urine collection was then carried on at known elevations at or above heart level, thus producing a back pressure

into the kidney. The U/B inulin ratios and the volume of urine formed were determined for each of the intervals of different pressure. The results are shown in Fig. 2, from which it may be seen that, following a preliminary equilibration phase, the volume of urine was inversely proportional to the hydrostatic back pressure on the kidney. When the volume of fluid was low, the U/B ratio tended to rise above one. This is suggestive evidence that filtration continued but that the reabsorption of water was a normal part of the kidney function under these circumstances.

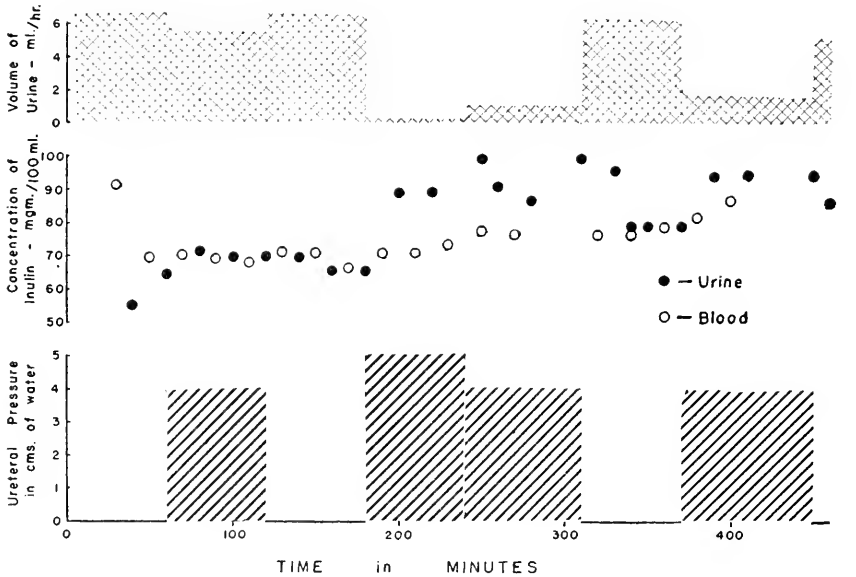

Fig. 2. The effects of ureteral back pressure on the rate of urine formation in the giant African snail.

Similar experiments have been performed on the cephalopod *Octopus hongkongensis* (Harrison, 1954; Harrison and Martin, 1955). In this case inulin solution was perfused continuously into the closed vascular system of the octopus. Urine samples were recovered as quantitatively as possible at 15- to 30-minute intervals for many hours, and blood samples were taken at corresponding but intermediate intervals. It was therefore possible to follow the blood and urine concentrations in this marine form for comparison with the terrestrial mollusc. The U/B ratio remained at approximately one, indicating that the inulin was probably filtered from the blood stream and excreted without change in the water content. Filtration should be relatively independent of the actions of tissue poisons and test showed that such was the case; neither phlorizin, DNP, nor bene-

mid exerted large effects on the U/B inulin ratio, though it was easy to demonstrate profound effects on the simultaneous excretion of other materials.

It would be of interest to understand what source of pressure might be available for filtration in the cephalopod. The renal appendages are venous structures lying on the main branches of the vena cava. They lie in a right and a left sac, each sac having a small tubular connection to the pericardial coelom surrounding the branchial heart and branchial heart appendage of the same side. The tube from the pericardial coelom empties near the orifice of the renal sac, making it possible that the pericardial coelom and the renal sac are emptied simultaneously by a long and vigorous respiratory movement of the animal, the contents of the pericardial coelom therefore not coming into contact with the renal appendages. If this is the case, our analyses of the activities going on in the renal appendages do not include any filtered material from the branchial hearts. Further investigation of this point is in progress; an effort is being made to collect the material from the pericardial coelom quantitatively under various experimental conditions.

If we therefore exclude the branchial hearts and the branchial heart appendages as a source of filtration, we must search again for a source of pressure. Observations on the living animal, and on the renal appendages *in vitro*, made by my colleague Dr. Florence M. Harrison, reveal a very promising mechanism. The blood vessels of cephalopods are notably contractile and show much rhythmic peristaltic activity. Each renal appendage represents a diverticulum from a branch of the vena cava. A small round or oval orifice penetrates the wall of the vena cava, leading into a labyrinth of progressively finer vessels. The peristaltic movements are not limited to the walls of the vena cava, but muscle tissue in the appendages also contracts, compressing a considerable volume of blood in the cul-de-sac represented by the appendage while the blood is forced back through the aperture into the vena cava. Upon relaxation blood flows again into the myriad of small vessels lined with renal epithelium. It is suggested that a fraction of the fluid is forced through the walls of the appendage, wherever the lining of cells is thinnest, with each contraction.

It is notable that in this survey of the molluscs we have now completed a full cycle. In the lamellibranch the filtration pressure was thought to originate in the auricular and ventricular activity of the heart and the actual site of filtration perhaps to be the tissues of the heart; in the gastropod the filtration pressure originated in the heart but the filtration site was in the capillaries and small sinuses of the arterial region; in the cephalopod the source of pressure may be contraction of muscle tissue related to that of the veins and the renal appendages are situated on the walls of the veins.

Arthropods

If we examine very briefly the origin and structure of the excretory organ of Crustacea, as representative of many arthropods, we find the origin to be from the primitive coelomoduct (Goodrich, 1945). It may consist of several parts, of which the first is a thin-walled end sac sometimes closed by a special sphincter or valve. Generally the thin, simple epithelium of this sac with its outer basement membrane is held open by attachment to surrounding structures by strands of connective tissue. This sac may offer an opportunity for filtration through the walls from the surrounding blood of the hemocoel. Probably of more importance for filtration is the direct arterial blood supply found in many species. Parry (1955) has injected and dissected the blood supply to the kidney in the prawn *Palaemonetes varians* and finds: "the main branch of the antennary artery on either side of the thorax leads directly to the end sac, where it suddenly splits up into numerous fine vessels which are lost in the walls of the end sac. Neither the labyrinth nor any other part of the gland appears to have any (arterial) direct blood supply . . ." The end sac communicates through the valve with a canal of greater or lesser length depending upon the species, a canal lined with thicker cells by means of which, it may be inferred, active changes may be brought about in the character of the urine.

The beginning of balanced studies to test for filtration as a factor in arthropod excretion may be said to have been made by Picken (1936). Measuring colloid osmotic pressure of urine and blood and the hydrostatic pressure of the body fluids of *Carcinus maenas, Potamobius fluviatilis,* and *Peripatopsis sedgwicki,* he concluded that a filtration pressure exceeded the colloid osmotic pressure in each of these animals so that filtration would naturally occur. It may not be safe to argue entirely from the hydrostatic pressure of the body fluid, but we may guess that the pressure in the arteries supplying the kidneys is even greater than this measured hydrostatic pressure. It seems at least likely that filtration may occur.

Some more recent investigations have produced evidence in favor of a process of filtration in the kidneys of other arthropods. It should be stated at once, however, that, until some further experiments are performed on the crayfish, all evidences for filtration in the fresh-water arthropods must be regarded with some skepticism. This is so because in the crayfish alone, among all the animals so far investigated, there appears to be evidence of the secretion of inulin. Maluf (1940, 1941a) interprets his evidence to indicate that all of the components, including water and inulin, of the urine of the crayfish are produced by secretion.

In a lengthy report Maluf (1941b) has described the handling of inulin, xylose, creatinine, and certain dyes by the antennal glands of large healthy

individuals of *Cambarus clarkii*. The essential data for our consideration are contained in Fig. 7 of his report, which is reproduced here as Fig. 3. It will be noted from the figure that the U/B inulin ratio, up to quite high blood-inulin concentrations, is well in excess of 2 and reaches as high as 5. Are these values the result of faulty technique? Maluf injected adequate amounts of inulin; it was distributed normally as indicated by the fact that computations made by the writer show a blood volume of 25% of the body weight, which checks well with Prosser and Weinstein's (1950) determination of the blood volume of this form; his analytical procedures were capable of delivering the accuracy claimed; the injected inulin may be nearly accounted for by the sum of the excreted inulin and that still present in the blood of the animal at the end of the experiment; the blood samples were not too large for good physiological results; and the urine flow corresponded to that obtained by another investigator working with the same species.

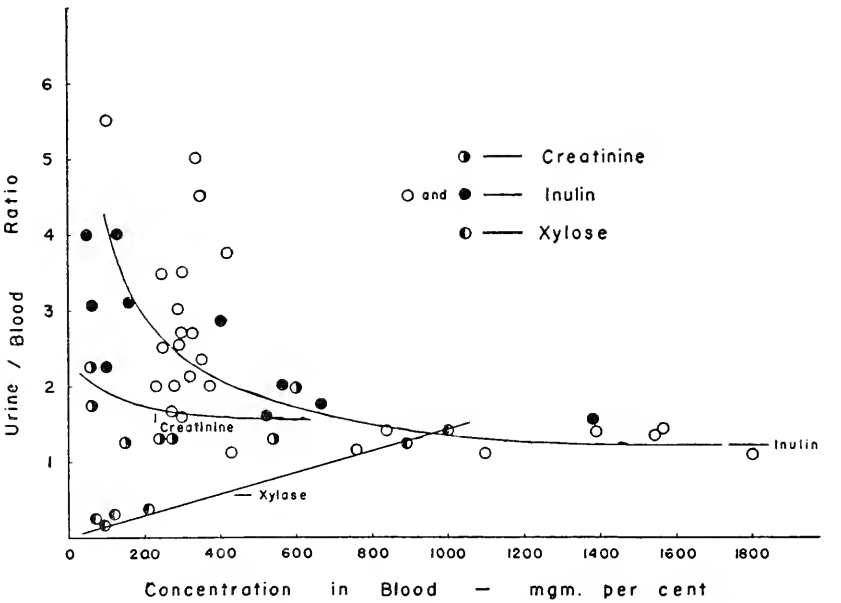

Fig. 3. Excretion of inulin, xylose, and creatinine by *Cambarus clarkii*. Redrawn from Maluf, 1941.

Two minor criticisms may be stated. In some experiments the excretory pores were sealed, so that abnormal pressures might have been built up in the bladders. Against this view is the fact that results of this series checked those of an earlier series in which the pores were not sealed. Second, it was necessary, in carrying out the blood collections during the experi-

mental period, to cut through a leg at the femur and to stop the bleeding by compressing the stump with a hot forceps. In some experimental animals handling produces a shock effect on urine formation, so that this method of blood collection may not be ideal. It does not seem possible to argue that there was any large effect, because reference to Table 1 shows no evidence that there was any suppression of urine formation in comparison with other fresh-water decapods. In his discussion of the shock effect Wikgren (1953) states that no marked shock effect was demonstrable in the crayfish. The result of all these comparisons is, then, that the observations of Maluf are essentially correct.

It does not follow that the interpretation is correct, and the alternatives are worth a brief examination. Maluf prefers to interpret the concentration curve of inulin as showing that at low blood-inulin levels the secretory process for inulin is more rapid than that for water, but that saturation of the mechanism begins to take place at higher blood concentrations. The data may equally be interpreted as the result of a filtration followed by reabsorption of water. The reabsorption of water from the kidney of a fresh-water animal seems at first glance to be highly improbable. Yet it may be essential that water accompany the very large quantity of salt that is reabsorbed by the kidneys of these animals, and that this reabsorption is related to the very low rates of urine flow which characterize the representatives of this group of fresh-water animals in contrast to the fresh-water and terrestrial molluscs and worms, as has been illustrated in Table 1.

Another result reported by Maluf seems to the writer rather to favor the view of water reabsorption, though it does not provide crucial evidence on the point. Why, we may ask, should one carbohydrate—inulin—be secreted, while another carbohydrate—xylose—is being reabsorbed. From experience with xylose in other animals it would appear that it is only a stereotype for glucose and that its reabsorption indicates an active glucose-reabsorbing mechanism. But if all the components of the urine are secreted, as in the aglomerular fish, why a specific reabsorbing mechanism for either glucose or xylose? The fact that the xylose U/B ratio curve rises to a value of approximately one at the higher blood concentrations receives a labored explanation by Maluf; but by the above interpretation this may carry the normal significance, simply that a transport mechanism has reached its maximum rate. It may be predicted on the new interpretation that phlorizin treatment will result in a curve for xylose like those for inulin and creatinine.

The creatinine curve, finally, does not contribute much towards making a choice of these alternatives. Most kidneys which have been studied are not indifferent to this substance, with both reabsorption and secretion

claimed in other animals. That the creatinine U/B ratios are lower than those for inulin would be interpreted on the filtration-reabsorption basis simply to mean that creatinine is less well rejected from the inflow of reabsorbed water than is inulin, and since it has a lower molecular weight this appears understandable. Because of the importance of the finding, Forster and Zia-Walrath (1941) carried out some experiments with inulin on *Homarus americanus* and found no evidence for secretion of inulin, reabsorption of glucose, or secretion or reabsorption of water. To compare a marine animal with a fresh-water one, however, does not answer our question satisfactorily and the reservation should be removed by further investigation of the crayfish. The search might be doubly rewarding if it proves not only that inulin is not secreted but indeed that there is water uptake from the kidney of a fresh-water arthropod. The search could not be negative, since further proof that inulin is secreted would be of general importance.

The studies with *Homarus americanus* have gone forward in the hands of Burger (1953, 1955a), who has carried out a careful analysis of kidney function in this very suitable animal. He finds U/B ratios for injected inulin of approximately unity, verifying Forster's work, with both urine and plasma concentrations falling with the same slope over periods of time up to 28 hours. In the absence either of a clear structural filter or a sizable pressure gradient, Burger (1955b) feels some reluctance to apply the term filtration to the process, though he agrees that the end result is the same.

It is not possible to pursue in any detail the phenomena of ionic regulation. Some of the studies directed to this end, however, may bring some additional evidence in favor of filtration as a process in the formation of urine in Crustacea. One case may be cited here because of the unusual capacity of palaemonid prawns to maintain their blood ions at a remarkably constant level in salinities equivalent to from 0 to 5% NaCl solutions. Parry (1954) says of her results:

> While the inorganic analyses of urine do not necessarily demonstrate the importance of excretion in maintaining the osmotic control of the animal, they do indicate how it maintains ionic control, and make possible some deductions as to the mechanism of urine production.
>
> The analyses of sodium, chloride, potassium, and calcium in urine are, on the whole, very similar to those of blood, and their concentrations would not be incompatible with the conception of the urine as an ultrafiltrate of the blood. The much higher concentrations of magnesium and sulphate in urine could result from some active excretion of these ions after the formation of an ultrafiltrate. The lower concentrations of sodium, and the higher concentration of chloride in the final fluid produced, might result from the necessity to balance the fluid ionically after the influx of magnesium and sulphate (and the ammonia which is presumed to be present). The comparatively constant proportion of sodium and chloride in blood and urine, inde-

pendent of the external salinity, suggests that the ultrafiltration is unaffected (except perhaps in its rate) by the external conditions or the internal conditions resulting therefrom.

Annelids

Bahl (1945, 1946, 1947) has made important contributions to our knowledge of the physiology of the excretory system of annelids. It cannot yet be said that adequate control of the blood concentration of test substances has been accomplished in any worm, and Bahl's results are somewhat complicated by the strikingly different chloride and osmotic pressure values between blood and coelomic fluid of the earthworm *Pheretima posthuma* upon which he made his physiological observations. This worm possesses nephridia of three types; according to Bahl these make up one of the most elaborate nephridial systems of all earthworms. Some are closed, possessing a flame, and of the closed nephridia some empty externally, others into the pharynx and buccal cavity. Some are open through a nephrostome so that coelomic fluid can pass into the lumen of the nephridium. Those with a nephrostome happen, in this form, to drain into the intestines—they are enteronephric. As a result of the number of nephridia and the location of their pores, it was not practical to collect fluid from the nephridiopores. Urine was collected either by keeping the worms in water or by allowing urine normally expressed to drip from worms held on a slanting glass dish. The atmosphere was saturated with water vapor to prevent evaporation of the urine formed. Fluid extruded from mouth or anus is properly included in work with this species as probably of nephridial origin.

Bahl was able to collect sufficient quantities of urine by this technique so that analyses could be made of the composition of the urine for comparison with those of blood and coelomic fluid. The results may be illustrated most readily with Table 2, adapted from Bahl (1947).

Inspection of the table shows a striking difference in the salt content of the blood and coelomic fluid, the latter being higher in Na, Ca, Mg, and Cl, while the blood has the higher content of K, PO_4 and SO_4. Even assuming a rather low molecular weight for the blood protein, it is still difficult to reconcile Bahl's list of salt concentrations with the freezing-point depressions given. It seems likely that other osmotically active substances were not accounted for among the substances analyzed.

It is of interest to try to understand the differences in concentration of coelomic fluid and blood, since Ramsay (1949a) did not observe a similar striking difference in the worm *Lumbricus terrestris*. In both studies the animals had been kept in tap water for several days before and during the study. There is ample evidence that earthworms can survive immersion for

long periods of time if toxic materials are absent (cf. van Brink and Rietsema, 1949), so we may assume the worms were in good physiological condition.

TABLE 2. THE COMPOSITION OF THE BLOOD AND COELOMIC FLUID IN COMPARISON TO THAT OF THE URINE OF *PHERETIMA POSTHUMA*

(From Bahl, 1947)

Constituent	Blood	Coelomic Fluid	Urine
Ammonia	1.5	1.0	1
Urea	0.8	1.0	1
Creatinine	7.0	5.5	1
Protein	121.0	16.0	1
Na	4.0	8.0	1
K	8.0	2.5	1
Ca	1.4	1.8	1
Cl	13.5	22.0	1
Mg	1.4	7.0	1
PO_4	16.0	1.8	1
SO_4	2.0	1.4	1
Glucose	0.1 mgm%	nil	nil
O.P. as freezing point depression	0.4–0.5° C	0.285–0.31° C	0.05–0.065° C

Let us assume that a blood filtrate enters the closed nephridia of *Pheretima*. Because of the immersion there might be an unusually large osmotic inflow of water, with the result that the nephridia would handle an unusual quantity of fluid, as much as half the volume of the body in 24 hours according to Bahl's measurements. To conserve salts the electrolytes would be pumped from the filtrate back through the nephridial cells. But, since the nephridium is bathed in coelomic fluid, the electrolytes would arrive not in the blood from which they originated but in the coelomic fluid; and only after an interval of time without intense water uptake would isotonicity be re-established between blood and coelomic fluid. In *Lumbricus* there are present only open nephridia into which coelomic fluid can drain; but the initial assumption that more blood filtrate enters the nephridia of *Pheretima* must be regarded with reserve, for Bahl believes there may be filtration into the open nephridia of *Lumbricus*. He states (1947, p. 141):

> In several earthworms there are characteristic vascular dilations or ampullae on the nephridial capillaries, the meaning of which has not so far been clear. Benham (1891) described them in *Lumbricus* and I have described them in *Lampito dubius* and again in *Hoplochaetella khandalensis*. It is probable that these dilations are really tiny "blood filters" through which the blood plasma is filtered out into the nephridia for excretion. No data are available on the blood pressure in the nephridial "artery," but apparently the rhythmically contractile dorsal vessel and "hearts" supply the necessary filtering force driving the blood plasma (minus the colloids) out of the blood capillaries and dilations into the nephridia.

An assumption of filtration into closed nephridia has been made but proof of this assumption is lacking. Neglecting the "blood filters" of Bahl, may there be another source of filtration pressure? In 1899 Goodrich made the suggestion that solenocytes may have the same function as Malpighian corpuscles of the vertebrate kidney and this suggestion proved useful; for water has been seen to accumulate in the contractile bladders of animals possessing protonephridia, water apparently derived from the protonephridia. But Goodrich speaks for the conservative view of the origin of such fluid when he remarks: " . . . the chief function of the 'flame' is to drive the fluid passing by osmosis through the thin wall of the chambers down the canal towards the nephridiopore." This view does not explain satisfactorily the appearance of the fluid in the nephridium. If, in accordance with the idea expressed by Goodrich, the fluid accumulates by osmosis we have to search for osmotically active substances in the lumen of the nephridium. Salts could be secreted into the lumen by active transport, there to be diluted by water of osmosis. Such a material might well be a potassium salt which could be reabsorbed and reutilized lower in the canal. Ramsay (1954) observed the potassium turnover in the Malpighian corpuscle of insects, and set up a deliberate test of this hypothesis; he was convinced that the results of his experiments could not support such a mechanism.

The tube surrounding the flame of the ordinary nephridium and in particular the tube of its derivative, the solenocyte, does not appear from its very thin nature to be particularly suited to a process of secretion, but is very well adapted to a process of filtration. The problem of finding a force responsible for filtration remains and leads us to the beating of the flame or of the flagellum. Carter (1940) examined and rejected the idea that the flame could exert enough pressure to overcome the colloid osmotic pressure of the fluid bathing the flame cells. Bahl (1945) argues that the pressure might be sufficient: "In the closed integumentary and pharyngeal nephridia the movement of the cilia in the ciliated tracts probably sets up a slight pressure which is enough to draw liquid by a process of filtration from the blood and coelomic fluid, through the exceedingly thin walls of the nephridium into the lumen of the intracellular canal." Pantin (1947) renews the discussion in the light of his findings that the activity of the flame cells is increased by the uptake of fluid in the terrestrial nemertine worm *Geonemertes dendyi*, and in the light of the visual observation of oscillations at the base of the cell set up by the action of the flame.

It is most attractive to view the flame cells and solenocytes in this way as sources of filtration pressure. If this interpretation is correct, the widespread importance of the process in higher phyla becomes interpretable as a natural succession to the filtration process so uniformly present in the

more primitive phyla. After reviewing a list including Platyhelminthes, Rotifera, Nemertea, Acanthocephala, Priapuloidea, Entoprocta, Gastrotricha, Kinorhyncha, Cephalochorda, some Archiannelida and Polychaeta, and larval stages of Polychaeta, Archiannelida, Echiuroidea, Mollusca, Phoronidea, and Cephalochorda, Goodrich (1945) says: "These organs (protonephridia) are in fact widely distributed and may be inferred to have been present in the common ancestor of all the Metazoan Triploblastica."

The point may be made here that by the indirect methods of renal physiology it may be possible to prove filtration by the solenocytes without an actual measurement of the pressure they are capable of exerting, just as the vertebrate renal physiologist has measured the blood flow through the kidney without operation, oncometer, or strohmuhr! Further work on these interesting forms is much needed.

Reabsorption

It would appear that a major supporting argument for the process of filtration is the presence of special mechanisms for reabsorption of materials from the kidney lumen. If urine were to be formed almost entirely by the process of secretion, as appears to be true of the aglomerular fish, it would be much more economical to secrete only those substances to be rejected from the body along with a minimum amount of water for their solution. A urine started as a filtrate, on the other hand, must contain the electrolytes that were in solution, as well as glucose, and if the urine starts as a coelomic fluid there may be considerable quantities of protein and other large molecules in the fluid entering the kidney. For the conservation of these materials a reabsorptive process is needed as it would not be in the secretory case. For an example, the aglomerular fish needs and has no mechanism for the reabsorption of glucose. This argument cannot be pushed too far, because the Malpighian body of the insect appears to employ secretion on a large scale but with evidences of reabsorption, the rôle of which is not yet understood. The evidences for reabsorption should be examined at least for the groups for which an effort has been made to demonstrate the process of filtration.

Molluscs

The mechanism of filtration into the pericardial sac of fresh-water lamellibranchs demonstrated by Picken, Florkin, and Potts would be wasteful of the solutes of the blood. Picken (1937) believed that much salt was reabsorbed as the filtrate passed through the kidney lumen. He says: "The pericardial fluid is in most cases approximately isotonic with the blood, but the urine is markedly hypotonic to the body fluid."

Confining our attention to the "Cambridge water" specimens, the

bloods ranged in NaCl-equivalent concentrations from 0.06% to 0.14%, mean 0.103%; the pericardial fluid from 0.03-0.13%, mean 0.091%; the urine from 0.04-0.11%, mean 0.071%. It may be seen that the means are rather close together and do not appear to justify the use of the term "markedly," since it is clear that much salt will be lost via the urine at these salt concentrations in view of the large daily urine volume.

Admitting the difficulty of collecting pure urine in these forms, the importance of the reabsorption of salt in meeting the osmotic problem is so great that one wishes there might be an extension of this work following the pattern suggested by Krogh (1939, pp. 61-64):

> As they stand Pickens' experiments would place the kidney tubules as being much less effective than the cells in the surface, but I venture to predict that this is only because they were not put to a crucial test. When animals are prevented from absorbing salts from outside, the kidneys will probably be able to produce a urine which is almost salt free. Otherwise the process of washing out with distilled water would be much more rapid than is actually the case.

Florkin and Duchateau (1949) have added some confirmatory evidence for reabsorption in *Anodonta*. Their chemical comparisons of blood and ampullar fluid were concerned with chloride, calcium, and inorganic phosphate, all of which proved to be less concentrated in urine than in the blood. The data speak clearly for reabsorption, particularly since the difference in Cl is marked, the urine containing only a little more than one-half the amount present in the blood. It is worthy of mention that the protein nitrogen of the urine was considerably higher than that of the blood. No interpretation of this observation was given, and it is possible that it represents a merocrine type of glandular excretion which is contributing protein to the urine in the process of urine formation. The advantage of collecting urine and conducting inulin clearance studies on this form must be pointed out, since it is possible that the uptake of salt is accompanied by the uptake of water. This would account for the observed increase in protein. The analyses of Potts for inulin cannot answer the question, since he did not collect urine but only assayed the water in which the animals were kept.

It has long been clear that fresh-water animals face a much more serious problem of ridding their bodies of water without losing salts than do brackish-water or marine animals. Reports of rates of urine flow, or rates of filtration, have been gathered together from the literature and are presented as Table 1. The ordinary rate of urine flow in the marine animals does not much exceed 5% of the body weight in a day, while that in fresh-water and terrestrial organisms can easily reach 50% of the body weight per day and, when filtration is allowed to take place unchecked, the very high rates represented by Pickens' figure may be obtained. Such rates

cannot be maintained for long periods of time by the animals, but represent observations of maximum rates which are useful. But the high rates of urine flow impose a severe task of salt reabsorption. Potts (1954c) has based an interesting computation of the amount of work required to bring about the reabsorption of salt on the analyses of *Anodonta* urine from Pickens' work and his own determinations of the salt content of *Anodonta* blood. He concludes that a lowering of the blood salt level may have been essential to the adaptation of fresh-water lamellibranchs to their new habitat because of the great surface which they expose to the medium. The move through brackish water may not only have increased the ability of the animals to take up salt but allowed for the adaptation of their tissues to the much lower salt content of this group. For an interesting comparison with the fresh-water crustaceans Potts' (1954a,b,c) papers should be examined.

In gastropods the collection of urine is relatively easy but we do not have, unfortunately, any comparable experiments on salt conservation. Freezing-point determinations show the urine of *Achatina fulica*, the giant African snail, to be hypotonic to the blood; the freezing point of blood was —0.462, that of the urine was —0.285. There has, however, been a careful proof of the reabsorption of glucose (Martin, Harrison, and Stewart, 1953). Urine obtained from the snail kidney even at higher rates of flow than can be considered normal shows a glucose concentration considerably below that of the blood. Values for the blood sugar of eleven animals ranged from 1.2-67.5 mgm% with an average of 20.7 mgm% and the corresponding urine-reducing substances in nine cases ranged from 0 to 35.0 mgm% with an average of 10.7 mgm%.

When an animal producing urine which shows this difference in glucose concentration from the blood is treated with phlorizin, the urinary glucose rises until it reaches the level of the blood glucose. If water reabsorption is taking place the urinary glucose may then rise above the blood glucose level, but simultaneous determination of inulin concentrations shows that the glucose does not exceed the inulin in excretion rate, and the result is best interpreted as a simple filtration followed by water reabsorption by a mechanism stable to phlorizin, but with the glucose reabsorption mechanism thrown out of action.

Another type of experiment was devised by my colleague, Stewart. In *Achatina* it is possible to tie a tube into the kidney lumen high on the structure, so that a material perfused into it will have to pass over many trabeculae of the kidney on its way to the excretory duct and may have adequate exposure to cells concerned with glucose reabsorption. When glucose solution is allowed to perfuse into the kidney in this way, or through the pericardial sac, the normal kidney extracts the added glucose, but after phlorizin the added glucose may be recovered quantitatively.

In the octopus, Mayer and Rathery (1907) concluded that reabsorption of glucose and urea was possible. Their experiments are not as physiological as might be desired, since they injected glucose or urea solutions into the renal sacs, ligated the urinary papillae, and left the animal undisturbed for 24 hours. At the end of this time either glucose or urea was present in the renal sac only in a trace. From our experiments (Harrison and Martin, 1955) and the report of von Fürth (1900), it would be anticipated that the renal sacs would be highly distended with the urine secreted by a healthy octopus during this period of time, and that dilution as well as reabsorption would have taken place. There is no reason to doubt, however, that the glucose was reabsorbed and deposited in the body tissues, and that the urea diffused into the blood and was excreted through the gills in the form of ammonia.

Harrison and Martin have performed experiments on glucose injected intravascularly and found that, as in the giant African snail, the filtered glucose is normally reabsorbed, but that in the presence of phlorizin the urinary glucose concentration rises until it reaches the level of the blood.

Arthropods

Because of their wide diversity of form and great success in invading every major habitat, a considerable variation in excretory function among the arthropods is to be expected; but members of this group appear to have developed sites of absorption other than the kidney which are so effective in handling the osmotic problem that reabsorptive processes by the kidney tissue may be rendered unessential.

The striking ability of the palaemonid shrimps to maintain their blood salt at a high level even in dilute media has already been noted. Parry (1955) continued his physiological investigations on this group by determining the rates of urine formation at the extremes of the osmotic range. A minimum output was found at a salinity equivalent of 50% sea water. Output increased almost linearly at lower concentrations with a maximum output at a salinity equivalent of 5% sea water, at which point the rate of urine production had increased tenfold. There was also an increased output at salt concentrations above 50%, reaching rates of about four times the minimum level when the salinity equivalents reached 100 and 120% of sea water. These observations were made on another species than *Palaemon serratus*, for which it was shown that the blood and urine remained isotonic over a considerable range of environmental salt concentration. It is clear that, if the blood salt were to be maintained at a high level while the animal was in 5% sea water, and the urine were to remain isotonic with the blood, and at the same time a very large volume of urine were to be formed, an enormous load would be thrown onto the salt-absorbing mechanisms.

These mechanisms are apparently absent from the kidney and so lie outside the range of this review; but this group of animals might profitably receive further attention, since this appears to be a very ill-adapted kidney for so remarkable an animal.

In another marine form, the lobster, evidence for filtration has been accumulated by Burger (1953, 1955a) and has been reviewed above. Burger has established the reabsorption of glucose with evidence parallel to that for the mollusc. A normal animal with a blood-sugar concentration of 24-40 mg.% shows no glucose in the urine. But when exogenous sugar raises the blood-sugar level to 100 mg.%, glucose begins to appear in the urine; reabsorptive capacity has been exceeded. Under the influence of phlorizin the reabsorptive mechanisms are blocked and glucose appears in the urine without the addition of exogenous glucose. Burger does not state whether, in this last case, the U/B ratio is one, as the filtration hypothesis implies. In the lobster, as in the palaemonids, the regulation of sodium and chloride ions does not appear to be a concern of the renal epithelium. Burger finds, however, that the divalent ions of calcium, magnesium, and sulfate are concerns of the kidney, though only the calcium appears to be reabsorbed in substantial quantity and this Burger feels is still unproved.

Annelids

In some members of this group of animals the problem of reabsorption is increased in complexity by the presence not only of filtering areas but of nephrostomes which can admit not only crystalloids to the epithelial lumen but colloids and formed bodies as well. As an added complexity some of the "open" nephridia drain into the gut, thus allowing for an additional site of reabsorption which might be preceded by a digestive process. We have, too, the added complexity of loss of fluid through the dorsal pores of the terrestrial forms, but quantitative studies of this route of loss are not available. In an earthworm presenting these complexities, *Pheretima posthuma,* evidences for filtration provided by Bahl (1945, 1947) have already been described. This author found significant evidence for reabsorption which covers several categories of compounds: proteins, hemochromogens, salts, and creatinine.

The results indicating the reabsorption of protein are not completely convincing. The blood of this species contains about 3.6% protein, a relatively high level, but it may be assumed that the protein of the blood is held back in the filtration process. Coelomic fluid may run into the open nephrostomes with little hindrance but in a quantity which, relative to the blood filtrate, is not known. The protein content of coelomic fluid is much less than that of blood and the urine contains only about 1/16 of the amount

present in coelomic fluid. It follows that, if the coelomic fluid contributes only 1/16 of the volume of the urine, we have no real evidence for the reabsorption of protein. But, if the contribution of the coelomic fluid is more than 1/16, we are still not able to judge whether the protein was absorbed in the nephridia or in the gut, into which the open nephridia drain in this form.

A different situation obtains in *Lumbricus* where the nephridia are "open" but empty to the outside. If some test substance can be found which leaves the coelomic fluid but does not enter from the blood, it may be possible to work out the relative contributions from these two sources. The substance should be one of large molecular weight and perhaps a protein would do. But it appears already that the native protein of the earthworm will not answer the question. Ramsay (1949a) found it convenient to heat-sterilize samples of blood, coelomic fluid, and urine of *Lumbricus terrestris* to be saved for later analysis. He noted that there was an abundant coagulum from the blood but a slight or nonexistent coagulum from urine and coelomic fluid. Quantative analyses for protein were not done on the small samples which had been collected for other purposes. If the protein content of the coelomic fluid in *Lumbricus* is very low, then, again on the partition hypothesis of the origin of urine from both coelomic fluid and blood, a small difference in the protein concentration of coelomic fluid and urine is to be expected and the amounts may be too low to answer our question quantitatively.

On the reabsorption of hemochromogen, on the other hand, Bahl's experiments seem very satisfactory, and conclude what he found to be a long labor. His discussion of earlier views of the function of the "ciliated middle tube" cannot be repeated here. Suffice it to say that the brown granules of the cells of this segment had previously been considered to be guanine or urates, whereas Bahl demonstrates by solubility properties and absorption spectra that the chief material is a hemochromogen. Because of the small accumulation in young worms and the large accumulation in adult worms, Bahl considers that this material is retired from service and that these cells represent an example of the classical "kidney of accumulation." A question might be raised as to whether or not a larger earthworm might not have accumulated, and be turning over, a larger store of iron-containing pigment. If this were so the reabsorption would be a useful, rather than a nonuseful, one. Only rate measurements would answer this question. A further question remains, however: Is the hemochromogen accumulated from the lumen side, having entered with coelomic fluid from the nephridiostome, or has it been picked up by "athrocytic" activity from the coelomic fluid bathing the outside of the nephridium? From Bahl's observations it appears possible to call this accumulation a "reabsorption,"

since it is only in the open, septal, nephridia that such accumulation is observed. The pigmented granules are completely absent in the closed integumentary and pharyngeal nephridia.

With respect to salts Bahl (1945) showed a very active reabsorption of chloride. He added comparative analyses of several other ions in 1947, and the data are of sufficient interest to the task at hand that they have been reproduced in Table 2 of this review. It will be noted that Na, K, Ca, Cl, Mg, PO_4, and SO_4 all are reabsorbed more or less actively. The differences in the handling of these ions is reminiscent of the observations made on other animals, where each ion appears to be conserved in a quantity apparently unrelated to other ions. As a result of the reabsorptive activity the urine is hypotonic to the blood. But perhaps as a result of the passage of some of the urine into the digestive tract, or perhaps through direct uptake of water from the nephridial lumen, the water conservation in this earthworm appears to be better than that of another species which occupies the same habitat during the wet months of the year. In the rainy season individuals of *Eutyphoeus waltoni* are found in large numbers in the same soil with *Pheretima*, but in the dry months *Eutyphoeus* burrows deep and goes into hibernation while *Pheretima* continues to be active in the relatively dry ground.

Finally, Bahl found a concentration of creatinine by the nephridium, the urine concentration reaching only 1/5 and 1/7 that of the coelomic fluid and the blood, respectively. He suggests no explanation for this observation, which is the reverse of that expected on any analogy to the vertebrate system. Such an active reabsorption of creatinine implies the presence of creatine in the animal, and perhaps the reconversion of creatinine to creatine. So interesting a result warrants further exploration in this group of animals.

A very interesting addition to the facts adduced by Bahl for *Pheretima* has been made by Ramsay for another earthworm. Using very refined micromethods Ramsay has collected urine from various segments of the nephridia of *Lumbricus terrestris* which had been quickly removed from experimental animals and kept in frog ringer solution on a microscope stage. By comparing the freezing point of the urine with that of the ringer solution surrounding the preparation, he has shown that the hypotonicity of the urine must originate here—the results do not reveal whether by the reabsorption of organic matter or salt or by the active secretion of water. If the variety of degrees of concentration of ions observed by Bahl also applies to *Lumbricus*, the addition of water does not seem a supportable hypothesis; the reviewer is of the opinion that reabsorption is more likely. Simultaneous measurements of inulin concentration in coelomic fluid, blood, and urine would be helpful; but the labor of the method is great and

it is to be hoped that some of the very large worms may be adapted to *in vivo* experiments similar to those with the giant African snail and with *Homarus*.

Secretion

Any inquiry into physiological processes soon reveals secretion by cells as a common phenomenon to be encountered in the study of almost any organ or system. Investigators very early recognized processes of secretion in excretory systems and a vast literature has appeared on the subject. Here we shall confine our attention to recent quantitative studies of secretory processes concerned only with excretion. To facilitate comparison with the other sections, the same order of treatment will be followed.

Molluscs

Martin, Stewart, and Harrison (1954) have applied the method of infusion into the blood of the giant African snail, with subsequent serial sampling of blood and urine, to the study of some of the classical compounds known to be secreted in other animals. Of their results only illustrative experiments may be cited here. The dye, phenol red, has long been used in such studies and has been concentrated by all the kidneys studied. In the snail kidney, too, this material is taken from the blood and concentrated in the urine. U/B ratios as high as 90 have been observed. Just as in the case of the vertebrates the transport mechanism depends upon an energy source, and the energy supply may be cut off in the snail by treatment with 2-4 dinitrophenol (DNP). After treatment with DNP the ratio falls very nearly to one, and excretion continues at the rate imposed by the filtration process.

In cephalopods the application of quantitative methods has a longer history. Mayer and Rathery (1907) made an unnecessarily traumatic, but nevertheless workable, approach to the problem of collecting blood and urine samples from the octopus. Perhaps because they were working wth animals a little too small for really easy sampling, they did not follow blood and urine concentrations at frequent intervals. Under these circumstances they missed the evidence for filtration and denied its existence, though they described crude experiments which indicated that reabsorption took place. Their evidences for the secretion of glucose, urea, and sodium chloride are not satisfactory because of the failure to correlate the times of sampling blood and urine. Had their work been only a little more refined, it might well have set a pattern even for vertebrate studies of the period.

Somewhat the same criticism can be made of Bruni's (1937) experiments. This worker made injections of various dyes intramuscularly in the

octopus and followed the changes of dye concentration in the urine. Though the pattern of excretion varied from dye to dye, Bruni concluded that the dyes were being excreted by filtration; but, since he did not carry out simultaneous analyses of blood and urine, we cannot rule out the probability that secretion was occurring.

Harrison and Martin (1955) and Harrison (1954), using the dye phenol red, have shown an active secretion of this substance which, as in the gastropod, may be inhibited by DNP.

Finally, for indirect proof of the secretion of inorganic materials claimed by Mayer and Rathery, we may cite the analyses of Robertson (1953) on blood and urine of the octopus from which he concludes: "The mechanism of ionic regulation of the plasma in cephalopods involves the continuous selective excretion of ions in the excretory fluids and controlled uptake of ions by the permeable surfaces."

From the experiments which have been described on molluscs it should be clear that substances which are actively transported and which require no chemical changes in their transport may be very advantageously studied in this phylum. Much work remains to be done to demonstrate what materials are transported and how their rates vary both independently and in competition with each other. It is to be hoped that such systems may be studied not only for the knowledge as it applies to this phylum, but that some of these systems may be employed in the larger task of demonstrating the specific cellular mechanisms of transport.

Arthropods

If we examine once again the paper of Maluf (1941b) on urine formation in the crayfish we find satisfactory evidence of the secretion of cyanol, a dye which he reports is not handled by the vertebrate kidney. Though his work was not done on a strictly quantitative basis, it is clear from the observation that the dye was many times as concentrated in the urine as in the blood that secretion was occurring. The study included a dye which is handled by the vertebrate kidney and the secretion of this dye, phenol red, was judged to be at about one-half the rate of cyanol. Neutral red appeared in the urine but did not appear to be concentrated there. These data confirm secretion but do not answer the profound question: Are all the components of crayfish urine secreted?

One of the crucial points on which a decision would rest would certainly be the secretion of water. In a somewhat earlier paper Maluf (1941a) reported cytological evidence in *Cambarus* for the secretion of water. It had been established already at this time that the urine is hypotonic to the blood. Of the alternative explanations Maluf preferred that in which all the constituents of the urine are secreted, the water being secreted in

largest amount. In the distal coil of the crayfish kidney Maluf described relatively large columnar cells with distinct mitochondriona and large clear apical granules which bulge into the lumen of the tubule. The evidence that water is moving outward is that, when the animal is placed in a medium of greater salinity, the rate of urinary flow falls and the osmotic pressure of the urine simultaneously rises. But the constancy in body weight indicated that the total volume of water in the animal remained about the same up to 272 mM NaCl per liter, an amount of salt which gave a fluid initially hypertonic to the blood. Maluf argued that this indicated that the decrease in urinary flow, with rising external salinity, was not due to a decrease in hemocoelic pressure which, assuming that filtration does occur, might cause a decrease in the rate of filtration. In his view, with the rate of urinary secretion depressed, the apical vacuoles of the tubule should disappear. Maluf showed that the vacuoles do indeed become markedly reduced in number after even 24 hours of this saline treatment, and often disappeared entirely after a few days of the treatment.

But Peters, who had demonstrated the hypotonicity of the urine to the blood in 1935, had interpreted his data to indicate that filtration occurs in this animal followed by reabsorption of salts. The apical vacuoles were taken by Peters to indicate an active absorption of salt from lumen to blood. We do not have at hand the critical evidence for a choice between these two views. There appear to the writer no good reasons for rejecting Peters' views, since it would seem that water might well accompany the reabsorption of salts, as it does in the vertebrates, for example. If less salt is reabsorbed, less water would be taken up and so the disappearance of the vacuoles may be interpreted as equally favorable to either point of view.

Burger (1955a) has carried out studies of the time course of excretion of substances injected into *Homarus*, including phenol red, bromsulfalein, and para-amino hippuric acid (PAH). Both PAH and phenol red were concentrated by the kidney, with the U/B ratios falling towards one as the blood concentration was increased. It was interesting that secretion into the gut appeared to be even more active than that into the urine; but, since the dye was then reabsorbed, this route played little active part in the excretion. The observation indicates simply that a transport mechanism useful in some other way to the animal is active in the hepatopancreas and perhaps epithelial cells of the gut.

Although insects are specifically excluded from this review, an observation made by Ramsay (1954) on the secretion of phenol red by the Malpighian tubule of the stick insect should be mentioned for its general interest. Since secretion appears to be the chief means of excretion in the insects, it is no surprise that phenol red should be secreted. What is sur-

prising is the observation that phenol-red secretion was stopped by a modest back pressure which simultaneously stopped the secretion of water. In view of the well-known capacity of secretory cells to secrete against high gradients and high pressure, this is a very interesting observation.

Annelids

Evidences for filtration and reabsorption in certain annelids have been described from the work of Bahl and Ramsay. The difficulties of quantitative blood and urine collection, and the relative certainty that secretory mechanisms exist, have so far prevented analyses of rates of dye excretion. Cordier (1934) published an extensive cytological study on the athrocytic excretion of various dyes injected into the coelomic cavity of the earthworm. His studies showed that a great deal of activity was involved in the processes, but no measurements of rate could be made. It is to be hoped that search will reveal forms suitable for quantitative work and that the knowledge of the excretory capacity of the annelids may be brought to a par with that of the other higher invertebrates.

Summary

This review is limited to a consideration of physiological studies in which some degree of knowledge of simultaneous concentrations in blood and urine is available. For the sake of brevity, studies on insects and studies on athrocytosis have been omitted.

Reliable data have accumulated for a filtration origin of urine in the molluscan classes Cephalopoda and Gastropoda, and a clear suggestion of filtration in Pelecypoda. In each class the filtrate undergoes chemical changes as it passes through the lumen of the kidney, with the reabsorption of salts or glucose or water established for at least one class of the phylum. There is active secretion into the urine of such foreign substances as phenol red and para-amino hippuric acid.

Appropriate studies of the Arthropoda are confined almost entirely to crustaceans. Although very satisfactory evidence for filtration has been presented for some marine members of this group, the fresh-water forms pose a very particular problem because of the claim that inulin, as well as all other ingredients of the urine, is secreted by the kidneys of the crayfish. If substantiated this would represent the first case in which such an activity of kidney cells had been demonstrated and would serve as a serious criticism to many conclusions based on studies with this substance. In the marine groups filtration is obviously accompanied by an active process of reabsorption of substances important to the economy of the animal, and several dyes are known to be secreted.

With the exception of some elegant studies by Ramsay, modern methods

have been difficult to apply to worms. Studies conducted on oligochaetes lead to the conclusion that filtration occurs, perhaps under the pressure differential set up by the flame cells. Open nephridia in many species add coelomic fluid to a blood filtrate, increasing the economy of a process of reabsorption. Quantitative evidences for secretion are lacking and an extension of the current inquiries promises to be fruitful.

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SOME FEATURES OF THE PHYSIOLOGY OF THE TUNICATE HEART

B. J. Krijgsman and Nel E. Krijgsman
University of Cape Town

It is well known that the heart of tunicates is an organ which, in structure and function, can hardly be compared with the heart of other animals. In contradistinction to that of vertebrates and most molluscs it has no strong muscular wall, and, contrasted with arthropods, it has no external filaments by which diastolic extension is achieved. Perhaps the most striking feature of the tunicate heart is the remarkable reversal of beat. In other groups reversal may occur occasionally and quite irregularly (Krijgsman 1952), but in tunicates this reversal of beat is a regular phenomenon.

Many problems of the tunicate heart still await solution, but certain points have been elucidated. We know that pulsation starts at one end and proceeds along the heart as a peristaltic wave, maintained by one layer of primitive muscle cells arranged nearly circularly. In spite of the fact that the constriction does not really close the heart and thus a certain leakage must occur, this type of propulsion gives rise to a one-way circulation as long as one-way beating lasts. This has been definitely proved by v. Skramlik (1929) with injections of India ink, thereby disproving the older investigations of Enriques (1904), who thought that there is merely an oscillation of the blood.

Another point which seems to be quite clear is the existence of two intrinsic pacemakers, one at each end of the tube-shaped heart. There are plenty of arguments for this thesis. First of all one observes a series of pulsations starting at one end. After some time, which may be minutes or hours, pulsations start from the other end. This may result in a true competition, or, more usually, the active end stops. Then there is a period of rest, after which the other end starts activity. This alternation in dominance was observed as early as 1822 by Kuhl and v. Hasselt and confirmed by all later workers. The fact that normally a pulsation starts at one of the ends of the heart indicates a localization of pacemakers at those points.

Further experiments have made it quite clear that this assumption is true. Local mild stimuli, for example local heating or cooling of an end, affects the frequency and even the dominance of that end. The most convincing experiments are probably those made by v. Skramlik (1926a,b), who saw that heating of a passive end can cause it to start activity and to gain dominance. Heating of an active end increases frequency, whereas

cooling may stop it. Heating or cooling of other parts of the heart only cause a change in amplitude. These results are similar to those obtained with the vertebrate heart; that is, local change in temperature of the pacemaker affects the frequency of the beat, but changes in other parts affect only the amplitude. This has been fully confirmed in our laboratory with *Ciona* (unpublished experiments).

Further proof for the peripheral position of the pacemakers has been obtained by ligaturing or cutting the heart in the middle. Contractions then start at each end. Finally we may mention the destruction of one end, which results in waves originating from the other end only (see Krijgsman, in press).

This well-known experimental work clearly proves the presence of localized pacemakers at each end of the heart. However, other portions of the heart can also show automatic activity; for, if the peripheral pacemakers are removed, the isolated central part can take up a slow beating after some time. Even small fragments of the central parts may show rhythmic contractions.

It therefore seems clear that all parts of the tunicate heart have the power of automatism. In normal circumstances these basic diffuse automatic properties are overruled by the centers at the ends, which induce their rhythm on the whole heart by their more powerful automatism.

We cannot agree with v. Skramlik (1926a,b, 1929, 1930b, 1933, 1938, 1941), who speaks of a third center of automatism in the middle of the heart. Although in abnormal circumstances one often observes peristaltic waves originating in the central region, we are reluctant to call this a pacemaker in the true physiological sense. After all, the peripheral pacemakers always dominate under normal conditions; the so-called central center does not appear to have any functional significance.

Whereas the points thus far discussed have been well established, there are other major problems which still remain unsolved. Such problems are, for example: (1) Has the heart myogenic or neurogenic pacemakers? (2) Is there an extrinsic regulation? (3) What is the cause of reversal? (4) Is there a specialized conductive system which propagates the stimuli produced by the pacemakers?

The answer to the question whether the pacemakers are of the neurogenic or of the myogenic type must be that we do not have sufficient evidence for either of these two possibilities. First of all, histological evidence is contradictory. Hunter (1902) was quite definite in stating that each end of the heart of *Molgula* possesses a ganglion consisting of a small number of nerve cells. Unfortunately his pictures are not convincing. Millar (1952), working with *Ciona*, found similar cells, but he is convinced that they are connective-tissue cells. Other workers also deny the

existence of nerve elements in the tunicate heart. Although it may well be that technical difficulties, for example refractory behavior of possible nerve elements, are responsible for negative results, the histological data available do not convince us, at the moment, that the pacemakers have a neurogenic character.

The same uncertainty applies to electrical stimulation of the heart. Most of the experiments were made by early workers. The data obtained do not help us, for they could refer equally well to either a neurogenic or a myogenic pacemaker.

Some data are available on the application of drugs on the tunicate heart. Unfortunately these results are confusing. Bacq (1934a,b, 1935), for instance, found no influence of acetylcholine or adrenaline on the heart of *Ciona*. Ebara (1953) and Waterman (1939, 1942, 1943) have detected an influence, but their results are not exactly compatible. Results obtained by the application of other drugs add to the confusion. We feel that much more work must be done along these lines before we can draw definite conclusions. The existing confusion may have arisen partly from the fact that the different aspects of the activity of the heart, that is, frequency, length of pulsation period, and duration of pause between the pulsation periods, have not been given due consideration as separate phenomena. A depressive drug, for example, first depressing a more sensitive center, might lead to longer pulsation series at the other one, thus wrongly suggesting a stimulation of the latter. As a matter of fact, the factors concerned cannot be studied independently if one center can obscure the response of the other. One therefore should work with hearts ligatured in the middle. Length of pause and duration of pulsation series could then no longer be obscured by competition. Such studies are in progress in our laboratory and seem to indicate a myogenic nature of the pacemakers of the *Ciona* heart. However, we want to express some reserve, since these experiments are not sufficiently advanced for a definite opinion.

All in all, we are reluctant to call the pacemakers of the tunicate heart either myogenic or neurogenic. One has to wait for more definite results in the field of histology and pharmacology.

The next problem which we wish to consider is the possible existence of extracardiac regulation. We can be brief on this point. Some fifty years ago Hunter (1903) claimed that he saw nervous connections between the pacemakers of the *Molgula* heart and the central nervous system. He alone is of this opinion, for no one else has ever seen extracardiac nerves in tunicates. The fact that some workers have claimed that the destruction of the central nervous system affects the heart beat must not be given too much weight, for several investigators have shown that all kinds of damage influence heart beat. Elimination of the central nervous system does

not appear to have a specific influence and thus does not prove that extrinsic regulation exists. As far as pharmacological evidence is concerned, we are again confronted with the contradictions already mentioned in the discussion of the neurogenic or myogenic nature of the pacemakers. We therefore feel that the data available are not sufficient to postulate the presence of cholinergic and/or adrenergic extrinsic nerves.

A question which deserves our attention is the cause of reversal. As we know, the periodic reversal of the direction of pulsation is a normal phenomenon in the tunicate heart. The possible cause and purpose of this reversal have long been a matter of discussion. A number of workers have advocated the so-called "back-pressure theory" (see Krijgsman, 1956). According to them the capacity of the capillary beds is so small that a gradual congestion builds up when the heart is pumping blood in a given direction. This should cause a reversal in order to obviate the back-pressure. Arrest of the leading center by a possible back-pressure is a tenable hypothesis, but one cannot understand why such a back-pressure could stimulate the resting center and thus cause a reversal of beat. Haywood and Moon (1950), who recently revived the back-pressure theory, clearly understood this and wanted to restrict the influence of the possible back-pressure to the arrest of the leading center. Haywood and Moon treated the subject in a mathematical way. Unfortunately, they introduced many simplifications in order to make such a treatment possible. Moreover, they adjusted their arbitrary constants to fit the observations, which makes the resulting agreement between theory and experiment rather less convincing.

Other workers maintain that the cause of reversal is an inherent property of the pacemakers. The leading center apparently cannot maintain its optimal frequency because of a kind of fatigue. There are several points in favor of this hypothesis:

(1) It has been shown by Quincke and Stein (1932) that the threshold for electrical stimulation of a pacemaker increases near the end of the pulsation period. This indicates a kind of fatigue, not inhibition by increasing back-pressure.

(2) If there is no back-pressure (isolated heart), reversal still occurs fairly regularly.

(3) An isolated center or halved heart shows, in spite of the absence of back-pressure, a pattern of alternating high and low frequency or temporary rest.

(4) The fundamental assumption of the "back-pressure theory" is a lower capacity of the capillary bed than that of the large vessels. This has never been proved.

(5) If a back-pressure should be built up, one would expect a gradually increasing expansion of the larger vessels and/or a change in the rate of flow in the course of a pulsation series. This has never been observed. On the contrary, careful workers such as v. Skramlik (1941), said that the flow of blood is a regular one-way movement as long as a pulsation period lasts.

(6) The "back-pressure theory" tries to give a causal explanation, if not of the start of activity of the resting center, then at any rate of the arrest of the leading center. However, there can be no certainty at all that increasing pressure causes arrest of the leading center. On the contrary, it has been found in the hearts of molluscs, arthropods, and vertebrates that the pacemaker is facilitated by increased tension of the heart muscles. Therefore we cannot accept, without experimental evidence, the postulate that a pacemaker of the tunicate heart slows or stops beating as a result of increased pressure. On the contrary, one could expect that the leading center would go on laboring, showing more or less isometric contractions. However, the back-pressure would then be reduced very quickly because of considerable leakage, and thus there would be no reason whatever for the cessation of the activity of the center.

(7) If an increasing back-pressure were the cause of arrest, the slowing or laboring of the leading center ought to be of a gradual nature, against the gradual increase in pressure. This has never been shown; several workers have observed that only the last or a few final beats are delayed, but prior to this there is a fairly constant rhythm (see Krijgsman, 1956).

(8) All authors who advocated back-pressure as a cause of arrest have assumed that this pressure actually builds up gradually, i.e., increases and increases until the heart can no longer counteract a final "critical" pressure. However, it seems most improbable that during the course of a pulsation series there is a continual increase in the back-pressure. On the contrary, in such a system a new steady state will be reached very soon. v. Skramlik (1930a), and Ebara (1951), observed that a pulsation series in the intact animal lasts a considerable time in certain species, sometimes even more than 3 hours. It would be contradictory to physical principles to assume that a back-pressure continues to increase during such long periods. Apart from this it has been observed that a heart ligatured in the middle continues to beat (Schultze, 1901; Nicolai, 1908; v. Skramlik, 1926a; Ebara, 1954) in spite of the fact that almost immediately a constant back-pressure is built up. Apparently the leading center can beat for a considerable time against a constant back-pressure. Thus a "critical" value of that pressure does not exist.

We feel that the "back-pressure theory" is based on mere assumptions,

some of them obviously incorrect. The "fatigue theory," on the other hand, is based on some sound experimental evidence. We therefore think that the "back-pressure theory" must be definitely discarded.

Now the question arises: What is this fatigue which causes periodical arrest of a leading center?

A pacemaker is a region which shows rhythmic spontaneous activity, by which the heart is induced to contract. If the pacemaker is neurogenic, as in most anthropods, its activity consists of nerve impulses. If the pacemaker is myogenic, it can be composed of modified muscle tissue, as in homoiothermic vertebrates, which cannot contract itself but sends impulses to the contractile system. Or it contracts itself, as in poikilothermic vertebrates and molluscs, and thus induces contraction on neighboring parts.

Let us assume that the pacemakers of the tunicate heart have a myogenic nature and contract themselves. After all, this assumption is in accordance with many observations. Now what is the cause of these rhythmic contractions of a pacemaker and why does it become exhausted after some time? Some authors state or rather imply that this fatigue is an exhaustion of the contractile power, in other words a prolonged refractory period of its muscular activity. As long as a pacemaker is working at normal frequency the other one would have no chance, because it is kept refractory all the time by the oncoming waves which induce it to contract before it can start spontaneous activity. However, this cannot be correct. We may mention a few arguments which militate against this explanation. An isolated center shows periods of activity, alternated by rest. These periods of rest occur in spite of the fact that no oncoming waves from the other side induce refractoriness. Further, the pause between reversals in the intact heart is shorter than the resting period in the halved heart. This has been shown by several workers and by experiments in our laboratory (see Krijgsman, 1956). Moreover, in the halved heart the pacemaker remains passive for some time after its contractile power has certainly been restored. And, finally, we may mention the fact that at the end of a pulsation series both pacemakers are equally exhausted as far as their contractile power is concerned, one by spontaneous, the other one by induced contractions. Thus during the rest period both have the same chance to recover. Why, then, should there be a regular reversal? One would expect a competition of the pacemakers at the end of the rest period. In short, regular reversal would be an incomprehensible phenomenon if interpreted in terms of contractility. The conclusion must be drawn that contractile power and pacemaker activity are two different things.

What, then, is the particular stimulus which releases the spontaneous activity of a pacemaker of the tunicate heart? In vertebrates and molluscs

there is evidence that this release is based on chemoreception of a metabolite (Krijgsman and Divaris, 1955), but nothing is known about possible chemical agents in tunicates. However, if we accept the principle as a reasonable one, we can develop a preliminary picture of the pacemaker activity. The threshold for a possible metabolite might rise during spontaneous activity until the pacemaker no longer responds to it. This, then, would be the "fatigue." During the subsequent rest the threshold could decrease until the metabolite again acts as a stimulus. Once we accept the probability that pacemakers are some sort of chemoreceptors, this line of thought seems adequate. Various sense organs show similar types of fatigue or adaptation with subsequent restoration. Hypothetical as this explanation may be, no other comprehensible picture of the periodic reversal of beat of the tunicate heart can be offered at the moment. It can only be determined by future work.

We do not know whether there is any significance in the periodic reversal of beat. One can see no reason why a permanent one-way circulation should not be efficient. Presumably the cause of the reversal is the weakness, that is, the periodic exhaustion of the sensory mechanism of the pacemakers, which has no physiological significance.

Finally we must touch upon the possibility of the presence of a conductive system in the tunicate heart. One is inclined to think in terms of certain stimuli of a more or less general nature, directly resulting from the contracting pacemaker, spreading into the adjacent region, thus evoking contraction of that area, and so on. Such stimuli might be, for example, the production of a potential difference, the release of ions or of a certain metabolite. Since the muscle fibers are circularly or spirally arranged and closely packed, such stimuli cannot be expected to be released by the ends of the muscle fibers only. On the contrary, one might expect that the stimulating factors impregnate adjacent regions by traveling in the wall parallel to the longitudinal axis of the heart. Thus they should stimulate the muscle fibers not in a polar but in a lateral way.

Unfortunately there are certain facts which militate against the acceptability of this point of view. We shall mention some of the most pregnant arguments.

Some workers, for example Hecht (1918), v. Skramlik (1926a, 1930a), Ebara (1954), and ourselves (unpublished experiments), have found that the velocity of the contraction waves in *Ascidia*, *Ciona*, and *Polycitor* is not the same in both directions. The figures obtained are significant and quite convincing. But how can we understand these findings without postulating the existence of a special conductive pathway? If contracting fibers could stimulate neighboring fibers directly—and most likely laterally—why then should the muscle fibers respond more slowly when struck from,

say, the right than from the left? A lateral polarity is a thing quite unheard of. We must also realize that, in the case of such direct stimulation by a proceeding wave, the pacemaker which has started the contraction cannot exert any more influence when the wave is on the way, for then the wave is out of reach of the pacemaker. The only alternative seems to be the assumption of a specialized conductive system, originating in the pacemaker regions, and running along the length of the heart. Such a conductive system could possess polarity, as in the vertebrate heart.

The assumption of a conductive system is strengthened by certain other observations. One is inclined to pinpoint this system at the longitudinal suture, along which the heart is connected with the pericardium. This suture has no muscular elements and thus does not contract. As shown in our laboratory with vital staining, it is a rather broad undifferentiated strand of transparent material, which contains some scattered cells of doubtful nature. Ebara (unpublished experiments) found the cells in the suture to form a longitudinal chain. Leaving the question open whether these cells represent a conductive system, we have to face the fact that, according to Ebara, local damage of the suture with a needle causes the peristaltic wave to stop at that point. Local damage of other parts, that is, muscular parts, of the heart does not stop the propagation of the peristaltic contraction. It seems clear, therefore, that the suture has some specific function, that is, it has something to do with the conduction of pacemaker impulses.

We have pictured some of the major problems with which the tunicate heart confronts us. Many questions still have to be solved, and suggestions put forward in this paper are partly of a hypothetical nature. However, we hope we have aroused interest and stimulated further research on the subject. A detailed review will be published elsewhere (Krijgsman, 1956).

Summary

Some major problems of the physiology of the tunicate heart have been discussed. The tube-shaped heart possesses two pacemakers, one at each end. Each of these pacemakers induces alternatively a series of peristaltic pulsations. There is not sufficient evidence for either a myogenic or a neurogenic nature of the pacemakers. Extrinsic regulation seems to be absent. The reversal of beat is caused by the "weakness," i.e., temporary exhaustion of the sensory properties of the pacemakers. The possibility of the existence of a specialized conductive system is discussed.

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THE RHYTHMIC NATURE OF LIFE

FRANK A. BROWN, JR.
Northwestern University

The living organism is never in true physicochemical equilibrium. It maintains a steady state which consists of continuous variations about some specific mean. For the most part our ignorance is great, not only as to the factors which regulate the mean level, but also as to those factors which continuously return the organism to the mean from its repeated fluctuations from it. It is not surprising that in such a system rhythmicities and cycles appear more the rule than the exception. Cycles such as contractile vacuole pulsations, spontaneous neuron discharge, and heart beat are well known and their mechanisms are being gradually elucidated by physiologists. These will not be the topic of this discussion. Rather, I shall consider rhythms and cycles of much lower frequencies, those that parallel or are even induced by rhythmic factors of the external environment directly or indirectly related to the changing relations of the sun and moon.

These are the daily, tidal, monthly, and annual cycles. Rhythms of these frequencies are, as we shall see, concerned in the maintenance of the organism's steady state over days, months, and even generations. Though the dependence of the invertebrate animals on such rhythms appears to be particularly heavy, the evidence now suggests that these animals are simply utilizing a nearly universal vital property. Since our knowledge of the nature of this property is being derived from investigations of a wide variety of animals and plants, I shall feel free to refer often to organisms other than the invertebrates.

Let us first view the problem in a very general light. Great strides were obviously made in man's never-ending attempts to become the master of his environment when he invented the clock, and perfected a workable calendar. Have you ever pondered what would be the outcome were all clocks and other time-measuring devices suddenly to become nonexistent in our civilization? There would be a complete collapse of most systems of communication and transportation. Our whole complex technological era would virtually disappear overnight. Precise timing enters into the manufacturing processes of innumerable essential materials which have become part of our daily mode of living. Even colleges and universities as we know them could not exist. Without clocks and calendars the probabilities are almost nil that I would have arrived here at this time to deliver this address or that anyone would have been in the room waiting to hear

it when I did arrive. Our civilization would be abruptly set backwards by centuries; we would revert to a pastoral mode of existence. Under such circumstances the earth would be inhabited by many more people than it could support at the greatly lowered level of operating efficiency and a new population equilibrium consistent with the new mode of life would soon be reached.

It seems probable that a comparable picture could be painted for innumerable other animals and plants, as the consequence of having their means of accurately measuring time suddenly abolished. There would be, undoubtedly, extinction of numerous species, and possibly even extinction of all of them, including man. I add man, because it is highly probable that he also depends upon the type of living clock I shall discuss. At best, there would be a tremendous alteration in the character of the animal and plant populations of the earth.

For his own clocks and calendars, man has utilized, insofar as he has been able, the natural periods of his cyclic external physical world. The interval between the times of the sun reaching its highest point in the sky or zenith on two consecutive occasions is the period of the solar day. The clocks of man divide up this period into 24 hours; each of these hours is in turn divided into 60 minutes, and each minute into 60 seconds. The period required for the earth to make one revolution about the sun is the year, which is divided by the calendar into 12 months of about 30 days each and about 52 weeks of seven days. The month is that simple fraction of the year which corresponds closely to the average lunar period from one full moon to the next. This lunar period is the synodic month of about 29.5 days.

Animals and plants appear similarly to have utilized the natural cycles of their physical environment in the development of their own clocks and calendars. Their natural periods, however, are chiefly the shorter ones which are responsible for rhythmic alteration in forces in the environment which are difficult or impossible for the organism to ignore. The solar day of 24 hours is associated with the cycle of change in illumination, temperature, and humidity of the day-night cycle. There are also daily rhythms of barometric pressure, the rain of cosmic radiation, and of other factors.

Another natural period which appears to have been conspicuously used by animals and plants in the measurement of time is the period of the lunar day of 24.8 hours. The lunar day is the period elapsing between two consecutive times when the moon is at its highest point in the sky. In other words, the moon reaches its zenith about 50 minutes later each day. Compared with the sun, the influence of the moon on illumination, temperature, and humidity is minor. And its influences in producing rhythms of barometric pressure and cosmic radiation are feeble compared with those

of the sun. In the intertidal regions of the shores of our oceans, however, the influence of the moon is more than twice as great as that of the sun. The tides of the ocean are produced predominantly by the gravitational influence of the moon and hence the tidal cycles are of lunar frequency. To a lesser extent the tides are affected by the gravitational attraction of the sun. At 15-day intervals the sun's and moon's influences are additive to produce the extra high, so-called spring tides. It is generally conceded that the greater part of the evolution of all animals and plants occurred in the littoral regions of the oceans. Hence, probably for many hundreds of millions of years, ancestors of all present-day living things were subjected to the rhythmic ebbing and flowing of the tides.

Organismic adaptation is one of the most fundamental of biological principles. Living things tend to become altered structurally and functionally in any environment in such a manner that they come to demand less energy exchange with their surroundings. It is adaptive that any organisms which find some phases of the environmental cycles more favorable than others develop their own intrinsic cycles of the same frequencies. To do so enables them to use the more favorable periods more advantageously and to prepare to defend themselves against the less favorable phases with less effort. I hope to be able to show you during the remainder of this lecture (1) that living organisms have actually become adapted to their rhythmic external environment, (2) that they have developed means of measuring off solar days, lunar days, and months, and possibly even the year, and (3) that they utilize these capacities very importantly and often critically in the temporal regulation of their normal activities.

Biologists have known for a long time that, like man, most animals don't carry on their various activities randomly throughout the 24-hour day but typically have a daily behavior pattern of a characteristic sort. Some are nocturnal like the cats, bats, owls, and earthworms; others are diurnal like the sparrows and butterflies; still others feed chiefly at twilight and dawn. Animals that live along the ocean shores in the intertidal regions are known to have behavior patterns that are cyclically repeated with the ebb and flow of the tides, with each cycle averaging about 12½ hours in length. Fiddler crabs scamper to feed at the water's edge only at ebb tides. Oysters and clams actively feed when covered by water at high tide. Some intertidal animals, particularly those that live so high up on the beaches that they are submerged only by the so-called spring tides, exhibit parallel 15-day or semilunar periods of activity.

Great numbers of lower animals and plants dwelling in the seas have bimonthly or monthly lunar breeding cycles, in which all the members of the species within any given region synchronously become sexually active. This synchrony is essential to the very maintenance of the species, since

it insures that a large number of males and females reach sexual maturity and discharge their reproductive cells to produce such high concentrations of them in the ocean as to provide a high likelihood that a sperm cell will reach an egg cell. The precision of the synchrony of breeding is no better illustrated than in the instance of the Atlantic fireworm (Huntsman, 1948). For three or four evenings each month during the summer at full moon, and 55 minutes after sunset, these worms, luminescing brightly, swarm in the waters about Bermuda. About a half an hour later only occasional stragglers are left. The Palolo worm of the Pacific Ocean swarms and breeds just as dawn is about to break at the third quarter of the October and November moons (Huntsman, 1948). The seaweed *Dictyota* releases its reproductive cells at full moon and high tide (Williams, 1905; Hoyt, 1927).

An even more spectacular example of a precisely timed behavior pattern is the breeding of a small pelagic fish, the grunion, of the Pacific coast of the United States (Clark, 1925). On three or four nights when a spring tide occurs in April through June, the male and female grunion swarm in from the sea just as the tide has reached its highest point on the beaches; they are tossed by the waves onto the beaches where they quickly deposit their eggs and sperm together in the sand. Then they flip back into the water and are off to sea again. The fertilized eggs develop in the moist sand and the young fish at the next spring tide, 15 days later, when the spot is again submerged by waves, leave the nest for an open-sea existence.

Another of the very interesting ways in which a biological clock may be used by organisms is in their normal navigation. Birds use, at least to some extent, in their homing or migration, the so-called "light-compass reaction." In this, the sun is kept at a fixed angle with respect to the long axis of the body. For short trips, the sun is sufficiently fixed so that this method permits flight in a straight line. On longer trips, as in day-long flights of birds in migration, the sun gradually moves through a considerable angle. Kramer (1952) has recently found strong evidence for some clock in the starling and pigeon which constantly corrects the orientation during the day for the normal positional changes of the sun. He studied the orientation of the birds in enclosures in which an artificial sun was held constant. During the day, the general orientation of the birds systematically shifted at a rate which one would have predicted on the basis of the birds' continuously correcting for the normal rotation of the earth. Pardi and Papi (1953) have shown that a small crustacean, *Talitrus*, navigates not only by the sun, but also by the moon, in migrations to and from the water's edge. These animals, too, appear to compensate continuously for the changing positions of the sun and moon in the sky.

Beling (1929), studying the time sense of bees, found that she could

train them to come to a feeding station at the same time on successive days but could not train them to come at different times on successive days. This suggested that the bee was being trained to migrate to the feeding station at some fixed time in a daily cycle.

In the human, the ability that many have to awaken at the same time morning after morning, the possession of such diurnally rhythmic changes as those of body temperature, blood sugar, and eosinophile count, and the precision of the monthly reproductive cycle lead one to believe that even man possesses a physiological clock and calendar.

The foregoing are merely a few examples of ways in which animals are normally dependent upon an ability to determine rather precisely the correct moment to carry out some activity.

The question which now occurs to us is the following: Are these activities set off in direct response to cyclically varying factors or signals in the external physical environment, or are there quite accurate clocks and calendars in all of these organisms which are capable of causing these critical events to occur just when they do, without any external cues required? If internal clocks are involved, the organisms would be able actually to anticipate and prepare for the occurrence of the environmental cyclic events; if clocks are not involved, there can be no such anticipation.

The first experimental evidence that animals possessed some means of measuring off 24-hour periods independently of the daily changes in light and temperature came from the work of the Polish biologist, Szymanski (1918). He found that 24-hour activity patterns continued to be exhibited and synchronized with the day-night cycle even when animals were kept in constant darkness and temperature. During the succeeding twenty or thirty years, through the work of numerous investigators, it became evident that persistent daily rhythmicity was very widespread among animals and plants (Welsh, 1938; Park, 1940; Caspers, 1951), being reported for animals ranging all the way from the simple one-celled protozoans, on the one hand, to the mammals including man, on the other. Among the plants, examples ranged from bacteria to flowering plants.

My own interest in the time-measuring mechanism responsible for these persistent daily cycles was aroused some years ago because of the obvious implication that such a clock, if there is one, must be relatively independent of temperature. Investigators appeared always to find the cycles to be rather precisely 24 hours in length, irrespective of the temperature at which the animals were kept. Furthermore, it was easily reasoned that, if a clock within the organism was really to be of any use, it must have the frequency of its daily cycles independent of temperature over the range to which the animals were normally subjected, in order to permit them to adapt their activities to the external daily cycle. And yet, in terms of con-

ventional physiology, this seemed quite unlikely. All of the known physiological processes (Belehradek, 1935), e.g., rate of embryonic development, rate of heart beat, metabolic rate, rate of muscle contraction, and even the conscious estimation of time in man (Hoagland, 1933) are accelerated about two to three times for each 10-degree C rise in temperature. If a metabolic clock were present, could it actually have this most unique property of temperature independence of the frequency of the daily cycles it regulated?

Starting with the temperature question, I commenced a series of studies in which I was assisted by a number of my present and former graduate students. Among those who have worked with me upon this problem are H. M. Webb of Goucher College, M. I. Sandeen of Duke University, M. Fingerman of Tulane University, G. C. Stephens of the University of Minnesota, M. F. Bennett of Sweet Briar College, M. N. Hines of Wooster College, W. J. Brett of Millsaps College, and R. O. Freeland, C. L. Ralph, Miss J. Shriner, and R. A. Brown at Northwestern University.

Initially for this study we wanted an animal which was cold-blooded, i.e., one whose body temperature varied with that of its surroundings, and which had a definite and easily measured daily behavior pattern. The common fiddler crab, which possesses a very striking daily variation in body color, appeared to be an ideal animal for the work. It becomes pale in the early evening as a result of movement of black pigment in each of the numerous highly branched pigment cells of its skin to the centers of the cells. At about daybreak the animals grow dark in color as the pigment commences to disperse into the branches of the pigment cells. They remain dark throughout the day. A quantitative method was developed for determining the exact stage of dispersion of the pigment at any given time of day. By placing a large group of animals in a photographic darkroom at constant temperature and then sampling a group from time to time, it was possible to follow the persisting daily variations in the pigment cells. The rhythmic change was studied continuously for about two months. During this time the rhythm not only did not weaken, but rather became stronger and stronger for about two weeks, and then continued unabated for the remainder of the time.

In the two months of observation, there was no measurable tendency for the rhythm to get out of phase with the outside day-night rhythm; in other words, the clock could not have gained or lost more than a few minutes in the two-month period. This rhythm continued to remain synchronized with the outside day-night cycles, whether the animals were in a darkroom at 26° C, 16° C, or even 6°C (Brown and Webb, 1948). If, however, crabs which were in a photographic darkroom were left six hours in sea water chilled to within a degree or two of freezing, and then

again warmed to room temperature, a daily rhythm was still evident, but now it was retarded in each phase by about a quarter cycle. In other words, the clock appeared to have been stopped or greatly slowed at the very low temperature, but when the temperature rose again, it resumed its normal 24-hour cycles but was now about 6 hours slow. The rhythmic change then continued indefinitely in its reset time with no tendency to return to the original time. By such low-temperature treatment, it was possible to get the cycle of the animals any desired degree out of phase with the normal one, and thereafter it was as stable in the new state as it had been in the original.

The clock can also be reset by illumination changes at sensitive times in the daily cycle (Brown and Webb, 1949). For example, fiddler crabs which have been kept in a very bright continuous illumination for about ten days stop changing color and remain continuously black. It can be shown that these animals possess a daily rhythm of sensitivity to stimuli which can reset the clock. If these rhythm-inhibited animals are placed in a darkroom either at 12:00 noon or 6:00 p.m., a normal rhythm of color change commences at once. If, however, they are placed in the darkroom at 6:00 a.m., the rhythm which now reappears in darkness is set *forwards* by about a quarter cycle, or about 6 hours.

If animals having a normal rhythm in darkness are illuminated on three consecutive days from midnight to 6:00 a.m., the phases of the cycles are set *backwards* by about a quarter cycle (Webb, 1950). If these same animals are exposed to illumination from 6:00 p.m. to midnight for three consecutive days, their cycles are set backwards still another quarter cycle, and the cycles are then inverted. Before the first shift in the cycle, illumination from 6:00 p.m. to midnight would have produced by itself no persistent shift.

One can also invert the phases of the rhythm by a few periods of illumination by night and darkness by day. The number of cycles of light change necessary to produce the inversion becomes fewer, the brighter the illumination.

It is impossible to impress upon the crabs any cycle other than the normal 24-hour cycle. Exposure to many 32-hour "days" of alternating light and dark finally produced a 96-hour cycle of change, the smallest common denominator of the imposed 32-hour and the normal 24-hour cycles. But just as soon as the crabs were returned to constant darkness they reverted to normal 24-hour cycles.

During this study of the persistent daily rhythm of color change in the fiddler crab, it became evident that the degree of darkening during the daytime varied from day to day (Brown, Fingerman, Sandeen, and Webb, 1953). Sometimes the time of greatest darkening occurred in the morning,

on other days at noon, and on still other days it occurred in the afternoon. Occasionally the crabs became their darkest both early in the morning and early in the evening. A study of the change in the time of day of greatest darkening showed that this occurred about 50 minutes later each day. Furthermore, when they were very dark about 7:00 in the evening, they were also very dark about 7:00 in the morning.

It is common knowledge, of course, that high and low tides in any given locality occur about 50 minutes later each day and that two low or two high tides on any given day are about 12½ hours apart. It was soon found that crabs in the photographic darkroom became their darkest at the times of low tide on the beach where they had been collected. When low tide occurred about 7:00 p.m. on any given day, there was also a low tide occurring about 7:00 a.m. In other words, when the crabs on their home beaches were actively foraging in the bright light at the water's edge with their bodies protected from both the sunlight and their predators by becoming their blackest, their captured relatives that had been in a laboratory darkroom for even as long as a month were also becoming *their* darkest.

One hypothesis accounting for the preceding results is that the crabs possess metabolic rhythms of two frequencies, one with 24-hour cycles and the other with 12.4-hour cycles. Rhythms of these two frequencies would be expected to coincide in phase about every 15 days. It was soon discovered that the crabs were, in parallel fashion, their darkest in the photographic darkroom at 9 o'clock every 15 days. This indicated quite emphatically that the frequency of the tidal or lunar rhythm of the crabs was quite precisely that of the tidal or lunar cycles of the external environment. The crabs in the darkroom therefore possessed a clock for measuring periods of semilunar length. A few simple experiments sufficed to show that the crabs' ability to measure off periods of lunar frequencies was independent of temperature fluctuations (Brown, Webb, Bennett, and Sandeen, 1954). To be of service to the crabs in their normal living, this was obviously necessary. If *our* clocks, for example, were to double their rates when the temperature rose 10° C, and halve their rates when it fell 10° C, they would be useless, indeed.

Are the tidal clocks of the crabs set by the tides on the beaches, or by the moon itself? To answer this we collected crabs from two different beaches a few miles apart; low tide on the Martha's Vineyard beach regularly occurred 4 hours later in the day than on the Woods Hole beach. The two groups of crabs, side by side in the same darkroom, each continued to signal the time of low tide on their own home beaches. Just as it had been possible to reset the 24-hour daily cycles by shifting the illumination periods, it turned out to be possible similarly to reset the 12.4-hour tidal cycles. The crabs behaved quite as if they normally used their daily cycle as a clock to

implement inherent tide tables. When their daily clock was set 5 hours slow, they signaled a low tide 5 hours late.

The clocks are inherited. This was clearly shown a number of years ago by Bünning (1935), and recently confirmed by Brett (1953) in our laboratory and by Pittendrigh (1954) using the fruit fly, *Drosophila,* which has been responsible for so much of our modern knowledge of heredity. The eggs of the fruit fly develop in one day into minute wormlike maggots or larvae which feed and grow for about 5 days. They then become inactive, secrete a pupal case, and lie dormant for about 4 days. The adult flies then emerge from the pupal cases in greatest numbers just after daybreak. Even when the larvae or pupae are placed in a photographic darkroom the great majority still emerge just after daybreak. Brett found, however, that, when eggs are laid and complete development occurs in darkness, the adult flies emerge randomly at all hours of the day. But one flash of light, as brief as a minute, given to the larvae or pupae is sufficient to cause the flies, days later, to emerge at the same time of day as the time of the light flash. It would appear, therefore, that the fly larvae had inherent 24-hour clocks but that the clocks were set for various times of day. The flash of light permitted all of the clocks in the population to be set to the same time, the flies treating the light flash as they would a dawn. The single flash of light obviously could not provide any 24-hour clue.

All this work on the crabs and flies required around-the-clock observations with several investigators spelling one another. In the tidal-rhythm study, about a quarter of a million separate observations were made and analyzed. To reduce the tedium we sought a method for continuous and automatic recording of rhythms other than those of color change. We had good reason to suspect that the same hormones that were responsible for the color changes in the crabs were important in regulating the rate of metabolism of the crabs. And, since in nature the animals tend to rest quietly at high tide and run about actively at low tide, a tidal rhythm of O_2-consumption might reasonably be expected.

A very simple, automatic, continuously recording respirometer was invented (Brown, 1954a). It consisted of a stoppered glass flask containing absorbents for carbon dioxide and other wastes eliminated by the crabs. Through the stopper passed a fine hypodermic needle leading into an oxygen-filled collapsible plastic sack. A crab was placed in each flask. The whole was then suspended into a bath, where it acted as a diver. The decrease in buoyancy of the diver as the oxygen was used was measured by a delicate spring scale which recorded continuously on a slowly moving strip of paper. Obviously, as fast as the crab used up the oxygen in the flask, fresh oxygen flowed down from the sack to replace it, and the diver became correspondingly heavier. For every cubic centimeter of oxygen

used by the crab, the diver increased in weight by one gram. Such a respirometer as this is very sensitive to barometric pressure and appropriate corrections had to be made for the pressure changes.

When the rate of oxygen consumption by fidder crabs was followed continuously for several months, there seemed superficially to be only random fluctuations, though large ones, from hour to hour and day to day. It was soon noted, however, that, whenever the barometric pressure was rising, the rate of oxygen consumption was reduced in proportion to the rate of rise; and, when the barometric pressure was falling, it was increased in proportion to the rate of fall. It has been known for some time that there are unequivocal daily and lunar rhythms of barometric pressure. Although the hour by hour and day by day changes tend to show a large random component, the barometric pressure tends to fall shortly after midnight, rise rapidly in the late morning, fall rapidly in the afternoon, and rise rapidly again in the early evening. The form of the daily rhythm becomes quite evident when one averages the values for each hour of the day for a period of two to four weeks; usually only 3 to 4 days suffice to give one the general form of the cycle. The fiddler crabs had a clear daily rhythm of O_2-consumption, with a low rate about 6:00 or 7:00 in the morning and another low rate about 6:00 or 7:00 in the evening; both are, on the average, times of rapid barometric pressure rise. In the afternoon, when the pressure tends to fall rapidly, there was the highest rate of O_2-consumption for the day in the crabs (Brown, Webb, Bennett, and Sandeen, 1955).

If now, instead of averaging values for each hour of the day for several *solar* days, a comparable average is calculated for the hourly values of 15 or 30 *lunar* days, it is apparent that the fiddler crabs also possess a lunar rhythm of O_2-consumption. In the lunar day of approximately 25 hours, a low rate in O_2-consumption preceded by 3 or 4 hours the time the moon was to be at its zenith in the sky, and another low rate occurred 3 or 4 hours before the time the moon was to be at its nadir. Here, then, was a rhythm of O_2-consumption in the crabs with cycles of tidal frequency. A rhythm of barometric pressure of lunar frequency, though existent, is of such low amplitude as to be incapable of resolution even with several months of pressure data for a temperate zone region.

The question then arose: Is this metabolic rhythmicity peculiar to fiddler crabs? Studies of the O_2-consumption of a salamander, a common seaweed, a snail, a sea cucumber, and even an Irish potato showed them all to possess similar daily and lunar rhythms of O_2-consumption and for the hourly fluctuations in rates of O_2-utilization a correlation with the rate and direction of the concurrent barometric pressure change, just as in the crabs (Brown, Freeland, and Ralph, 1955; Brown, Webb, Bennett, and Sandeen, 1955).

The common earthworm behaved differently. Ralph, working in my laboratory, found them to have both daily and lunar rhythms of O_2-consumption, but the cycles were upside down relative to those of other animals and plants. In other words, their O_2-consumption was higher when barometric pressure was *rising* and lower when it was *falling*.

Carrots also possessed daily and lunar rhythms of O_2-consumption, but they were different even in form from the others. The carrot had a lowered rate of O_2-consumption whenever the barometric pressure was changing in either direction and the amount of reduction reflected directly the rate of pressure change. The carrot, in other words, tended to show *its* low rate of metabolism not only when the potato or fiddler crab did, but also showed a low rate when the potato or fiddler crab showed a high rate.

These extensive studies of O_2-consumption clearly indicated that, even in a photographic darkroom, at unvarying temperature and humidity, the animals and plants were able to receive and show a metabolic response to some rhythmically varying external factor. The factor was obviously some general cosmic one, inasmuch as it could be readily shown that, when O_2-consumption increased in one living thing in one darkroom in our laboratory, there was, simultaneously, far more commonly than pure probability would dictate, an increase in another species in another darkroom in another part of the building, if the species was one exhibiting the same sign of correlation with barometric pressure. Whatever the forces operating, the walls and ceilings of a reinforced concrete and brick building were no barrier to them.

Although it seems quite clear that fluctuations in some external factor induce changes in oxidative metabolism of organisms, the sign of the response of the organism to this factor may differ not only with species, but even in the same species from time to time. Not only did the earthworm show in 1954 an essentially inverted relationship relative to most other organisms investigated concurrently; but the mean daily cycles of the potato, *Fucus*, and two species of fiddler crabs, obtained during spring and summer months of 1955, were nearly complete inversions of those obtained in 1954. It can be shown that the signs also change from time to time over shorter periods and that the mean daily cycle form reflects simply the predominant sign for the period of the analysis. The cause of the sign changes is completely obscure at present.

Among the possible external rhythmic factors of which one thinks at once are barometric pressure changes. The normal range of variation in barometric pressure is more than an inch of mercury. Those who have lifted a small bottle of mercury can well appreciate how heavily a 1-inch-thick blanket could lie upon one. On the other hand, the air above us serves as an indispensable absorber of much of the continuous rain of cosmic

radiation towards the earth. Life as we know it would not survive without such a protective screen. When the barometric pressure drops, the screen is thinner and less effective and more radiation reaches the earth's surface. Therefore, since there are daily and lunar rhythms of barometric pressure, due partly to the gravitational pull of the sun and the moon on the earth's atmosphere, so are there also daily and lunar rhythms in the intensity of the cosmic-ray rain. Supporting but not proving an hypothesis that these rhythms in cosmic radiation may be at least partly responsible for the rhythms observed in organisms is the fact that we have been able to show within the past year that, when the intensity of cosmic-ray rain is experimentally altered through the use of screening lead plates, fiddler crabs show a response to the change in intensity (Brown, Bennett, and Ralph, 1955). Even more support has come from recent experiments with a barostat. Under conditions of constant pressure the rhythms are still observed. In addition, very clear 27-day cycles have been found in the oyster and quahog (Brown, Bennett, Webb, and Ralph, 1956). The most regular period in the rhythmic emission of cosmic radiation from the sun is known to be a 27-day period (Bartels, 1934). Also, the physicist Simpson (1954) has very recently described 27-day cycles in cosmic radiation. One final suggestion that cosmic radiation is an operating factor is the fact that the observed, slow, multi-day drifts in rates of O_2-consumption in the several organisms studied (Brown, Webb, Bennett, and Sandeen, 1955; Brown, Freeland, and Ralph, 1955), show forms with a very suggestive close relationship with the gradual barometric pressure changes, but commonly anticipating the latter by one or two days. Barometric pressure changes are now known to be significantly influenced by cosmic radiation reaching the earth's atmosphere during the preceding day or so.

We do not yet know the nature of the external factor or factors which are producing the change in metabolism. However, since there is a correlation with barometric pressure, it is apparent that, under conditions which biologists have hitherto considered as constant ones, living things are still able to receive rhythmic signals with cycles of daily, lunar, monthly, and even annual lengths. For example, there is a clear monthly rhythm of barometric pressure. The moon is above the horizon at noon during that half of a month straddling new moon, and below the horizon at noon during that half straddling full moon. The daily rhythm of barometric pressure is different when one calculates it using daily data for a fortnight straddling full moon than when one uses a fortnight of data straddling new moon. Over a full moon the pressure at night is relatively lower than for the period over a new moon, and consequently the extent of the morning pressure rise is greater, on the average. Correspondingly, the rhythms of

metabolism in all animals and plants studied are different for the two fortnights of a month.

Whenever a biologist discovers some strikingly new phenomenon, he immedately seeks ways by which to assure himself that the phenomenon is a real one, and not the result of some error arising in the pecularities of his apparatus or methods of analysis. This is especially true when one deals with respirometers and rates of O_2-consumption.

It was gratifying, therefore, when it was discovered that by simply attaching threads to oysters and clams and measuring their tendency to open their shells and hence ventilating their gills, one could find striking daily and lunar rhythms resembling even in some detail the daily and lunar rhythms of O_2-consumption in the fiddler crabs, seaweed, salamanders, and potatoes (Brown, Bennett, Webb, and Ralph, 1956; Brown, 1954b). And when a rat was placed in an apparatus to measure its spontaneous running activity and under conditions to randomize its normal overt daily running period, the same form of daily rhythm was found again. The rat resembled the earthworm, however, in showing greatest activity in the early morning and early evening, instead of lowest at these times as in the majority of the other species studied. The rat also possessed a lunar-day cycle remarkably similar to that of the potato, and also 27-day and synodic monthly cycles.

Fiddler crabs, placed in automatic recorders of running activity, continue to exhibit at least for many days extremely conspicuous overt tidal cycles of running. It is interesting to note here that the fiddler crab has, thus, two simultaneous cycles of two frequencies, lunar and solar; one is the predominant regulator of activity and the other of color change.

It seems clear, from a consideration of the information I have described, that cycles of daily and lunar frequencies are continuously imposed upon living things from the physical world. But you will recall that the daily cycles of color change in the fiddler crab in the darkroom could be reset to any hour of the day and would then persist indefinitely. Therefore there could be no direct, simple relationship between the signals coming from the external environment and what the crab was doing at any given time. The crab must have also an internal clock of some kind. Some brief experiments have given us reason to believe that, when one resets the time of the color-change clock, the imposed O_2-consumption clock is *not* reset, but continues responding directly to the outside factors. The color-change clock appears capable of being set in any relationship to the metabolic clock. This may perhaps be compared to the factory worker who, in changing his time of work from the day to the night shift, does so without resetting his watch.

We have been able to separate to some extent the "inner" and "outer"

clocks. Maynard Johnson (1939), working a few years ago at Harvard University, showed that mice, in constant darkness, tended to run at the same time every day. But when they were kept in continuous light the clock which controlled the activity of the mice ran fast about an hour a day. The mice would run about an hour later every day. We have found that we can similarly speed up the internal clock of the rat by keeping it in continuous light, and have shown that another clock, the imposed one, is continuing to run unaltered. Both clocks regulate the running activity. An "inner" clock regulates the repeated day by day running; an "outer" clock controls the amount of running at any given hour of the day.

Two other experiments are very instructive. One of them was performed upon oysters. The oysters were collected in New Haven Harbor in Connecticut and shipped to Evanston, Illinois in a lightproof container. The container was opened in a photographic darkroom. There the oysters were found to have a daily rhythm in opening their shells and the daily rhythm, in turn, showed a monthly variation. The oysters also had a tidal rhythm of opening; for the first two weeks they opened their shells most at the time of high tide in New Haven Harbor. The form of the tidal rhythm then changed, and for each of the next two fortnights they opened their shells most at the times of lunar zenith and lunar nadir in Evanston, which occurred more than three hours later in the day than the time of high tide in their home waters. One interpretation of this change in the tidal cycles in Evanston is that the opening of the shells at the time of high tide, advantageous for their normal feeding, was a learned behavior using the lunar-day clock. The learned behavior was forgotten in a couple of weeks away from the ocean tides and the oysters reverted to a simple direct response either to barometric pressure change itself or to some other factor whose intensity varied with the pressure.

The second experiment was one designed to discover whether a fiddler crab could measure a 24-hour period by means of an internal clock even when there was no possible external indication of this period. There was only one way to provide the proper experimental conditions. A group of fiddler crabs was sealed in each of two opaque wooden tubs at Woods Hole, Massachusetts (Brown, Webb, and Bennett, 1955). One tub was left at Woods Hole, while the second was carried by airplane to Berkeley, California. On the next day, at a prearranged time, the two lots of crabs were synchronously opened in darkrooms in the two parts of the country. The crabs were studied for six days to determine the settings of their color-change clocks. The clocks of the crabs in California remained essentially synchronized with those of the Woods Hole crabs. This they had been able to do even though every conceivable factor varying normally in a 24-hour cycle as a result of the rotation of the earth had, during the day of the trip,

been stretched out to a cycle which was 27 hours and 20 minutes in length and the lunar day stretched to 28 hours and 10 minutes. The traveling crabs had ignored these long cycles of all external factors and had, nonchalantly, measured off normal 24-hour and 24.8-hour periods. There was obviously, therefore, an internal clock which in darkness possessed considerable precision.

The crabs in California showed no tendency to shift their color-change clock to California time during the six days they were watched. Using their internal clock they had apparently shifted the time relationships of the color-change clock to the metabolic clock and the new relationship was permanent. Had the crabs been permitted to experience two or three normal light cycles in California, they would undoubtedly have reset their color-change clocks to the sun time of their new longitude.

Renner (1955) has more recently demonstrated the existence of an autonomous inner clock in bees. Bees were trained in Paris, France to come to a feeding station at a particular time of day. Then they were sealed and rapidly carried to New York. There, when the container was opened in a room which was a duplicate room to the Paris training room, they continued to feed on their original Paris time.

It seems highly likely in the present state of our knowledge that both internal and the imposed daily and lunar clocks are necessary to provide animals and plants with such precisely timed overt rhythms as those observed for color-change, feeding, running activity, and reproduction. The imposed clock would clearly be fully temperature-independent and would account for the precision of the frequency of the cycles over long periods in constant darkness at various temperatures. On the other hand, the imposed clock will not produce an overt rhythm in which a specific thing is done by the animals or plant at, for example, every low tide or each morning. Variations in barometric pressure and all forces correlated with it are too randomly variable from hour to hour or day to day to assure this. Only on the average over a few days can these last be integrated into daily cycles of the order of sharpness of the day-night light changes. On the other hand, the internal clock must be a metabolic one and, therefore, probably only more or less accurate and more or less temperature-independent.

Evidence being obtained currently in our laboratory is clearly indicating that the organism is, in fact, integrating three to five days of fluctuation in some still unknown external factor whose intensity is correlated to some degree with barometric pressure, and using this product of integration in determining the form of its own metabolic cycles. Such a mechanism, the detailed operation of which is still far from known, provides the organism with an effective pacemaking stimulus to assure the long-term pre-

cision of its daily cycles and simultaneously provides them, for reasons we have explained, with an effective monthly pacemaker. In a sense, then, the internal clock not only regulates the rhythmic phenomena of the organisms but simultaneously functions as a self-correcting mechanism for retaining its mean, very precise 24-hour periodicity.

There is further suggestion from some of our analyses of fluctuations in the rate of ventilation in quahogs, that the internal clock is used in the mechanism responsible for metabolic homeostasis. There appears to be a strong tendency for the rate of metabolism at any given period of a day to be higher or lower in compensation for variations from the mean which occurred for that daily period during the preceding two or three days. The mean daily state so attained possesses a cycle form so characteristic of a population for that period that two random samples, of nine quahogs each, can in less than two weeks yield mean daily cycles exhibiting a coefficient of correlation of more than 0.9.

Summary

It seems highly probable at this time that all living things are continuously responding to some external factor or factors which have variations reflecting the positions of the sun and moon relative to the earth. The patterns of these external factors are known to show variations of several frequencies varying from the relatively short primary solar and lunar cycles, on the one hand, to the much longer 27-day, monthly, and annual cycles, on the other. At least many living things appear also to possess an internal clock or clocks capable of measuring off at least a few primary solar and lunar cycles independently of the external cycles. The internal clocks appear to be the ones concerned with the regulation of those overt rhythms in many activities which clearly repeat themselves with great precision from one day to the next or from one low tide to the next. The internal clocks may probably also have an essential role in that integration of the several cycles of the external factors which is necessary to resolve the rhythmic character of these latter. The evidence suggests that these two kinds of clocks, internal and imposed, normally work hand in hand in living animals and plants to help assure that they time their multifold activities to permit them to live more successfully in their essentially hostile, rhythmic environment.

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