STUDIES ON PERMEABILITY IN RELATION TO NERVE FUNCTION

I. AXONAL CONDUCTION AND SYNAPTIC TRANSMISSION

by

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INTRODUCTION

Cellular boundaries are endowed with the ability either to permit or to prevent the entrance and leakage of various compounds and metabolites. This makes possible the elimination of waste products and the supply of substances important for ionic equilibrium, energy requirements, and other vital functions of the cell. There are many indirect indications for the selective permeability of the membranes covering the cell. The great importance of this property for the understanding of cellular mechanisms and of the action of compounds applied externally, which includes most pharmacological effects, has long been recognized. Nevertheless, surprisingly little is known in regard to the factors which determine and affect permeability of cellular boundaries. Direct measurements are extremely difficult. The introduction of isotopes as research tool in biology, mainly due to the work of HEVESY[1] and SCHOENHEIMER AND RITTENBERG[2], has opened a new pathway to the approach of the problem, but the obstacles to be overcome are still tremendous. The lucid appraisal of the field by KROGH[3] in his Croonian lecture shows that in spite of some progress in recent years this aspect of cellular function is in its initial phase.

The permeability of the surface membranes of the nerve cell is of particular interest. Physiologists of the last century have already postulated that changes in permeability must be intimately associated with the function of the neuron, *i.e.*, with the propagation of the nerve impulse. DU BOIS-REYMOND who first established conclusively that nerve activity is associated with flow of current devoted much time to testing the possibility that the source of the electromotive force for the electrical manifestations observed may be ionic concentration gradients between the interior of the cell and its outer environment[4]. When, in the later part of the nineteenth century, physico-chemical investigations revealed the marked potential differences which may be produced by semipermeable membranes, the existence of such membranes was postulated as a basis for the electrical manifestations during the passage of the nerve impulse. OSTWALD[5] wrote in 1890: "An den halbdurchlässigen Membranen kommen weit grössere Potentialdifferenzen zustande als in gewöhnlichen Flüssigkeitsketten. Es ist vielleicht nicht zu gewagt schon hier die Vermutung auszusprechen, dass nicht nur die Ströme in Muskeln und Nerven sondern auch namentlich die rätselhaften Wirkungen der elektrischen Fische durch die hier

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erörterten Eigenschaften der halbdurchlässigen Membranen ihre Erklärung finden werden". From the discussions of DU BOIS-REYMOND, HERMANN, OSTWALD and others concerning the mechanism underlying the generation of the electric currents during nerve activity there finally emerged the membrane theory formulated by BERNSTEIN early in this century[6]. This theory forms the basis of all modern concepts of conduction and has been an extremely useful working hypothesis. Essentially the theory assumes that the nerve fibre in resting condition is surrounded by a polarized membrane, selectively permeable to potassium ions. The concentration of these ions inside the nerve fibre is high compared with that outside. There is, therefore, a tendency for the potassium ions to move to the outside, but they are kept back by the negative ion for which the membrane is impervious at rest. There thus develops a positive charge on the outside surface of the membrane and a negative charge on the inside. When a stimulus reaches the surface, a breakdown of resistance occurs; the permeability for the negative ion is increased, resulting in a depolarization. The depolarized point of the membrane is negative to the adjacent region; whereby a small electric current, the "Strömchen" of HERMANN, is generated. This current in its turn stimulates the adjacent region, leading there to a depolarization. The same process is repeated in successive parts of the nerve fibre and in this way the impulse is propagated along the axon.

Recent developments have made necessary a modification of the membrane theory in its original form. It has been shown by CURTIS AND COLE[7] and by HODGKIN AND HUXLEY[8] that during the passage of the impulse there occurs not only a depolarization but an actual reverse of the charge. This result was obtained in experiments on the giant axon of Squid by the introduction of an electrode into the interior of the axon and by direct determination of the potential across the membrane. The spike potential was found to be markedly greater than the potential difference in rest, in some cases it was nearly twice as great. There are some technical difficulties which make the exactness of the absolute values uncertain, but the fact that the charge is reversed during activity appears to be unquestionable and well established. It follows that the assumption of a simple depolarization cannot be maintained. The process responsible for the generation of the flow of current is complex and is not merely an abolition of the resting potential.

The availability of radioactive ions made possible the study of the movement of ions across the neuronal surface membrane. Such investigations were initiated during the last two years by HODGKIN AND HUXLEY[9] and KEYNES[10] in England and by ROTHENBERG in this laboratory[11]. The results will be fully discussed in the following paper. They show that sodium and potassium ions are being constantly exchanged, the latter at least to some degree between the inside of the axon and its outer environment. The ionic equilibrium is a dynamic and not a static condition. The conclusion is similar to that encountered in many other fields where radioactive or stable isotopes were used (SCHOENHEIMER[12]).

During activity the outflow of potassium and the influx of sodium are greatly increased. The data of the two laboratories are in good agreement and supplement each other. According to the Cambridge group about $2 \cdot 10^{-12}$ mole of potassium leaks per cm^2 surface per impulse; ROTHENBERG's experiments indicate that the influx of sodium is about $4 \cdot 10^{-12}$ mole per cm^2 per impulse. The question how this movement of the two species of ions in opposite direction may account for the reverse of the charge is still open. No satisfactory hypothesis has been advanced so far. It is obvious, however, that events must take place in the active membrane, the site of the electrical manifestations,

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which make this accelerated ionic flow possible, and others which restore the resting condition. Experimental evidence that such events actually take place during the passage of the impulse has been obtained by observations of COLE AND CURTIS[13] carried out with the giant axon of Squid. These investigators measured the impedance changes with alternating current of varying frequency applied across the nerve fibre. The impedance was always reduced during the passage of the impulse. Analysing their results, they concluded that the membrane resistance breaks down during activity from about 1000 ohms per square centimeter to about 40 ohms per square centimeter.

The assumption of a process in the membrane responsible for the electrical manifestations is not in contrast but in full agreement with all classical views. As was stated by KEITH LUCAS AND ADRIAN[14] more than 30 years ago, all facts indicate that the energy for the propagation of the nerve impulse cannot be derived from the stimulus itself as in the case of a sound wave. According to the English investigators the energy must be supplied locally by a "propagated disturbance". The most likely assumption as to the nature of the "propagated disturbance" is that of a series of chemical reactions producing a change of the proteins or lipoproteins of the membrane and resulting in an increased permeability. Some kind of trigger mechanism must be responsible for the change by which the ionic concentration gradient, inactive in rest, becomes effective. This concentration gradient appears to be the most probable source of the electromotive force. The change in the membrane required for this process must be, from the thermodynamic point of view, associated with an irreversible loss of energy. The reversal will require energy supply which can be conceivably derived from chemical reactions only. It is remarkable that KEITH LUCAS (*l.c.*) in logical conclusion of his views postulated that conduction must be associated with heat production, although at that time all attempts to demonstrate it had failed. In 1926, however, A. V. HILL and his associates were able to demonstrate heat production associated with nerve activity after they had developed the recording instruments to an amazingly high degree of perfection[15]. In the same year evidence was obtained by GERARD AND MEYERHOF that conduction is accompanied by extra oxygen uptake[16].

These investigations have established the experimental basis for the assumption that conduction is associated with chemical reactions. The finer mechanism, however, remained unknown. A. V. HILL's LIVERSIDGE lecture: *Chemical Wave Transmission in Nerve*, delivered in 1932, was a challenge to biochemists to approach this central problem of neurophysiology[15a]. Without a satisfactory answer as to the nature of the chemical changes generating the flow of current, no decisive progress in the understanding of the mechanism of nerve function will be achieved. The difficulty of finding this answer is easily understood if we consider the information obtained by the physical recordings. The initial heat per gram nerve per impulse in a frog sciatic nerve is of the order of magnitude of 10^{-8} gcal. The chemical reactions involved in the primary event must take place within one-tenth of a millisecond or less. Reactants in a process of such a high speed, metabolized in amounts of such a small order of magnitude, cannot be measured directly.

OTTO MEYERHOF's pioneer work on muscular contraction has shown how much information as to the mechanism of cellular function may be obtained by the study of enzymic reactions and by correlating them with events recorded with physical methods on the living cell. By the successful linking of cellular metabolism and function MEYERHOF's work opened new pathways and was perhaps still more revolutionary than in other fields.

It was under the inspiration obtained in Professor MEYERHOF's laboratory that

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the writer has tried to approach the problem of nerve metabolism in relation to function in a way similar in principle to that which had proved so satisfactory and valuable in the study of muscular contraction. It is a particular pleasure and privilege to pay tribute to Professor MEYERHOF at the occasion to which this volume is dedicated by reviewing some aspects of this work.

Role of Acetylcholine in Conduction

Since the discovery of the powerful pharmacological effects of acetylcholine by REID HUNT AND TAVEAU[17] early in this century, the compound has attracted the attention of physiologists. Observations of MAGNUS, DALE, LOEWI, CANNON and many others suggested that acetylcholine may be released from nerve endings and act as a "mediator" of nerve impulse to the effector organ. There were many difficulties and contradictions and the theory of chemical mediation encountered increasing opposition (FULTON[18], ECCLES[19]).

During the last 14 years the writer and his associates have offered evidence indicating that the theory in its original form has to be modified. Based on the approach outlined above, a great variety of facts have accumulated suggesting that the release and removal of acetylcholine are intracellular processes[20–23]. They seem to be closely associated with the alterations in the active membrane which occur during the passage of the impulse. The transmitting agent is the flow of current but in the chain of events which generate the "Strömchen" the acetylcholine-esterase system appears to play an essential role.

The important data have recently been summarized at a Symposium on the physiological role of acetylcholine[23]. A more detailed and comprehensive presentation may be found in the textbook on Hormones[24]. It may suffice to mention here briefly a few essential facts, supporting the assumption of the necessity of acetylcholine in conduction.

Studies on the enzyme which hydrolyses acetylcholine, acetylcholine-esterase, have revealed the following features: 1. The reaction occurs at an extremely high rate, the "turnover number" is 20 000 000 per minute or even higher, indicating that one molecule of ester may be hydrolysed in 3–4 millionth of a second[25] or possibly even faster (unpublished data). This high speed is pertinent for any assumption correlating a chemical reaction directly with the electrical manifestations of conduction. 2. Acetylcholine-esterase is present in all conducting tissues throughout the whole animal kingdom[26, 27]. 3. The enzyme is localized exclusively in the surface where the bioelectrical phenomena occur. This is in contrast to many other enzymes required for conduction, as for instance the respiratory enzymes[28]. 4. The concentrations of the enzyme are adequate to account for an amount of acetylcholine metabolized which is compatible with the assumption of an essential role in conduction. 5. The enzyme in conducting tissues has a number of properties by which it may be easily distinguished from other esterases occurring in the organism[26, 29]. Only in erythrocytes the same type of esterase is found. Since the physiological substrate is known to be acetylcholine, the use of the term acetylcholine-esterase for this enzyme has been recently proposed[30].

All these features of acetylcholine-esterase, however suggestive, would not yet permit the assumption of its essentiality for conduction. The enzyme activity has, however, been correlated in many ways with the electrical events of conduction. In experiments on the electric organ of *Electrophorus electricus* a direct proportionality has been established between the voltage of the action potential and the concentration of

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acetylcholine-esterase over a wide range, varying from 0.5 to 22 volts per cm[31]. No other enzyme tested shows any parallelism. The result supports the assumption of a close relation and interdependence between these electrical and chemical processes.

Using the same material, it has been shown that the energy released by the breakdown of phosphocreatine is adequate to account for the total electrical energy released by the action potential. It appears probable that phosphocreatine acts, as in muscle, only as a reserve for energy rich phosphate and that the breakdown of adenosine triphosphate (ATP) precedes that of phosphocreatine. In contrast to muscular contraction, however, it appears for many reasons unlikely that ATP may be the primary reaction associated with conduction [23, 24]. If the postulate that acetylcholine may be directly associated with conduction is correct, the hydrolysis of the ester should precede the breakdown of ATP and the energy released by the latter used for the synthesis of acetylcholine. In accordance with this postulate, an enzyme, choline acetylase, was extracted from brain which in cell free solution synthesizes acetylcholine using the energy of ATP[32, 33]. It was the first demonstration that acetylation, occurring so frequently in intermediate metabolism, requires ATP energy and, more generally, that ATP energy may be used outside the glycolytic cycle, in which its crucial role had been shown, first by MEYERHOF and his associates and later extended by the work of PARNAS, the CORIS, NEEDHAM, SZENT-GYÖRGYI and many others.

Finally it has been shown with a great variety of conducting tissues, nerve and muscle, that inactivation of acetylcholine-esterase by specific inhibitors results in an abolition of conduction[27, 34]. This effect is easily reversible with compounds which inhibit the enzyme reversibly. With DFP, an inhibitor which inactivates the enzyme irreversibly, the abolition of conduction becomes irreversible. However, the irreversible inactivation of the enzyme is a relatively slow process. Its rate depends on a great number of factors[35]. Therefore, this compound was particularly suitable for testing the essentiality of acetylcholine in conduction. A striking parallelism has been established in nerves exposed to DFP between the progressive inactivation of acetylcholine-esterase and the abolition of conduction as a function of time and temperature. In no way is it possible to dissociate conduction from acetylcholine-esterase activity[36, 37]. Claims to the contrary were shown to be due to the use of inadequate techniques. The minimum amount of enzyme required for unimpaired conduction is relatively small, about 10% of the total activity present. Considering the smallness of the initial heat, the remaining activity is, however, still adequate[38]. The excess is not unusual and is in accordance with the experience with other enzymes, but it led to some misinterpretations in the early phase of the investigations.

The view that the acetylcholine-esterase system is essential in conduction appears to be well established. The precise function of the ester is, however, unknown. It is possible that, during activity, a higher rate of collision of sodium or potassium ions with the acetylcholine-protein or lipoprotein complex leads to a release of the ester. This process may be an essential factor in the alterations of the membrane proteins leading to an increased permeability. The possibility of a rapid removal of the active ester by acetylcholine-esterase which would restore the resting condition permits such an assumption. No other process is known to have the necessary speed. An electrogenic action of the ester may be demonstrated in electric tissue, as will be discussed later. In connection with the great number of other electrical and chemical observations the hypothesis appears worthy of consideration. In this connection, the experiments reported in the following paper on

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the effect of inhibitors of acetylcholine-esterase on the ion permeability are also of interest although still far from conclusive.

It was mentioned above that the esterase in the red blood cell has the same characteristic features as the esterase in conductive tissue. There, too, the enzyme is localized exclusively in the surface membrane[39]. It is therefore of interest that GREIG AND HOLLAND[40] have described observations suggesting that inhibitors of choline ester splitting enzymes may affect the permeability of red blood cells. If this hypothesis be confirmed, it will be another support for the assumption of a similar function of acetylcholine in the neuronal surface membrane. Analogies as to the permeability of these two types of cells have long been known to physiologists.

Difference between conduction and synaptic transmission

In view of the evidence that acetylcholine has an essential function in conduction it appears necessary to reconsider the role of the ester in synaptic transmission. It is the purpose of this article to analyse the question how the earlier observations, suggesting the theory of chemical mediation, may be integrated into the picture resulting (I) from the enzyme studies and (II) from the attempt to correlate the chemical and physical events of nerve activity.

The theory of chemical mediation was based essentially on two facts: 1. the stimulating effect of acetylcholine in relatively small amounts (a few μg) upon synaptic junctions, and 2. the appearance of acetylcholine in the perfusion fluid of such foci following nerve stimulation. The complete inertness of the fibre to acetylcholine even if applied in high concentrations (up to 20 g per liter) was considered as definite proof that the physiological function of the ester is limited to the synapse.

a) Impermeability of the axonal surface membranes to acetylcholine. Studies on the permeability of the axonal surface membranes have thrown new light on this problem and have provided a satisfactory explanation for the discrepancy between the earlier observations and the conclusions necessitated by the enzymatic studies. The investigations were carried out on the giant axon of Squid. This material is unusually favourable in view of the large diameter (0.5 to 0.7 mm) of the axon. It is possible to extrude the axoplasm from the cell interior of this preparation without contamination by substances attached to the outside surface. The axoplasm thus obtained may be analysed for compounds to which the axon has been exposed for various periods of time. In this way the inside concentration of these compounds and if desired the rate of penetration may be determined.

It was found that those inhibitors of acetylcholine-esterase which alter and abolish conduction, like eserine and DFP, penetrate into the axoplasm, although the rates of penetration of the different compounds may vary considerably[37]. In striking contrast to the compounds mentioned prostigmine, an extremely potent inhibitor of acetylcholine-esterase, does not affect conduction even in high concentrations (10^{-2} M)[34]. This compound was not found in the axoplasm, although the methods used were highly sensitive and adequate to detect an extremely small fraction of the concentration of the compound present on the outside. The experiments show that the axonal surface membranes are impervious to prostigmine and, moreover, that the site of the acetylcholine-esterase associated with conduction must be inside a structural barrier which makes the enzyme inaccessible to the inhibitor. Eserine is a tertiary amine and lipid soluble, prostigmine is a quaternary ammonium salt and lipid insoluble. It appears likely that the difference

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in chemical structure and properties is responsible for the difference in permeability of these two types of compounds. Possibly the lipid membrane, known to surround all axons, whether myelinated or not, may be the structural barrier.

Acetylcholine like prostigmine is a methylated quaternary ammonium salt. The failure of acetylcholine to affect conduction was explained by the assumption that the axonal surface membrane may be impervious to the choline ester. This assumption has been tested directly in the following way. The axons were exposed to acetylcholine labelled with N^{15}. High concentrations (20 gram per liter) were used. When the axoplasm was tested for the presence of N^{15}, only insignificant traces were present. These traces, moreover, were largely accounted for by the contamination of the acetylcholine used with tertiary amine containing N^{15}. Tertiary amine labelled similarly with isotopic N penetrated rapidly and an equilibrium between the inside and outside concentration was obtained within 60 minutes[41]. Fig. 1 demonstrates the results obtained.

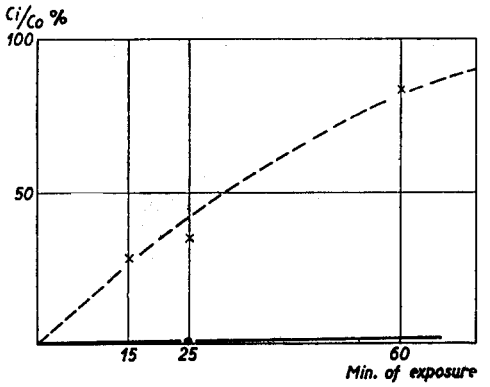

Fig. 1. Rate of penetration of trimethylamine and acetylcholine labelled with N^{15} into the interior of the giant axon of Squid. The ratio of the concentration of the N of these compounds inside (Ci) to that outside (Co) is plotted against the time of exposure in minutes. The dotted line indicates the rate of penetration of N on exposure to trimethylamine (286 μg N per ml), the straight line, that of the N found on exposure to acetylcholine (1430 μg N per ml of which 55 μg were non-quaternary N)[41].

The experiments show conclusively that the axonal surface membranes are impervious to acetylcholine. They explain why the fibre remains inert when the ester is applied externally, even in high concentrations. The fact that the action of the ester is limited to the synaptic junction indicates that the active membrane may be reached at these foci even by those compounds which do not penetrate into the interior of the axon or the muscle fibre. The peculiar ability of the synapse to react to compounds which do not affect axonal conduction appears thus to be due to a difference in anatomical structure. This applies also to curare which, as recent observations have shown (KING[42], WINTERSTEINER AND DUTCHER[43]), has as active principle a methylated quaternary ammonium salt. The observation of CLAUDE BERNARD that this compound acts exclusively on the neuromuscular junction and does not affect nerve or muscle fibres was for a century the basis underlying the assumption that the neuromuscular junction has special properties. It seemed to support the view that the fundamental mechanism of transmission may differ from that of conduction.

On the basis of the investigations described, the schematic presentation of the neuromuscular junction in Fig. 2 may serve as illustration of the situation. Only the compounds on the left side are capable of acting everywhere, because they may penetrate through the structural barriers. In contrast, the compounds on the right side act only upon the post-synaptic membrane which appears to be either less or not at all protected. The nerve ending itself, although not surrounded by myelin, appears also to be protected by a structural barrier since, according to BRONK[44], it is inexcitable even by relatively high concentrations of acetylcholine in the perfusion fluid.

Recently it was found that tetraethyl pyrophosphate (TEPP) does not affect con-

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duction[45]. TEPP is an extremely potent inhibitor of acetylcholine-esterase, much more powerful than eserine, prostigmine and DFP. TEPP inactivates the enzyme irreversibly like DFP but this effect is immediate, in contrast to the slowly progressive action of DFP[46]. Nevertheless, in a frog sciatic nerve exposed to TEPP in concentrations (2 mg per ml) several thousand times as high as those required to inactivate completely and irreversibly the enzyme in solution, conduction remains intact. This suggests that the acetylcholine-esterase retains its activity. Under the same conditions DFP which penetrates into the interior abolishes conduction and enzyme irreversibly, although it is thousand times less potent as inhibitor. The only apparent explanation for the failure of TEPP to penetrate into the axon is its insolubility in lipid. Since this property applies also to methylated quaternary ammonium salts, the assumption gains further support that the structural barrier may be a lipid membrane surrounding nerve and muscle fibre but absent at the post-synaptic membrane of synaptic junctions. But whatever the anatomical location and the chemical nature of the barrier may finally turn out to be, it is of decisive importance to recognize its existence. The barrier has not been identified morphologically but has to be postulated on the basis of the physico-chemical and enzyme studies described.

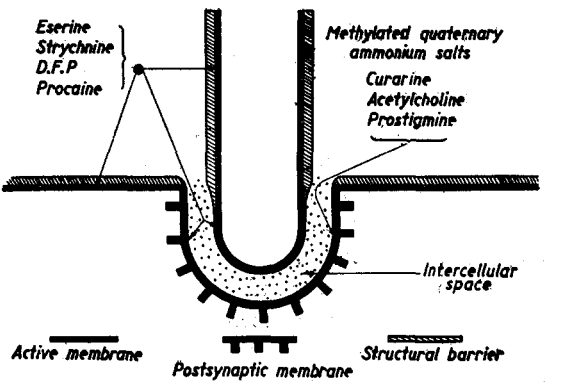

Fig. 2. Scheme of the neuromuscular junction. A structural barrier protects nerve and muscle fibre against the action of methylated quaternary ammonium salts. These compounds act only on the postsynaptic membrane, which apparently is either less or not at all protected. Other compounds, like eserine, DFP, strychnine, and procaine, being able to penetrate through the structural barrier, act upon the active membrane of nerve and muscle fibre[23].

It has been reported that intact nerves may split at least 25% or more of the acetylcholine which may be hydrolyzed during the same period by the ground nerve[47]. On the basis of this result, it was concluded that acetylcholine may penetrate into the interior. Since it has been shown that acetylcholine does not penetrate into the axon, even if applied in high concentrations, the more likely conclusion from this observation is the location of part of the enzyme outside the barrier. It has never been claimed that all the esterase present is inside and necessary for conduction. The experiments reported[47] were carried out with the manometric technique in which the CO_2 output is measured. There has recently been introduced by HESTRIN a new simple and rapid chemical method which makes possible a direct determination of the acetylcholine removed by hydrolysis[48]. This method is based upon the reaction of O-acyl groups with hydroxylamine in alkaline medium. It is more specific than the manometric method, especially when large amounts of tissue are necessary and simultaneous chemical reactions cannot be excluded. Using this method it has been found that the acetylcholine-esterase activity of the ground nerve is about twice as high as the manometric method indicates. The intact nerve splits acetylcholine at a rate which is only a small fraction (about 5 to 7%) of the total activity[45]. This activity is suppressed by prostigmine which like acetylcholine does not penetrate into the interior. Complete inhibition of this enzyme activity does not affect conduction. The meaning of the small amount of esterase on the outside

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of the barrier is not clear. The activity may be due to an unspecified esterase other than acetylcholine-esterase or to the presence of small blood vessels, microscopic muscle fibrils or cut nerve fibres where the surface may be reached by the ester. This is, however, entirely irrelevant for the major problem involved.

The elucidation of the situation became possible by the fortunate circumstance that so many different kinds of extremely potent inhibitors of acetylcholine-esterase were available: reversible and irreversible types of inhibitors and in each of the two groups compounds which penetrate and others which do not penetrate. This combination made it possible to find a satisfactory answer to some of the most pertinent questions involved: 1. the necessity of acetylcholine-esterase for conduction; 2. the existence of a barrier for methylated quaternary ammonium salts, and 3. the localization of the enzyme in respect to the barrier.

Even if a compound affects both axon and synapse, there may still be a great difference as to the concentration required. Chemical substances may act upon the apparently unprotected active surface of the post-synaptic membrane in concentrations much smaller than those necessary for affecting the nerve or muscle fibre. An interesting illustration is provided by the experiments of ROEDER and his associates[49], who found that DFP abolishes synaptic transmission in much lower concentrations than those which affect conduction. DFP is very lipid soluble and may therefore accumulate in the myeline sheath to a certain concentration before penetrating into the aqueous interior of the fibre in concentrations sufficiently high to inactivate the enzyme and, consequently, to abolish conduction. At the time when conduction disappears, the concentration of DFP is small in the axoplasm compared with that in the outside fluid[37]. This finding supports the assumption that the concentration of DFP at the site of action may be small and is consistent with the potency of the compound as inhibitor of acetylcholine. The necessity of a high outside concentration may be attributed to the relatively slow rate of penetration. In the case of eserine, the distribution between inside and outside at the same period, *i.e.*, at the time when the action potential has disappeared, is very different. The rate of penetration will be determined by the properties of the various chemical compounds on the one hand and by the properties of the various surface membranes. Additional factors may be of importance, such as the affinity of the compound to the enzyme, its potency as inhibitor and the kinetics of the inhibition. In view of the complexity of the process, it is not surprising that in applying potent inhibitors of acetylcholine-esterase, the phenomena observed may differ sharply in so many respects, although the underlying cause is the same chemical reaction.

The action of procaine, one of the compounds marked on Fig. 2, requires comment. The blocking of conduction by this and other similar anaesthetics cannot be explained in terms of acetylcholine-esterase inhibition. These compounds are weak inhibitors of acetylcholine-esterase, although other esterases may be affected more strongly[50]. THIMANN[51] has pointed out that these compounds have some resemblance in structure to acetylcholine, but are tertiary amines. They will, therefore, easily penetrate into the interior and they may act competitively with the ester on some proteins or lipoproteins of the membrane. Since apparently they do not depolarize the membrane[52], it is possible to assume that they form a complex but, in contrast to acetylcholine, they do not change the condition of the protein. However, they may prevent the action of the ester released and thereby block conduction, whereas otherwise the resting condition may remain unchanged. This is consistent with the apparent failure of cocaine,

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described in the following paper, to produce a significant change in permeability.

b) Release of acetylcholine during activity. In view of the permeability studies described, the limitation of the action of acetylcholine to the synapse, if the ester is applied externally, cannot be used as an indication for a special role at this junction, as was proposed by the theory of chemical transmission. For the same reason, the second fact on which the hypothesis was built has to be reconsidered. The appearance of acetylcholine in the perfusion fluid of the synapse following nerve stimulation must be attributed to the absence of an insulating membrane. If acetylcholine cannot pass through the structural barrier into the interior, it will not be able to leak from the inside to the outside in stimulated nerve and muscle fibres. The only site where such leakage will be possible is the postsynaptic membrane. However, even at the synaptic junction the ester does not appear under physiological conditions. DALE and his associates have repeatedly emphasized that the ester appears in their experiments only if the normal mechanism responsible for the rapid removal of the ester, *viz.*, acetylcholine-esterase, is largely inactivated by the presence of eserine. Even in presence of the drug, the amounts leaking out were extremely small, about one hundred-thousandth of that required to set up a stimulus. On the basis of more recent experiments, in which acetylcholine was applied directly to the motor end plate, the difference was of the same order of magnitude. Such a difference is not easily explained in terms of chemical mediation. It is true that in LOEWI's original observations on the frog heart, no eserine was present. However, considerable difficulties were encountered by him as well as other investigators when they tried to reproduce the appearance of the ester. For this reason, LOEWI's theory was repeatedly criticized[53, 54]. When a heart preparation has been perfused for a certain period of time with Ringer's solution, the post-synaptic membrane may not be in a completely normal condition and may therefore permit leakage of the compound, which under physiological conditions may be rapidly inactivated. The condition of the membrane may depend upon a variety of factors, such as the length of the perfusion period, the composition of the perfusion fluid, the condition and the species of the frog used, etc. Variations of these factors may explain the difficulties encountered by a number of investigators who tried to reproduce this observation. The same consideration may be applied to the finding of KIBJAKOW[55], who in 1932 described the appearance of acetylcholine in the perfusion fluid of the synaptic ganglion in absence of eserine. His observations were questioned by DALE's school, but it is conceivable that with the less perfect perfusion technique in KIBJAKOW's experiments, the active membrane suffered more damage and thus permitted the leakage of traces just in the measurable range. So far there is no conclusive evidence that the appearance of the ester outside the cell is a physiological event.

It is an interesting psychological phenomenon, encountered frequently in the progress of science as well as in the work of individual investigators, that certain observations are neglected or even discarded because they are inconvenient, puzzling and do not fit into preconceived ideas. Later, when the views have changed, the facts may suddenly gain significance and it becomes possible to integrate them into the general picture. The release of acetylcholine at the synapse assumes a new aspect if considered in connection with other pertinent observations which at the time of their presentation did not find sufficient attention.

In 1933, simultaneously with or even prior to the finding of DALE that acetylcholine appears in the perfusion fluid of the sympathetic ganglion or of the neuromuscular junc-

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tion, CALABRO[56] had shown that, following prolonged stimulation of the rabbit vagus, an acetylcholine-like substance is released from the cut end into the surrounding fluid. BINET AND MINZ[57] found, in 1934, that from the transsected surface of nerves a compound is released which increases the sensitivity of the leech muscle to acetylcholine. CALABRO's findings were confirmed and extended by BERGAMI[58] and by BABSKI AND KISLJUK[59, 60]. In 1937 VON MURALT[61] described a difference of the acetylcholine content between stimulated and unstimulated nerves. In view of the possibility of a very rapid disappearance of the active ester, he developed a special technique by which he "shot" the nerves into liquid air. Tested by bioassay after a short period of extraction the amount of acetylcholine was 0.2 μg per gram in the stimulated as compared with 0.12 μg per gram in the control nerve. In a large series of experiments the difference between stimulated and control nerve was later found to be 0.09 μg per gram[62]. However, the difference between the two nerves disappears if extraction is continued for a longer period of time. There is, therefore, some uncertainty as to the interpretation. It is conceivable that the acetylcholine released from its complex is present in a free form and therefore diffuses from the frozen tissue during extraction more rapidly than that part of the acetylcholine which is bound to protein or lipoprotein.

Even in sensory nerves release of acetylcholine has been demonstrated by BRECHT AND CORSTEN[63] from the cut end after stimulation. These investigators used a remarkably sensitive method, the contraction of the frog lung in presence of eserine, and hereby succeeded in detecting the ester released. The amounts are still smaller than those released from motor nerves, but this difference appears consistent with the smaller rate of metabolism indicated by the lower concentrations of acetylcholine-esterase and choline acetylase[27]. It is significant that the release of acetylcholine has been demonstrated in parasympathetic, motor and sensory nerve fibres. The situation is pertinent in connection with the finding that the enzymes which form and hydrolyse acetylcholine are present in all types of nerves and that the inactivation of acetylcholine-esterase invariably leads to abolition of conduction.

The facts described support the assumption that there is no difference in principle between the release of acetylcholine at the synapse and in the axon, except that in the latter case the ester cannot pass through the structural barrier. They make it appear still more probable that this release is an intercellular process and that the appearance outside the cell at the synapse must be attributed either to the poisoning of the enzymic mechanism, normally preventing the leakage or to some other damage of the active surface where it is least protected and most vulnerable. At the time when these findings were described, acetylcholine was considered to be a chemical mediator and since chemical transmission in the axon is inconceivable, it was difficult to integrate them into the general picture. Little or no attention was consequently paid to these findings. VON MURALT has been very cautious in his statements as to the possible significance of the release of acetylcholine in the nerve fibre. He called the ester an "Aktionssubstanz", meaning that it may be important like many other substances for nerve activity in the axon as well as at the synapse. This caution was well justified at a time when nothing was known about the high speed of the reaction, the effects of acetylcholine-esterase inhibitors on conduction and the great variety of other factors known today. These facts had to be established before it became possible to assume a direct association of the ester with the generation of the electric currents which propagate the impulse. In the light of recent developments, however, the situation

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has changed. The demonstration of the release of acetylcholine in the axon appears as relevant as that at the synaptic junction and requires a modification of the original interpretation.

The structural barrier for acetylcholine present in the fibre and its absence in the post-synaptic membrane may be considered as the main reason that the attention of many physiologists was focused for such a long time on the synapse only. Very little is known concerning the properties of the barrier and the factors affecting it. The observations on the permeability of neuronal surface membranes described in this and the following paper are only an initial phase in the attempt of analysing the problem. Its importance can hardly be overemphasized, not only for the understanding of the cellular mechanism but of the pharmacology and pathology of the nervous system as well. The development of new drugs may be greatly facilitated if the structural factors determining the permeability and the rate of penetration are known. In many cases an action may be desirable, preferably or exclusively, on the synapse, in others, upon both axon and synapse.

The existence of structural barriers and the great variations of their properties may account for the many obstacles encountered and the many contradictory reports when the two criteria of chemical mediation were applied to different types of synapses. The unnumerable differences of anatomical structure, the biochemical composition of the surrounding medium and many other accessory conditions must be essential in determining the action of acetylcholine when applied externally. These variations do not permit the assumption that the fundamental physico-chemical mechanism of the propagation of the nerve impulse may not be the same. In view of the physico-chemical properties of acetylcholine and similar N-methylated compounds, the difficulties will become nearly insurmountable in the study of brain and spinal chord which contain large amounts of lipid. It is not surprising that the painstaking efforts to demonstrate or to disprove the "cholinergic" nature of synapses in brain and spinal chord have resulted in a most unsatisfactory and confusing picture.

In contrast the conflicting results obtained when the "cholinergic" nature of synapses, especially in brain, is tested by the usual criteria of chemical mediation, the approach based on the study of the enzymes connected with acetylcholine metabolism and their correlation with function did not encounter comparable difficulties. All results obtained in this way indicate the generality of the role of acetylcholine in all conducting tissues, including that of brain and spinal chord[24].

c) Basic similarity between conduction and transmission. At the Symposium on the synapse, in 1939, ERLANGER[64] scrutinized the problem whether the electrical characteristics of synaptic transmission are basically different from those which may be observed on the axon. His data indicate that the electrical phenomena considered to be pecularities of the synapse may be demonstrated on fibres, *viz.*, latency, one-way transmission, repetition, temporal summation and facilitation, and transmission of the action potential across a non-conducting gap. The facts based on the electrical signs of nerve activity make it unnecessary to assume that any condition exists at the synapse which differs in principle from that found in the peripheral axon, except in quantitative respect.

Ten years have passed. During that time extensive investigations have been made on the electrical characteristics of transmission across the natural and artificial synapse (ephapse). From the work of many investigators, mainly ARVANITAKI[65, 66], BULLOCK[67], ECCLES[19], GRANIT AND SKOGLUND[68] and others considerable evidence has accumulated

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in support of ERLANGER's views that the basic mechanism of transmission and conduction is the same, the propagating agent being in both cases the flow of current. According to ECCLES[19], impulses travelling down the pre-synaptic fibre, generate a current which produces in the synaptic membrane of the post-synaptic cell an anodal focus with cathodal surround; this is followed in a second phase by a more intense cathodal focus with anodal surround. The cathodal focus sets up a local response from which a catelectrotonus spreads over the post-synaptic cell membrane. The catelectrotonus, the end plate potential, sets up a propagated impulse in the post-synaptic cell as soon as a certain threshold is reached. The sequence of events is similar to that observed on artificial synapses and on a single unit preparation of the synapse, the stellate ganglion of Squid (BULLOCK[67]). Since the electrical signs and the biochemical data favor the assumption that the mechanism of transsynaptic transmission is basically the same as that of conduction, it follows that the role of acetylcholine in these mechanisms is most likely the same. In both cases the propagating agent is the flow of current, but the release and the removal of acetylcholine must be essential events in the alteration of the pre- and post-synaptic membrane during the flow of current across the synaptic region and the generation of the end plate potential. It would be difficult to picture these currents as being different in nature from those in the axons. A few biochemical data may be mentioned in this connection which support the assumption of a high rate of acetylcholine metabolism in the post-synaptic membrane of the motor end plate. COUTEAUX AND NACHMANSOHN[69, 69a] found that, following the section of the sciatic nerve of guinea pigs, the high concentration of acetylcholine-esterase of the motor end plates of the gastroncemius decreases only slightly. Within three to four weeks after the operation one-fourth or possibly less of the enzyme concentration had disappeared. Then the activity remains constant for many months. This result suggests that three quarters of the enzyme or more is localized in the post-synaptic membrane, the "sole plate" of KÜHNE, a pure muscular element which persists after the disappearance of all nerve elements.

The electric organs have physiologically evolved from striated muscle. The electric plates are homologous with the motor end plate. The discharge of these organs is homologous with the end plate potential. Recent studies of COUTEAUX[69b] have revealed that the post-synaptic membrane of the motor end plate is morphologically a very peculiar structure. By using Janus green or methyl violet, he demonstrated a striking similarity with the electrolemma of the electric plate surrounding the nerve endings. The direct proportionality between the voltage developed during the discharge and the concentration of acetylcholine-esterase observed in the electric tissue suggests a high rate of acetylcholine metabolism associated with the end plate potential.

These findings alone without all the other evidence accumulated would not necessarily imply that the acetylcholine is released in the post-synaptic membrane itself. The following observations are, however, of interest in this connection. The discovery of the extraordinarily high concentration of acetylcholine-esterase in electric tissue made possible the assumption that acetylcholine might be the agent that produces the depolarization presumably occurring during the action potential. The possibility of a depolarizing action of acetylcholine has been considered by DUBUISSON AND MONNIER[70] and COWAN[71]. In 1938, when the prerequisite for such a theory, namely the high speed of destruction of the active agent appeared established, AUGER AND FESSARD tested the effect of eserine on the discharge of the electric tissue of *Torpedo marmorata*[72]. As may be seen in Fig. 3

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the height of the potential is markedly depressed in presence of eserine. The duration of the descending phase is considerably prolonged. This effect of eserine on the end plate potential is consistent with the assumption that the appearance and the removal of acetylcholine within the post-synaptic membrane may be essential for the generation of the potential.

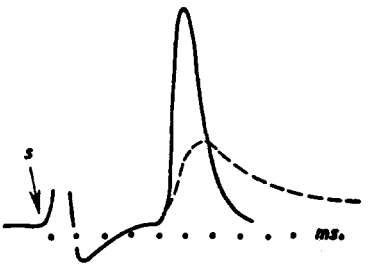

Fig. 3. Effect of eserine on the discharge of electric tissue of *Torpedo marmorata*. The fully drawn line shows the discharge in absence, the dotted line in presence of eserine[7].

In view of their corresponding biochemical and bioelectrical findings, FESSARD AND NACHMANSOHN decided then to test whether acetylcholine injected into the electric organ may produce an action potential. Such an electrogenic effect might be expected if acetylcholine is the compound which is responsible for the alterations of the membrane, occurring during the discharge. In experiments carried out at Arcachon in 1939 on *Torpedo marmorata*, in which they were joined by FELDBERG, they were able to demonstrate that acetylcholine has an electrogenic effect[73, 74]. The arterial injection of acetylcholine caused potential changes similar to the natural discharge. However, the changes were small and slow and very large amounts were necessary for the effect. Fig. 4 illustrates the effects of acetylcholine injected in amounts varying between 5 and 200 μg. 5 μg had no effect. With 200 μg the potential difference was about 0.7 millivolts and the descending phase had not yet reached the base line after several seconds. If the acetylcholine is injected in presence of eserine, preventing a too rapid destruction of the ester, the effects are greatly enhanced. Fig. 5 shows that under these conditions an effect may be obtained even with 2.5 μg of acetylcholine. With 10 μg the potential change produced is greater than 3 millivolts, although the duration is still about 3 seconds.

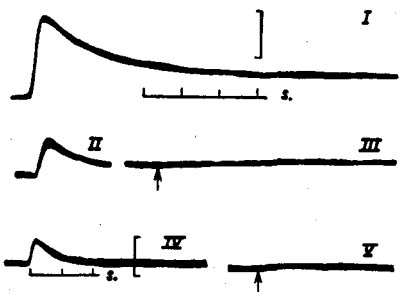

Fig. 4. Potential changes produced by intraarterial injection of acetylcholine into the electric organ of *Torpedo marmorata*. I, II, IV and V correspond to the injection of 200, 100, 20 and 5 μg of the ester; whereas at III only perfusion fluid was injected. Between II and III the sensitivity has been increased fourfold. 0.5 millivolt indicated at I, 0.1 millivolt at IV. Time in seconds.

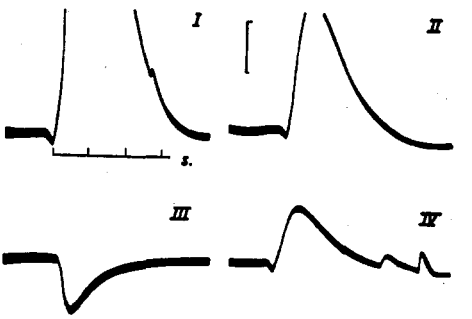

Fig. 5. Potential changes produced in the same way as in Fig. 4 but in presence of eserine. I, II and IV correspond to the injection of 10, 5 and 2.5 μg of acetylcholine; at III only perfusion fluid was injected. 0.5 millivolt indicated at II. Time in seconds.

The experiments show that the ester may produce an alteration of the membrane preceding the flow of current. They support the view that the ester plays an essential role in the generation of the current and make it difficult to assume that the release of

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acetylcholine may occur in the recovery period. In that case it would be hard to understand how the compound produces current. Although the potential changes resemble the normal discharge, there is, however, a most striking contrast in two respects: the smallness of the voltage and the 1000 fold increase of the duration. The normal discharge occurs in 2 to 3 milliseconds; the voltage of a single unit is about 100 millivolts. Although a quantitative evaluation is impossible since the number of units in series reached by the intraarterial injection is uncertain, the discrepancy as to duration and strength is enormous, even in presence of eserine. The method used is crude compared to the effect which might be expected if the compound were released from the nerve ending. In that case it would reach the opposite surface much faster, but in view of the relatively large amounts injected, of which apparently at least a fraction reaches the active membrane, the response is small beyond all proportion. It thus becomes difficult to conceive that physiologically the substance is released from the nerve ending and, penetrating the intercellular space, produces the end plate potential. This difficulty does not arise if it be assumed that the release and the removal of the ester are intracellular events which do not involve any diffusion but occur in the post-synaptic membrane and generate there the flow of current.

If locally supplied energy is necessary for the small electric currents which propagate the impulse along the axon as postulated by KEITH, LUCAS, and ADRIAN, it appears almost certain that such energy will be required for the generation of a potential in the second unit. The flow of current reaching the post-synaptic membrane may result in a release of acetylcholine which may act as a trigger in the chain of events and supply the energy for building up the end plate potential. It is remarkable that exactly this mode of action has been proposed by LAPICQUE[75] in 1936—"l'état d'excitation suscité dans la sole nucléée peut y déclencher une réaction auxiliaire venant fournir le supplément de puissance requise. Tel serait le rôle de l'acétylcholine; c'est exactement le rôle que joue l'amorce dans la technique des explosifs . . . La production de l'acétylcholine serait, dans cette conception, située, non entre le nerf et le muscle, mais dans le muscle lui-même, auquel appartient sans conteste la sole nucléée. Il s'agirait donc strictement parlant, non d'un intermédiaire dans la transmission de l'excitation entre nerf et muscle, mais d'un premier stade, formant relais dans l'excitation musculaire pour assurer sa généralisation à toute la masse du myone".

The electrogenic effect of acetylcholine injected into the electric tissue is another illustration of the fact that the post-synaptic membrane is not protected against the ester. It is interesting that the effect of curare on electric tissue was a controversial issue for a long time. Recently, however, AUGER AND FESSARD[72] have shown that the effect of curare is regularly reproducible if the permeability factor is taken into account and the drug is applied in adequate form.

Curare, being a methylated quaternary ammonium salt, may act upon the protein of the active membrane as a competitor of acetylcholine. The effect persists since the compound cannot be hydrolyzed but must be removed by diffusion. If the rapid removal of acetylcholine is inhibited by eserine, the result is strikingly similar to that obtained with partial curarization of the end plate, as the experiments of AUGER AND FESSARD have shown. The depression and prolongation of the potential in Fig. 3 must obviously be attributed to the persistence of acetylcholine and with still higher concentrations of eserine a complete "curarization" will be obtained.

As pointed out by ERLANGER, conduction along the axon and transmission across

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synapses may vary as to quantitative aspects. This is not surprising in view of the discontinuity and other structural differences. Although the time relations are similar, there is a synaptic delay of the order of a millisecond. This may be the result of several factors, as *e.g.*, the decreased diameter of the nerve fibre near the ending which may lead to a decreased rate of conduction. Exact measurements of these various factors are difficult, due to obvious technical reasons. However, the quantitative differences between intracellular and transsynaptic propagation are well in the expected range, and none of them requires the assumption of a fundamentally different mechanism.

In conclusion, no convincing evidence exists supporting the idea that acetylcholine assumes a function at the synapse entirely different from that in the axon, *i.e.* is released from the nerve ending, penetrates the intercellular space and acts on the post-synaptic membrane, thus substituting the flow of current as a "chemical mediator". A fundamental rule of scientific thinking requires that one should not assume two different principles without necessity. WORK AND WORK[76] have recently quoted the excellent formulation of this rule by DAVID HUME in his *Treatise of Human Nature*: "To invent without scruple a new principle to every new phenomenon, instead of adapting it to the old; to overload our hypothesis with a variety of this kind, are certain proofs, that none of these principles is the just one, and that we only desire, by a number of falsehoods, to cover our ignorance of the truth". Neither the so-called "electrical" nor the "chemical" concept of synaptic transmission is satisfactory. The interpretation proposed harmonizes both concepts by integrating the progress achieved concerning the structure, the biochemical data and the electrical signs of activity.

The earlier observations on acetylcholine deserve credit for having drawn the attention of physiologists to this compound in connection with nerve activity. However whereas, the ester was first associated with one type of nerve endings, then with a few others, the study of its role by the combination of chemical and physical methods has shown its essentiality in the conduction of nerve and muscle impulses throughout the animal kingdom. The type of approach applied by OTTO MEYERHOF to studying muscular contraction has proved valuable in obtaining a better understanding of fundamental principles underlying the mechanism of another cellular function vital for life.

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