CVIII. THE MECHANISM OF THE BIOLOGICAL SYNTHESIS OF ACETYLCHOLINE

I. THE ISOLATION OF ACETYLCHOLINE PRODUCED BY BRAIN TISSUE *IN VITRO*[1]

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As a result of our work on choline-esterase, the enzyme responsible for the destruction of acetylcholine, we were led, some 2 years ago, to commence an investigation which had as its objective the elucidation of the mechanism whereby acetylcholine is synthesized in the animal organism. This objective has not yet been achieved. In view, however, of the recent publication of Quastel *et al.* [1936] on the same theme, it appears to us desirable to publish the results which we have already obtained.

Possible mechanisms of synthesis

In attacking the problem in question we made the provisional assumption, in support of which there exists a considerable body of evidence in the literature, that the substance liberated at the terminations of cholinergic nerves and responsible for the transmission of impulses from these nerves to the effector organs is actually acetylcholine, and that its formation occurs in the nervous tissue. Three different synthetic mechanisms of two fundamentally different types appeared to be possible. In the first place, it was conceivable that the acetylcholine was produced from choline and acetic acid by esterification under the influence of choline-esterase. This enzyme is known to exist in nervous tissue [Stedman & Stedman, 1935, 1] and there is some evidence that it is able to exercise a synthetic action. Thus, Ammon & Kwiatkowski [1934], in confirmation of Abderhalden & Paffrath [1926], have shown that when certain biological material containing choline-esterase is incubated with sodium acetate and choline chloride, a small amount of acetylcholine is formed. The concentrations of choline and acetic acid required under these conditions for the formation of even physiologically appreciable amounts of acetylcholine are, however, necessarily so high as to place the process outside the range of physiological possibilities. Unless the esterification can be conceived of as occurring in a non-aqueous medium, an explanation of the synthesis of acetylcholine by this mechanism is, in our view, out of the question.

The second type of mechanism considered was one in which choline, or some derivative of choline, interacted with a normal metabolite to yield acetylcholine. Two such metabolites, namely pyruvic and acetoacetic acids, appear to be theoretically capable of thus participating in the synthesis of acetyl-

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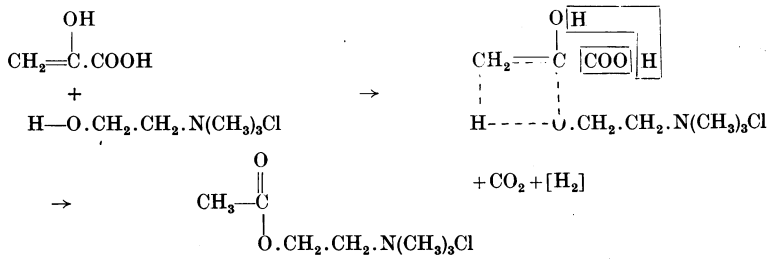
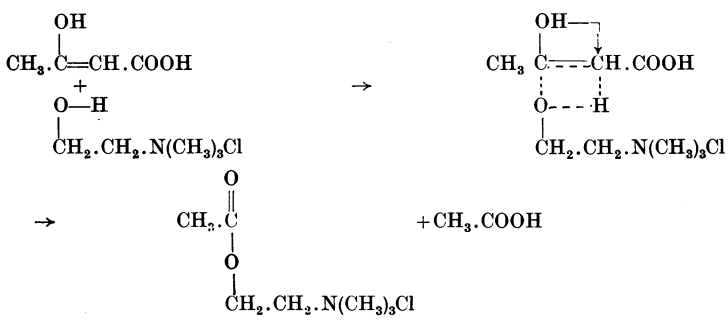
choline. In the case of pyruvic acid, it is conceivable that the reaction might take the following course:



This involves the formation of a loose additive compound between choline and pyruvic acid in its enolic form, which then undergoes oxidation and decarboxylation with the production of acetylcholine. The hypothetical mechanism in the case of acetoacetic acid is even simpler. With this possible precursor, it is only necessary to postulate the similar formation of an additive compound between the enolic form of the acid and choline, when acetylcholine would be readily formed provided that the hydrogen of the enolic hydroxyl group migrated to the position which it occupies in the ketonic form of the molecule. This is illustrated in the following scheme:



Synthesis of acetylcholine by brain tissue in vitro

It was assumed that such processes, should they actually occur, would take place under the influence of one or more enzymes, and it was therefore necessary, in order to test the various hypotheses experimentally, to obtain a preparation in which the synthesis of acetylcholine could be demonstrated *in vitro*. Brain tissue was first examined, and it was, in fact, found that when minced brain from the cat, guinea-pig or ox, the only species which we have so far examined, was incubated at 37° in the presence of eserine, the addition of this substance being necessary in order to inhibit the action of choline-esterase, a substance was formed which, judged by its action on the eserinized back muscle of the leech, closely resembled acetylcholine in its properties. The amount produced was small, but could be considerably increased by grinding the minced tissue, before incubation, with chloroform. This is illustrated by an experiment in which an ox brain, obtained from the slaughter-house, was minced and thoroughly ground in a mortar to render the material as uniform as possible. It was then divided into portions of 20 g., of which three were ground respectively with 5 ml. of chloroform containing 5 mg. of eserine, 5 ml. of water containing 5 mg. of eserine sulphate, and 5 ml. of water containing 5 mg. of eserine sulphate,

followed, in this case, by the addition of a few drops of chloroform. After incubating this material for 4 hr. at 37°, each sample was stirred up with 100 ml. of alcohol. After standing overnight, it was filtered, the solid residue being washed with alcohol, and the filtrate and washings were evaporated to dryness *in vacuo* at a low temperature. The residue from this process was now thoroughly ground up with sufficient water to bring the volume to exactly 50 ml., filtered, and the filtrate tested on the eserinized leech preparation. The results obtained are shown in Fig. 1, from which it is clear that considerably more acetylcholine

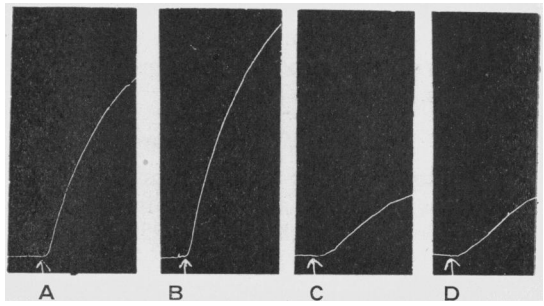


Fig. 1. A shows response of leech muscle to acetylcholine bromide 2×10^{-8}; B to 0·05 ml. of extract from chloroform preparation; C to 0·05 ml. of extract from water preparation; and D to 0·05 ml. of extract from water + chloroform preparation.

was produced in the tissue which was ground with chloroform than in that ground with water. It is further evident that the addition of a few drops of chloroform to material, which had been previously ground with water, has no significant influence on the amount of acetylcholine produced. Further investigations therefore were always carried out with the chloroform preparation, our usual procedure being to grind a minced ox brain (about 200 g.) with 50 ml. of chloroform containing 50 mg. of eserine. Weighed portions of the mixture were then employed for various experiments. It should, perhaps, be stated here that the subsequent addition of water or aqueous solutions to such chloroform preparations does not entirely abolish the effect of the chloroform, although the yields of acetylcholine are somewhat diminished.

That the acetylcholine is actually produced during incubation and is not preformed in the brain tissue follows, we believe, from experiments in which a comparison was made of the acetylcholine content of the tissue before and after incubation. Thus, 20 g. of a chloroform preparation of ox brain were incubated for 4 hr. while a similar sample of the same material was treated, immediately after preparation, with alcohol. The activities of the final extracts, which were prepared by a method, which is rather more elaborate than that employed above and is described below in connexion with the isolation of acetylcholine, were then compared on the eserinized leech muscle (Fig. 2)

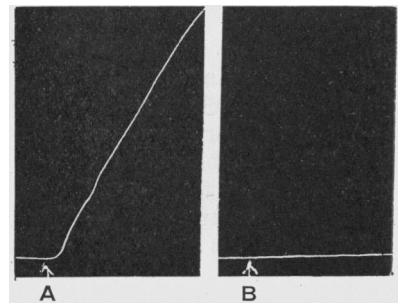


Fig. 2. Response of leech muscle to 0·05 ml. of extract from incubated material (A); and to 0·05 ml. of extract from non-incubated material (B). Final extracts measured 50 ml.

The amount of acetylcholine in the control is evidently negligible compared with that in the incubated material. This result does not imply that no acetylcholine was present in the whole brain. During the process of mincing preformed acetylcholine is brought into contact with the choline-esterase of the brain, by which it is rapidly destroyed.

In view of the successful outcome of the above experiments we have not carried out any extensive investigations with other tissue. Preliminary experiments have, however, indicated that treatment of minced spleen (horse), heart (cat) and gut (cat) by the procedure used in the case of brain results in the production of little or no acetylcholine. We cannot assert that no acetylcholine is produced by these tissues *in vitro*, but the amount, if any, which is formed is negligible in comparison with that produced by an equal weight of brain tissue under the same conditions.

Isolation of acetylcholine

As far as we could judge from our experiments with the leech preparation, the substance prepared as described above certainly resembled acetylcholine very closely. We were able to show that its activity on the leech muscle was potentiated by eserine, and, further, that its activity was destroyed by a preparation of choline-esterase obtained from horse serum by the method which we have previously described [1935, 2]. Nevertheless, it appeared to us desirable to place its identity beyond doubt by its actual isolation. In order to determine if this was practicable, a biological assay of the material produced was carried out, as a result of which it was concluded that 200 g. of ox brain, when incubated under the conditions described above, would produce an amount of the active material which, in its action on the eserinized leech muscle, was equivalent to approximately 1·5 mg. of acetylcholine bromide. Since the average weight of an ox brain is about 200 g. it was evident that 30 brains would yield about 45 mg. of the bromide or 36 mg. of the chloride, an amount which we considered was just sufficient to permit of its actual isolation if the substance were, in fact, acetylcholine. In order to add a margin of safety we therefore decided to attempt the isolation from 40 ox brains, which, provided they all behaved in a uniform manner, should yield 48 mg. of acetylcholine chloride.

Before attempting the isolation there was, however, one obvious difficulty which required investigating. In the material from which we hoped to isolate acetylcholine, there would be present not more than 48 mg. of the chloride of this substance, and this would be admixed with 2 g. of eserine. Since all the precipitants, such as Reinecke's salt and chloroplatinic and chloroauric acids, which are of service in the isolation of acetylcholine, also react with eserine to yield insoluble salts, it was clear that some other process would have to be employed to remove the alkaloid. Normally, in order to separate eserine from a quaternary salt, it would suffice to shake out the former with ether or chloroform from alkaline solution. In the present instance, the sensitivity of acetylcholine to alkalis had to be borne in mind. Nevertheless, it was found that, after making the solution very faintly alkaline to litmus by the addition of saturated aqueous sodium bicarbonate, the eserine could be completely removed from it by extracting it six times with chloroform; moreover, biological assays indicated that this could be done, provided that all operations were carried out as rapidly as possible and the solution was again rendered faintly acid on their completion, without the occurrence of any appreciable decomposition of the acetylcholine. This method was therefore adopted in the isolation now to be described.

The brains were obtained in pairs from the slaughter-house as rapidly as possible after the animals were killed. Immediately on their arrival at the laboratory they were minced separately. Each brain was then thoroughly ground with a solution of 50 mg. of eserine in 50 ml. of chloroform, and the mixture incubated for 2 hr. at 37°. On removal from the incubator 500 ml. of alcohol were added, the mixture thoroughly stirred and shaken and left overnight. The alcoholic extract was then removed by filtration, the residual fibrous tissue again extracted with alcohol, and the solvent removed from the combined filtrate and washings by distillation *in vacuo* at a low temperature. In this distillation the temperature of the bath was usually maintained at 35° and in no case allowed to rise above 40°. The temperature of the contents of the flask was, therefore, never appreciably greater than that of the room. If, as frequently happened, it was necessary to store the semi-solid brown residue, this was done in a vacuum desiccator over sulphuric acid. In the next stage of the process, the residues from two brains were intimately ground with about 150 ml. of 10% aqueous trichloroacetic acid, the mixture well shaken and kept in the refrigerator for a few hours in a moderately narrow cylinder. The precipitate usually settled sufficiently to enable the bulk of the extract to be removed with a pipette. The remainder was separated by centrifuging the residual material, the precipitate being finally washed with a further small quantity of the trichloroacetic acid. The bulk of the acid was then removed from the combined solutions by extracting them four times with ether, after which eserine was shaken out from the aqueous solution by the method described above, the final acidification being made with tartaric acid. The solution was now evaporated to dryness by distillation *in vacuo*, with the precautions previously described. Extraction of the residue with cold alcohol eliminated a further quantity of insoluble material, the filtrate being evaporated to dryness in a vacuum desiccator, in which the residue was subsequently temporarily stored. When about 10 brains had been treated in this way the solutions obtained by dissolving these residues in the minimum volume of water were combined, treated with a slight excess of a saturated solution of Reinecke's salt, and placed in the refrigerator for a few hours. The precipitated reineckate was then filtered, washed successively in the customary manner [see e.g. Dudley, 1933] with water, alcohol and finally ether, and stored until all of the brains had been treated in the same manner. The combined reineckate from the 40 brains weighed about 2·5 g. It was decomposed with silver sulphate and barium chloride and the solution containing the chloride of the recovered base concentrated by distillation *in vacuo*. The residual syrup was dissolved in alcohol and treated with an excess of an alcoholic solution of chloroplatinic acid. After standing in the refrigerator overnight, the chloroplatinate which had separated was filtered and washed with a little alcohol. It weighed 1·45 g. On crystallization from about 5 ml. of water, 60 mg. of material were obtained. This was recognized as the double chloroplatinate of choline and acetylcholine by comparison with an authentic specimen of the substance prepared according to details given by Dudley [1929] from a mixture of choline and acetylcholine chlorides. Both specimens crystallized in the form of octahedra which melted separately, and when intimately mixed, at 262° with decomposition. This comparison completely satisfied us as to the identity of the material isolated from the brain tissue. Nevertheless, in view of the fact that the decomposition point of the double chloroplatinate to some extent depends upon the rate of heating, it was decided to convert the bases into chloroaurates, from which pure acetylcholine chloroaurate could be isolated. For this purpose, the elegant method devised by Dudley [1929] was employed. The remainder of

the double chloroplatinate was dissolved, with warming, in 2 ml. of water, the operation being carried out in a small centrifuge tube. It was then decomposed by shaking it with finely divided metallic silver, centrifuged and the residue washed with 0·5 ml. of water. On treating the combined solutions with concentrated aqueous gold chloride, an immediate precipitate was formed. After standing in the refrigerator for 2 hr. this was filtered without washing, and, after drying, weighed 75 mg., the abnormal yield being due, no doubt, to contamination with free gold chloride. It was then dissolved in 2 ml. of warm water, filtered through a hot-water funnel from a little insoluble matter, the filter being washed with a further 0·5 ml. of warm water, and the filtrate left in the refrigerator for 2 hr. The crystalline material which separated weighed 20 mg. and melted at 152°. It was evidently not yet quite pure. A further recrystallization from hot water was therefore carried out, when there were obtained 15 mg. of a substance forming irregular plates which were indistinguishable under the microscope from the crystals of an authentic specimen of acetylcholine chloroaurate. This material, as also a specimen of acetylcholine chloroaurate placed in the same bath, melted at 165° and no depression occurred when approximately equal weights of the two were intimately mixed. The gold content of the chloroaurate obtained from the brain tissue corresponded, moreover, with that required for acetylcholine chloroaurate. (Found: Au, 41·0%. $C_7H_{16}O_2NAuCl_4$ requires Au, 40·6%.) There is thus no doubt that the substance formed when brain tissue is incubated is acetylcholine. The yield obtained, based on the weight of the double chloroplatinate isolated, corresponded only with 18 mg. of acetylcholine, whereas a rough calculation had indicated that 48 mg. would be formed in the material used. In view of the instability of acetylcholine and of the many operations involved in its isolation, the loss which occurred is not, perhaps, greater than would be expected. For instance, biological assays indicated that the conversion of the acetylcholine into the reineckate and its recovery therefrom resulted, in our hands, in a loss of no less than 25% of the active material.

It was mentioned above that the crude chloroplatinate first isolated weighed 1·45 g. but only yielded 60 mg. of the double chloroplatinate of choline and acetylcholine. The remainder of the material was not, in this case, further investigated. That it consisted mainly of choline chloroplatinate follows, however, from a previous abortive attempt to isolate acetylcholine. Starting with 37 ox brains and treating them exactly as described above, the syrup recovered from the reineckate was, in this instance, dissolved in a small volume of water and treated with aqueous gold chloride. The chloroaurate which separated weighed 1 g. and, although biological tests proved that it contained some acetylcholine, was found to consist largely of choline chloroaurate, for, after two recrystallizations from water it melted with decomposition at 266° and contained the correct percentage of gold. (Found: Au, 44·3%. $C_5H_{14}ONAuCl_4$ requires Au, 44·5%.) The chloroaurate is not a suitable salt for use in the isolation of acetylcholine from a large excess of choline, and it therefore seemed preferable, in this case, particularly in view of the losses by decomposition which had occurred during the recrystallization of the gold salt, to start the new experiment described above rather than to attempt to convert the chloroaurates into chloroplatinates.

Influence of conditions on amount of synthesis

Before, and concurrently with, the work resulting in the isolation of acetylcholine, we carried out many experiments with the object of gaining some insight into the mechanism of its formation. Some of these were only of an

orienting type, designed to discover the best experimental conditions for further work. In view of their provisional nature, these will only be briefly referred to.

The first point which required examination seemed to be the effect of the time of incubation on the yield of acetylcholine. Unfortunately, the results obtained with different preparations have not been entirely consistent. They do, however, agree in showing that the yield is not appreciably, if at all, increased by continuing the incubation beyond about 4 hr. In one experiment a decided maximum was reached after $1\frac{1}{2}$ hr., and this was followed by a moderately rapid fall. In others, however, the maximum yield was not produced until 3 hr. had elapsed, when the quantity present only diminished slowly with time. On the whole it was decided that the best results would be obtained if a 2-hr. period of incubation were used, although, in some of the earlier experiments, 4 hr. had been chosen. In connexion with the time of incubation, it should be pointed out that, at 37°, the spontaneous hydrolytic decomposition of the acetylcholine would not be negligible; this, no doubt, accounted for the diminution in yield which was observed on prolonging the period of incubation.

As was to be expected, the *p*H of the mixture undergoing incubation was not without influence on the yield of acetylcholine. Using an incubation period of 4 hr. and a sodium phosphate buffer, the best results were obtained in the region of neutrality; at *p*H 6·0 and 8·0 the yield was much depressed. Nevertheless, as compared with preparations to which 10 ml. of water were added instead of an equal volume of the buffer solution, the effect of the phosphate was to diminish the yield whatever the *p*H. Further experiments were therefore carried out without any attempt to control the acidity.

In some of our earliest experiments the brain tissue was incubated in evacuated vessels. Somewhat better results were, however, subsequently obtained in the presence of air, and the process of evacuation was therefore discontinued. No conclusion can, however, be drawn from this as to whether the process occurring in our chloroform preparation and resulting in the production of acetylcholine requires oxygen; the process of evacuation which we employed was, perhaps, not such as to ensure the complete exclusion of oxygen from the reaction vessel.

Effect of addition of various substances

A number of substances, when added in amounts of 50 mg. to 20 g. of the chloroform preparation, exercised little, if any, influence on the production of acetylcholine. Among these substances are glucose, potassium chloride and glycine, all of which produced, if anything, a slight inhibition. Experiments with choline and sodium acetate indicated that these substances may be placed in the same category. Fig. 3 illustrates the results in the case of choline. In this experiment, the minced brain (about 200 g.) was ground with 50 ml. of chloroform containing only 10 mg. of eserine, while the amount of choline chloride added to the 20 g. sample was restricted to 10 mg. This was dissolved in 1 ml. of water, a similar volume of the solvent being added to the control. The restriction of the amount of choline used was made because larger quantities, which may themselves be without action, in the dilution finally employed, on the leech preparation, do actually produce some effect in the presence of acetylcholine. Fig. 3 shows that choline exerts no influence on the yield of acetylcholine. The slightly greater response of the leech muscle to the extract containing choline, if it can be regarded as significant, must be attributed to the action referred to above. Fig. 3 also demonstrates the potentiation by eserine of the action of the substance produced *in vitro* on the leech muscle. When the tissue is

incubated with larger amounts of eserine (50 mg. per brain), this effect is not so marked owing to the greater rapidity with which the eserine, which passes into the final extract, then exercises its effect on the muscle. It can, however, be demonstrated in such cases by removing the alkaloid from the extract by the

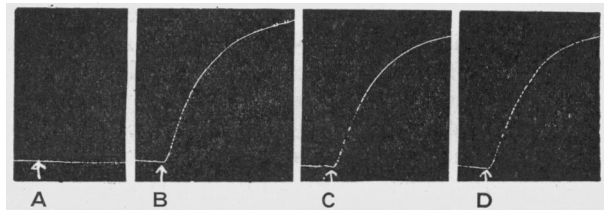


Fig. 3. Effect of addition of 0·025 ml. of final extract from preparation containing added choline before (A) and after (B and D) treatment of leech muscle with eserine. Control added at C. Final extracts measured 10 ml.

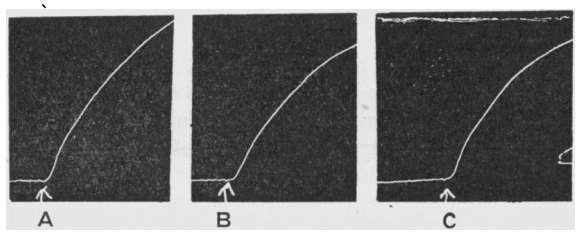


Fig. 4. Response of leech muscle to 0·05 ml. of final extract from preparation containing added sodium acetate (B), and to control (A and C). Final extracts measured 50 ml.

process previously described. The results obtained with sodium acetate are illustrated in Fig. 4. In this experiment 50 mg. of sodium acetate dissolved in 1 ml. of water were used. No effect, apart from a possible slight inhibition, can be observed.

Effect of addition of sodium pyruvate and acetoacetate

In view of the possibility that sodium pyruvate or sodium acetoacetate might act as a precursor of acetylcholine, special attention has been paid to the influence of these substances on the yield of acetylcholine in the chloroform brain preparation. The effect of the former substance is shown in Fig. 5. In this experiment, 50 mg. of sodium pyruvate, dissolved in 5 ml. of water, were added to 20 g. of the chloroform preparation, which was then incubated for 4 hr. The control was treated similarly, 5 ml. of water being used in place of the solution of pyruvate. It is evident from the tracing that the pyruvate has produced an inhibition of the synthesis of acetylcholine which is virtually complete. This particular experiment was carried out as long ago as November 1935. It has been repeated on many occasions since, with the same result. There appears, therefore, to be no doubt as to the power of sodium pyruvate to inhibit the formation of acetylcholine in the chloroform preparation. Included in Fig. 5 is a tracing showing the effect of 0·05 ml. of lactic acid, neutralized with sodium hydroxide, and diluted to 5 ml. on the same leech preparation. An inhibition is again evident although it is not so marked as with the pyruvate.

In examining the effect of acetoacetic acid on the synthetic process, a solid preparation of sodium acetoacetate, obtained from the ethyl ester, has been

employed. Iodimetric estimations of the acetone produced on hydrolysis of this preparation indicated that it contained 70 % of sodium acetoacetate. Its effect on the synthesis of acetylcholine by the chloroform preparation is illustrated in Fig. 6. A solution of 50 mg. of the acetoacetate in 1 ml. of water was

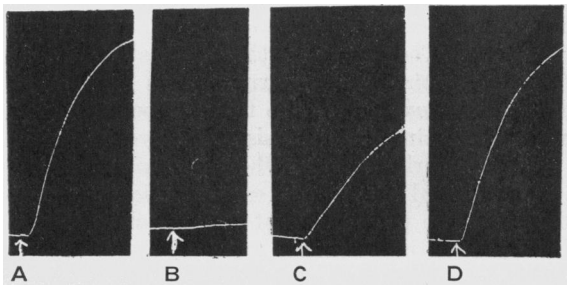


Fig. 5.

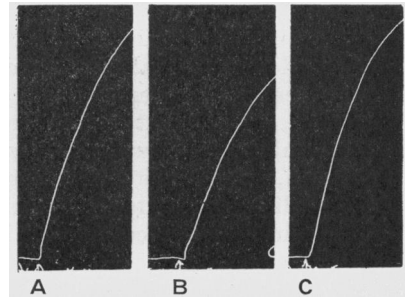


Fig 6.

Fig. 5. Response of leech muscle to 0·025 ml. of final extracts from control (A and D), and from material containing added sodium pyruvate (B) and sodium lactate (C). Final extracts measured 10 ml.

Fig. 6. Response of leech muscle to 0·05 ml. of final extracts from material containing added sodium acetoacetate (A and C) and from control (B). Final extracts measured 25 ml.

used, the period of incubation being 4 hr. The control was treated with 1 ml. of water and 50 mg. of the sodium acetoacetate were added to it after the reaction had been stopped by the addition of alcohol. It is evident from the tracing that an increased synthesis of acetylcholine has occurred in the material containing the acetoacetate. It must be admitted that the effect is not large, probably corresponding to an increase of not more than 50 % in yield, but similar results have been obtained in a number of experiments. In some of these the amount of acetoacetate used was reduced to 20 mg. and in others a shorter period of incubation of 2 hr. was employed. In all cases, however, the response of the leech muscle was increased by the addition.

Discussion

The first isolation of acetylcholine from animal material was that of Dale & Dudley [1929] who obtained it from the spleen of the horse. As this result demonstrated conclusively that acetylcholine occurs in the animal organism it constituted, in our opinion, almost overwhelming evidence in favour of the view that the substance with identical physiological properties which is known to be present in nervous tissue is, in fact, acetylcholine. Certain authors have, however, apparently accepted this conclusion with reservations, for the substance in question is sometimes referred to as a choline ester or as an acetylcholine-like substance. The caution, which has at times been exercised in this respect, is no doubt connected with the facts that the function of acetylcholine in the spleen is unknown and that its occurrence in this organ is apparently confined to ungulates. The isolation of acetylcholine from mammalian nervous tissue, effected in the present investigation, must, we believe, finally remove any doubts of this kind which may have remained.

It is, however, not so much the actual isolation of acetylcholine from ox brains which we wish to emphasize in connexion with our work, but the fact that

the material so isolated was produced *in vitro*. Mincing the brain tissue causes, as we have shown, a complete destruction of any acetylcholine which it may have contained. On incubation of this minced tissue, under the conditions we have described, acetylcholine is again formed. Its formation in this way has been repeatedly demonstrated by the action of extracts of the incubated material on the eserinized leech muscle, and this result has been finally confirmed by its isolation and chemical identification. The mechanism whereby the synthesis is effected is, presumably, enzymic in nature. The details of the process cannot, however, yet be regarded as elucidated, although our experiments are, we believe, not without importance in this connexion. Of the three hypothetical mechanisms which we have advanced to explain the synthesis, two have, in our opinion, been definitely excluded by our experiments. Neither sodium acetate nor sodium pyruvate, two of the hypothetical precursors, when added to our brain preparation, causes any increase in the production of acetylcholine. In the case of sodium pyruvate the effect is, on the contrary, to inhibit to a high degree the formation of acetylcholine which occurs in its absence, a behaviour which appears to establish quite conclusively that sodium pyruvate itself cannot act as a precursor. Our third hypothetical mechanism has, however, received some experimental support. The addition of sodium acetoacetate to a brain preparation causes an increased production of acetylcholine. Similar results have been obtained on a number of occasions, but the increased yield of the ester has, in all cases, been of the same order of magnitude as in the experiment quoted, namely, approximately 50%. We expected, when once the true precursor was found, that it would be possible to increase the yield to a much greater degree than this. That we have been unable to do this with acetoacetate causes us, not to abandon the hypothesis that this substance is an actual precursor, but rather to believe that some other factor, hitherto not considered, is also involved. For example, in the mechanism which we have outlined, it is clearly necessary for the acetoacetate to exist in the enolic form. If the enolic hydroxyl were esterified with, for instance, phosphoric acid, the enolic form would be stabilized and such an ester might conceivably represent the true precursor of acetylcholine.

There is, however, another point which must not be overlooked. The limiting factor may be the precursor of the choline moiety of the molecule. We have provisionally assumed that this precursor is choline itself, although no evidence has been produced in support of this assumption. The addition of choline to our brain preparation has not resulted in any increased production of acetylcholine, but this result must to some extent be discounted for two reasons: such additions have, on account of the method of assay employed, been restricted to very small amounts, and these have not been much, if at all, greater than the quantity of free choline present in extracts from the incubated material to which no such addition had been made. That there is no deficiency of free choline in the incubated brain follows from the fact that we were able to isolate 50 times as much choline as of acetylcholine from such tissue. Nevertheless, it is possible that choline itself is not a precursor of acetylcholine but that, as we have suggested in connexion with acetoacetate, some derivative of it yields the choline directly to the second precursor.

Some reference must, finally, be made to the experiments of Quastel *et al.* [1936]. The methods employed by these authors differ fundamentally from those which we have used. Whereas we have employed tissue which has been minced and thoroughly ground with chloroform, a process which doubtless destroys most, if not all, of the cells present, Quastel *et al.* have worked with brain slices,

which can be regarded as representing surviving tissue. Such discrepancies as have appeared between the results yielded by the two investigations can almost certainly be attributed to this difference in technique. The acceleration of the formation of the "choline-ester", which, in the light of our investigation, must now be regarded as acetylcholine, observed by Quastel *et al.* on the addition of, amongst other substances, glucose, pyruvic and lactic acids is quite contrary to our findings. The explanation of the discrepancy will, we believe, probably be found in the fact that the substances which increase the formation of acetylcholine in surviving brain tissue also increase the metabolism of the tissue. While it is at present impossible to dogmatize on this matter, we are inclined to think that such substances, by the mere fact of increasing the metabolism of the brain tissue, or possibly by extending the period of its survival, may cause an increase in the rate of formation of acetylcholine without themselves contributing the actual precursors.

Summary

Acetylcholine is produced in minced brain tissue when this material is incubated at 37° in the presence of eserine. By grinding the minced tissue with chloroform before incubation, the yield of acetylcholine is greatly increased. That the substance produced *in vitro* under these conditions is actually acetylcholine has been shown, not only by its action on the eserinized leech muscle, but by its actual isolation as the double chloroplatinate of choline and acetylcholine, from which acetylcholine chloroaurate was subsequently prepared.

The above chloroform brain preparation has been considered suitable for investigating the mechanism of the biological formation of acetylcholine. Three hypothetical mechanisms for this synthesis have been formulated, involving respectively acetic, pyruvic and acetoacetic acids as precursors of the acetyl moiety of the molecule. Of these, the first two have been rejected, since sodium acetate is without influence on the production of acetylcholine in the chloroform brain preparation, while sodium pyruvate exercises a considerable inhibition on the process. Sodium acetoacetate, on the other hand, increases the yield of acetylcholine by approximately 50%; this substance, or one of its derivatives, is therefore provisionally considered to be a possible precursor.

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