XXXIV. ON THE MECHANISM OF ACETYLCHOLINE FORMATION IN BRAIN *IN VITRO*¹

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It was shown by Quastel *et al.* [1936] that when brain cortex slices are allowed to respire in a medium containing eserine a substance having the chemical and pharmacological properties of a choline ester is formed and diffuses into the medium. That the substance is acetylcholine is made almost certain by the subsequent isolation by Stedman & Stedman [1937] of acetylcholine as the chloroaurate from minced ox brain. It is proposed, therefore, from now on to refer to the substance in question as acetylcholine.

Quastel *et al.* showed that the rate of formation of acetylcholine by brain cortex slices depends greatly on the metabolic conditions of the tissue. It is greatest in the presence of glucose and of oxygen. Under anaerobic conditions there is no accumulation of acetylcholine; all that is produced may be shown to be preformed in the tissue slices. The absorption of oxygen by the tissue is not in itself sufficient to bring about synthesis of acetylcholine, for respiration in presence of sodium succinate fails to increase the acetylcholine formation. Respiration only in the presence of substances important in carbohydrate metabolism, such as glucose, sodium lactate, sodium pyruvate, results in a large increase in the yield of acetylcholine by brain tissue slices. Addition of acetate ions is without effect on the acetylcholine synthesis.

In the absence of eserine no acetylcholine formation can be detected. This is clearly due to the very active choline esterase present in brain tissue. Calculations show that 1 g. (wet wt.) rat brain cortex will break down 28 mg. acetylcholine chloride in 1 hr. at 38.4°; guinea-pig brain cortex has a similar activity. The eserine added to tissue slices must be sufficient to inactivate the choline esterase.

Neither kidney, liver, spleen nor testis tissue slices have any measurable activity in forming acetylcholine under conditions most favourable for its production from brain. Rat diaphragm has about one-third the activity of rat brain.

This investigation took on a new aspect when we discovered that there exists in brain a substance, not pharmacologically active so far as the eserinated leech preparation is concerned, which breaks down under a variety of conditions to form acetylcholine. It is with the consequences of this finding that the present communication is concerned.

Technique

Estimation of acetylcholine. Acetylcholine was estimated by measuring the contraction of the eserinated longitudinal muscle from the dorsum of a leech as recorded on a slowly revolving smoked drum. The contraction was compared with those caused by known quantities of acetylcholine chloride. Results are given in terms of acetylcholine chloride per g. wet wt. of tissue. Details of the estimation are given by Quastel *et al.* [1936].

¹ The substance of this paper was communicated to the Physiological Society on 11 December, 1937.

Preparation and examination of brain tissue. The brain was removed from the animal (rat, guinea-pig) as soon as possible after death. It was then minced, or otherwise prepared, as described later and known quantities were placed in manometric vessels of the Warburg respiratory apparatus, containing phosphate-Locke media. The vessels were filled with O_2 and estimations of the O_2 consumption were as usual. The experimental period varied from 1 to 3 hr. at 37° , after which the material in the manometric vessel was tested for its acetylcholine activity.

Media. Solutions were made up to have an osmotic pressure approximately equal to 0.16 *M* NaCl. The final volume of the medium was made up to 3.0 ml. with 0.16 *M* NaCl after all other desired substances had been added. The *pH* was maintained at 7.4 except where otherwise stated in all experiments to be reported. The phosphate-Locke medium which was generally used was composed as follows: NaCl, 0.13 *M*; KCl, 0.002 *M*; $CaCl_2$, 0.001 *M*; sodium phosphate buffer *pH* 7.4, 0.03 *M*. To this was added glucose when occasion demanded. All acids were employed as neutral Na salts.

Eserine sulphate (0.2 ml. 1 in 400) was added to the medium in the manometric vessel usually either at the commencement or at the termination of the experiment; it was placed in the side-tube of the vessel and tipped into the vessel at an appropriate time.

Demonstration of a combined form of, or a precursor of, acetylcholine in fresh brain tissue

If fresh brain be well minced and ground with water, eserine added and the mixture kept at room temperature or at 0° for an hour the centrifugate shows acetylcholine activity. This represents the free acetylcholine present. If the mixture, however, after an hour, or immediately after addition of eserine to the tissue, be shaken with chloroform and kept at room temperature for 30 min. the amount of free acetylcholine present is greatly increased [cf. Stedman & Stedman, 1937]. The experimental procedure is to shake vigorously 5 ml. of the suspension of brain tissue with 0.2 ml. chloroform, keep the mixture at room temperature for 30 min., remove chloroform¹ by distillation *in vacuo*, make up the volume to the original 5 ml. and centrifuge. The centrifugate is now examined for acetylcholine activity.

Some typical results are shown in Table I, where notes on the experimental procedure are recorded. It will be seen from Exp. 1 that the effect of chloroform treatment on a suspension of macerated guinea-pig brain tissue in water containing eserine at 18° is to raise the content of free acetylcholine from 0.6 to 2.1 γ per g. wet wt. of tissue. It is worth recalling in this connexion that Kwiatkowski [1935] gives 0.6–2.3 γ for the amount of acetylcholine in 1 g. guinea-pig brain. It is clear from Exp. 1 that chloroform exercises its effect rapidly at room temperature. Exp. 2 shows that the effect of chloroform treatment of fresh brain tissue at 18° is greater the sooner the tissue is mixed with the chloroform. This must mean that the substance liberating acetylcholine after treatment with chloroform is unstable at 18° . This fact will be considered again later. Exp. 3 shows the effect of immediate treatment with chloroform at room temperature on brain macerated with eserine water, a value of 4 γ /g. acetylcholine being obtained.

The action of chloroform is clearly demonstrated if the brain tissue suspension is kept at 0° before it is shaken with chloroform. This is seen in Exp. 4. Ox and

¹ Chloroform must be removed as traces of it interfere with the activity of an eserinizied leech preparation.

Table I

Exp.	Species of brain	Treatment	Acetylcholine chloride found $\gamma/g.$
1	Guinea-pig	Ground with 10 vol. water at room temperature (18°). Divided into a number of equal parts (approx. 5 ml.): (A) One part treated with eserine and left at 18° for 90 min. Centrifuged and acetylcholine estimated ... (B) Second part treated with eserine and left at 18° for 90 min. Then shaken with 0.2 ml. chloroform. Chloroform removed, suspension centrifuged and acetylcholine estimated	0.6 2.1
2	Guinea-pig	Ground with 10 vol. water containing eserine sulphate (1/4000) at room temperature. Divided into equal parts: (A) One part left at room temperature (18°) for 1 hr. and then treated with chloroform at room temperature for 90 min. Acetylcholine estimated in centrifugate after chloroform removal (B) Second part treated at once with chloroform at room temperature for 90 min.	1.8 3.0
3	Guinea-pig	Ground with 10 vol. water containing eserine sulphate (1/4000) at room temperature. Divided into equal parts: (A) One part left at room temperature for 90 min. ... (B) Second part treated at once with chloroform and left at room temperature for 90 min.	2.0 4.0
4	Guinea-pig	Ground with 10 vol. ice-cold water and divided into equal parts: (A) Eserine added at once to one part and left at 0° for 60 min. Treated with chloroform and left at room temperature for further 90 min. Chloroform then removed (B) Eserine added at once to second part and left at 0° for 60 min. Then placed at room temperature for further 90 min.	4.0 <1.0
5	Ox	Ground with equal vol. of saline containing eserine sulphate (1/4000) at room temperature and divided into equal parts: (A) One part left at room temperature for 90 min. ... (B) Second part treated at once with chloroform and left at room temperature for 90 min	1.0 6.0
6	Rat	Minced at 0° , well mixed with 30 vol. phosphate-Locke-eserine solution and divided into equal parts: (A) One part left at room temperature for 60 min. ... (B) Second part treated at once with chloroform and left at room temperature for 60 min.	1.5 3.7

rat brains show phenomena precisely similar to that with guinea-pig brain (Exps. 5 and 6).

These results demonstrate that there exists in fresh brain tissue a substance which when shaken at room temperature with chloroform liberates acetylcholine. This substance is itself, as repeated experiments have shown, pharmacologically inactive so far as the eserinated leech preparation is concerned. It seemed possible that the chloroform might act by destroying the brain cells and allowing freer diffusion of acetylcholine contained in the cells into the outer medium. This possibility was shown to be incorrect by the facts:

(a) Long standing of eserized brain tissue at 0° or at room temperature does not result in the acetylcholine content of the medium reaching the value which is obtained after chloroform treatment.

(b) The effect of chloroform is apparently greater when it is shaken with an eserized brain suspension soon after the preparation of the latter than when it is shaken with a preparation which has stood for an hour at room temperature (Exp. 2, Table I).

(c) Minced brain tissue kept at room temperature or at 37° for 1 hr. in the absence of eserine still shows acetylcholine formation after treatment with chloroform (Exps. 1 and 2, Table II). Free acetylcholine in the tissue would have been destroyed under these conditions by the choline esterase present.

It is obvious from the appearance of the brain suspension after chloroform treatment that denaturation or coagulation of the tissue proteins has taken place. The experiment gives rise to the impression that there is a definite association between the denaturation of tissue protein and the liberation of acetylcholine and it seems a reasonable view that the acetylcholine-precursor is a protein complex.

Synthesis of acetylcholine-precursor by brain in vitro

When minced brain respires at 37° in a glucose-phosphate medium synthesis of the acetylcholine-precursor, or of the combined form of acetylcholine, takes place. This is very clearly shown in Exp. 3, Table II. Respiration of the minced brain in a phosphate-Locke medium or a phosphate-Locke-glucose medium took place for 2 hr. at 37°, eserine being present at the start of the experiment, or in other experiments being added during or at the end of the experiment. At the termination of the experiment the 3 ml. of suspension in each manometer were made up to 5 ml. with eserine water, divided into two equal parts, one part kept

Table II

Rat brain tissue was minced at 0°, well mixed with 3 vol. sodium phosphate buffer 0.2 *M*, pH 7.4, and divided into a number of equal parts.

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Exp.	Brain initially frozen before mincing:	Acetylcholine chloride (γ/g.)	
		Total	Free
1	(A) One part of tissue suspension mixed with NaCl-KCl-CaCl ₂ solution (to give final concentration of salts quoted on p. 244) in Warburg manometer vessel and respiration allowed to proceed for 2 hr. at 37°. Eserine was added 30 min. after thermal equilibrium	3.0	2.4
	(B) As above but glucose (0.02 <i>M</i>) added to the medium	7.2	5.0
	(C) As in (A) but eserine added at the end of the experiment (2 hr.)	1.0	0.4
	(D) As in (B) but eserine added at the end of the experiment (2 hr.)	3.6	1.2
	<i>Brain not frozen before mincing:</i>		
2	(A) One part of tissue suspension mixed with NaCl-KCl-CaCl ₂ solution and respiration allowed to proceed as in Exp. 1 (C), eserine being added at the end of the experiment (2 hr.)	<1.0	<1.0
	(B) As in (A), but glucose 0.02 <i>M</i> added to the medium	7.6	3.0
3	(A) One part of tissue suspension was placed in Warburg manometer vessel in phosphate-Locke medium and respiration allowed to proceed for 2 hr. at 37°. Eserine added at the commencement of the experiment	2.9	1.0
	(B) As in (A) but glucose 0.02 <i>M</i> added to the medium	23.0	11.5

at room temperature for 30 min. and the other treated with chloroform and then kept at room temperature for 30 min. After removal of the chloroform the suspensions were centrifuged and the acetylcholine contents of the centrifugates estimated. The value obtained after treatment with chloroform gives the "total" acetylcholine and the value obtained where no chloroform was added gives the "free" acetylcholine (Table II).

It will be noted from Exp. 3 (A), Table II, that the values of acetylcholine obtained after incubation at 37° in absence of glucose are rather lower than those obtained from fresh brain tissue which has not been incubated at 37° (see Exp. 6, Table I). In presence of glucose, however, the "total" acetylcholine is greatly increased, a value of 23.0 γ /g. being recorded (Exp. 3 (B), Table II).

This can only mean that the complex which breaks down to acetylcholine is synthesized *in vitro* in presence of glucose, the values obtained after incubation in a phosphate-Locke-substrate-free medium being simply the values of the remaining initial or preformed acetylcholine¹ complex in the tissue. Moreover, the result shows that the complex can be synthesized when eserine is present in the medium.

It may now be demonstrated that the acetylcholine-precursor can be synthesized in absence of added eserine. This is shown by Exps. 1 and 2, Table II. In Exp 1 (C) and (D) and in Exp. 2 eserine was added at the end of the experiment, i.e. after 2 hr. respiration at 37°. The suspensions were then analysed for "total" and "free" acetylcholine by the method described earlier. It will be observed that in both experiments the presence of glucose has considerably increased the "total" acetylcholine. Synthesis of the precursor obviously occurs in the absence of eserine. It will be noted that the amount of synthesis recorded in Exp. 2, Table II, is considerably greater than that shown in Exp. 1. The value given in Exp. 2 is the one usually found; that in Exp. 1 is low for a reason which will be given later.

Considering the very great choline esterase activity of fresh brain it is astonishing that free acetylcholine can be found in brain tissue after incubation for 2 hr. at 37° in absence of eserine. In this time and at this temperature 1 g. rat brain can break down over 50 mg. acetylcholine. The only reasonable interpretation of the formation of acetylcholine, under the experimental conditions quoted, is that a precursor of acetylcholine, not attacked by choline esterase, is first formed and that this is comparatively stable under the optimal respiratory conditions obtaining in the presence of glucose and oxygen.

If acetylcholine were first formed and this then combined with a tissue constituent to form the "acetylcholine-precursor" a high rate of acetylcholine synthesis would be necessary to compensate for the hydrolytic activity of choline esterase and enable synthesis of the precursor to take place. A sufficiently high rate of synthesis of free acetylcholine, in the presence of eserine, has never been observed in our experiments. Again it will be shown that the effect of added acetylcholine on the synthesis of the "precursor" in brain tissue is not nearly so great as that of added glucose (see Exp. 6, Table XIV). These observations make it clear that acetylcholine cannot be first formed. The comparative stability of the precursor is shown by the fact that in the presence of eserine free acetylcholine does not rapidly accumulate. Eserine, it should be pointed out, does not inhibit appreciably the breakdown of acetylcholine-precursor to free acetylcholine (see Table X).

The view that an acetylcholine-precursor is first synthesized by brain tissue under optimal respiratory conditions, and that this is comparatively stable

¹ This statement must be qualified by the fact that fresh brain contains a little glucose which may cause some synthesis according to its final concentration in the experimental medium.

under these conditions not only explains the facts given so far, but also explains why, considering the intense choline esterase activity of brain, any acetylcholine can be found at all in extracts of fresh brain tissue.

Some properties of acetylcholine-precursor¹

1. *Acetylcholine-precursor breaks down to acetylcholine under acid conditions.* On bringing fresh brain tissue, or tissue which has been incubated in a phosphate-glucose medium in presence of O_2 , to pH 3.0 and leaving the tissue at this reaction at room temperature for 30 min. the acetylcholine-precursor is broken down to acetylcholine. Subsequent treatment with chloroform has no further effect in increasing acetylcholine formation. These results may be seen in the typical experiments recorded in Table III or Table IV.

Table III

Rat brain was chopped or minced at 0°, mixed with 2 vol. saline and divided into a number of equal parts. These were placed in Warburg manometer vessels in phosphate-Locke-glucose medium and allowed to respire in O_2 at 37° for 2 hr. after which eserine was added to each vessel.

	Acetyl- choline chloride γ/g.
(A) One part left at room temperature for 60 min.	3.4
(B) One part treated at once with chloroform and left at room temperature for 60 min.	7.5
(C) One part brought to pH 3.0 by addition of HCl, left at room temperature for 30 min., neutralized and left for further 30 min.	7.5
(D) One part brought to pH 3.0 as in (C), left at room temperature for 30 min., neutralized, then treated with chloroform and left for further 30 min. ...	7.5

Table IV

Guinea-pig brain was chopped and minced with 2 vol. water, added in Warburg manometer vessels to a phosphate-Locke-glucose medium and allowed to respire in O_2 at 37° for 2 hr., after which eserine was added. The mixture was cooled in ice and divided into several equal parts.

	Acetyl- choline chloride γ/g.
(A) One part left at room temperature for 1 hr.	1.8
(B) One part treated with chloroform and left at room temperature for 1 hr. ...	4.2
(C) One part was brought to pH 3.0 with HCl, left at room temperature for 30 min., neutralized and left for further 30 min.	3.9
(D) One part was brought to pH 3.0 with trichloroacetic acid, neutralized after 45 min. and left for further 15 min.	4.3

Estimation of "total" acetylcholine. Acid conditions clearly greatly increase the velocity of acetylcholine formation from the "precursor". In measuring "total" acetylcholine the most convenient method now adopted is

¹ It would be convenient to give this substance a name indicating that it gives rise to acetylcholine. The following names have been considered by us: cholinogen, acetylcholinogen, cholin-esterogen, cholygen (since acetylcholine may also be written cholyacetate), but we feel it would be wiser at present to refer to the substance as acetylcholine-precursor on the understanding that this does not refer also to the substances (e.g. choline) which may give rise to the "precursor" itself.

to decrease the pH of the tissue to 3.0 with *N* HCl, leave the suspension at room temperature for 30–60 min., neutralize, centrifuge and examine the centrifugate for acetylcholine. This method gives approximately the same results as chloroform treatment and is much more convenient. Treatment with trichloroacetic acid gives the same results as with HCl, but use of this reagent is not recommended as trichloroacetate ions appear to have a deleterious effect on the leech preparation.

2. *Acetylcholine-precursor is not diffusible or dialysable.* The acetylcholine-precursor may be shown to be almost entirely in the residue obtained after centrifuging minced brain tissue which has respired under optimal conditions. Typical results are shown in Table V.

Table V

Rat brain 1.3 g. was minced, mixed with 2 vol. saline and allowed to respire in a phosphate-Locke-glucose medium for 2 hr. at 37°, after which eserine was added. The total volume (12 ml.) was made up to 24 ml. with ice-cold eserine water and centrifuged.

Residue made up to 24 ml. with eserine saline (1.7 γ /g.) Brought to pH 3.0 (7.2 γ /g.)	Centrifugate (2.9 γ /g.) Brought to pH 3.0 (3.2 γ /g.)
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The centrifugate shows an acetylcholine activity which is scarcely increased by acid treatment. The residue, on the other hand, shows a small free acetylcholine activity which is greatly increased by acid treatment. This and other experiments leave no doubt that the “precursor” is either not water-soluble or that it adheres strongly to the tissue debris. All attempts to separate it by dialysis through a cellophane membrane have so far failed.

3. *Acetylcholine-precursor is stable at 0° but unstable at higher temperatures.* If brain tissue containing acetylcholine-precursor is kept at 0° for 1 hr. there is no fall in “total” acetylcholine (see Table VI), showing that the precursor is relatively stable at this temperature. On keeping the tissue for 1 hr. at 18° there is a considerable fall in the amount of “precursor”. This is probably partly due to formation of acetylcholine which is hydrolysed by choline esterase present. There is a further fall if the tissue is kept for 1 hr. at 37° (Table VII). No glucose was added to the tissue in these experiments, and the respiration of the tissue was feeble or negligible.

Now though there is a fall in the amount of acetylcholine-precursor at 37°, in the absence of glucose, there is no corresponding increase in free acetylcholine (Table VII) and it is obvious that this breakdown is of a different type from that giving rise to acetylcholine. A possible explanation of this phenomenon will be given later.

Reference may be made at this point to the experiments of Corteggiani [1937] who has found that there is a complex in brain tissue which on heating to 70° for 3 min. gives rise to free acetylcholine. Probably the complex in question is identical with our acetylcholine precursor. It is to be expected that if denaturation of the tissue, as produced by chloroform, releases acetylcholine from the precursor, denaturation accomplished by heat treatment will have a similar effect. Doubtless the action of alcohol or of acid alcohol in liberating free acetylcholine, described recently by Loewi [1937], is also due to a denaturing effect on the tissue proteins.

Table VI

Guinea-pig brain was minced at 0°, mixed with 10 vol. ice-cold water, and divided into a number of equal parts.

	Acetylcholine chloride (γ /g.)	
	Total	Free
(A) One part kept at 0° for 5 min., eserine added and kept at 0° for 60 min.	4.0	<1.0
(B) One part kept at 0° for 60 min., eserine then added	4.0	<1.0
(C) One part kept at room temperature (18°) for 60 min., eserine then added	2.0	<1.0

Table VII

Guinea-pig brain was minced with 10 vol. water, and divided into a number of equal parts.

	Acetylcholine chloride (γ /g.)	
	Total	Free
(A) One part kept at room temperature (18°) in presence of eserine for 60 min.	2.1	0.6
(B) One part kept at 37° in presence of eserine for 60 min.	0.6	0.6

Effects of chloroform. As already stated, chloroform will accomplish its effect of releasing acetylcholine from the acetylcholine-precursor simply by shaking with the tissue at room temperature. This occurs also at 0°. If minced brain tissue, however, is shaken with chloroform and kept at 37° for 90 min. there is little or no liberation of extra acetylcholine. Typical results are shown in Table VIII. Now it is possible that this negative, or much diminished, effect of chloroform at 37° may be due to destruction of the acetylcholine in presence of chloroform even in presence of eserine. This suggestion is definitely excluded by the results of the experiment given in Table IX. In this 5 γ acetylcholine chloride were added to minced brain in presence of eserine and chloroform at 37° for 90 min. and after this period were, within the experimental error, entirely recovered.

Reference can now be made to the experiments of Stedman & Stedman [1937] who claim that acetylcholine is synthesized on incubation of ox brain in presence of chloroform at 37°. It may be shown that the amount they obtained (1.5 mg. acetylcholine bromide per 200 g. wet wt. brain $\equiv$ 6 γ acetylcholine chloride per g. wet wt.) after incubation is the amount found on treating ox brain at once with chloroform and leaving at room temperature for 90 min. (Exp. 5, Table I). As has already been shown this represents the preformed or initial value of "total" acetylcholine. Hence the acetylcholine found by the Stedmans, under their experimental conditions, can scarcely be regarded as due to synthesis *in vitro*; it is the product of breakdown of the acetylcholine-precursor present initially in the brain tissue they used. The fact that the Stedmans found it necessary to incubate the ox brain tissue with chloroform at 37° to obtain the full output of acetylcholine is due, it appears to us, to the possibility that insufficient chloroform was added to accomplish full denaturation of the tissue protein at room temperature and that incubation at 37° accelerated this process. It is, however, just possible that in view of the large quantities of minced tissue (20 g.) which the Stedmans used for their incubation experiments there may have been sufficient glucose present already in the tissue to accomplish some synthesis, even with the chloroform present.

Table VIII

6 g. fresh ox brain were ground with sufficient saline-eserine (1/4000) to make final volume 12 ml. This was divided into several equal parts (2.0 ml.).

	Acetylcholine chloride (γ /g.)
(A) One part mixed with phosphate-saline-eserine and left at room temperature for 90 min.	1.0
(B) One part mixed with phosphate-saline-eserine, shaken with chloroform and left at room temperature for 90 min.	6.0
(C) One part mixed with phosphate-saline-eserine and left at 37° for 90 min.	<1.0
(D) One part mixed with phosphate-saline-eserine, shaken with chloroform and left at 37° for 90 min.	<1.0

Table IX

Guinea-pig brain was ground with 2 vol. of phosphate-saline solution and divided into several equal parts.

	Acetylcholine chloride found
(A) One part treated with eserine, 5 γ acetylcholine chloride added and left at 37° for 90 min.	5.0 γ
(B) One part treated with eserine, 5 γ acetylcholine chloride added, shaken with chloroform and left at 37° for 90 min.	5.0 γ
(C) As in (A) but no acetylcholine added	<1.0 γ
(D) As in (B) but no acetylcholine added	<1.0 γ
(E) As in (B) but no eserine added	Nil

It is plain from Table VIII that synthesis of acetylcholine does not take place in the presence of chloroform under the experimental conditions cited, and it may be concluded that most, if not all, of the extra acetylcholine found by Stedman & Stedman after incubation of brain tissue with chloroform is due to the breakdown of the initial or preformed acetylcholine-precursor.

Effects of various concentrations of eserine on the synthesis of acetylcholine-precursor. Although, as has already been shown, synthesis of acetylcholine-precursor takes place in absence or presence of eserine, there is as yet no information to indicate whether the presence of eserine exercises any inhibitory effect on the synthesis. Experiments devised to settle this point were carried out with eserine present at concentrations varying from 1 in 120,000 to 1 in 1200. The results are cited in Table X.

Table X

A fresh rat brain was chopped finely, mixed with 3 vol. saline and divided into several equal parts.

	Acetylcholine chloride γ /g.	
	Total	Free
(A) One part was analysed immediately for free and "total acetylcholine after addition of eserine (1/4000)	2.9	<1.0
(B) The remaining parts were incubated in phosphate-Locke-glucose medium in presence of eserine at various concentrations at 37° for 2 hr. They were then analysed with the following results:		
Eserine concentration present:		
1/120,000	20.7	11.5
1/24,000	23.0	11.5
1/6,000	23.6	—
1/1,200	23.0	13.8

It will be seen that the amounts of "total" as well as of free acetylcholine formed as a result of synthesis in the brain tissue are not appreciably affected by the very wide range of variation of eserine concentrations. Had the eserine any inhibitory effect on the synthesis this would certainly have been detected at the highest concentrations of eserine used. Concentrations of eserine greater than 1/1200 cannot be used in these experiments *in vitro* owing to the fact that complications arise due to pigment formation from eserine as a result of oxidation with a concomitant fall in respiration of the brain.

When no eserine is added at all during the entire experiment no "free" acetylcholine can, of course, be detected. Treatment with acid (pH 3.0) even in the absence of eserine does result, however, in the liberation of a little acetylcholine which may be detected by the eserinated leech preparation. This result is clearly due to the fact that acid conditions inhibit choline esterase activity or render the enzyme inert. The amount of acetylcholine so produced is small compared with that formed by treatment with acid in presence of eserine.

Effects of metabolites and aerobic conditions on the synthesis of acetylcholine-precursor. On allowing minced brain tissue to respire in the presence of various substrates it is immediately clear that synthesis of acetylcholine-precursor takes place in the presence of glucose, sodium lactate and sodium pyruvate. Typical results of the total acetylcholine produced will be found in Table XI. The "free" acetylcholine produced is usually of the order of half the "total" estimated after acid treatment. Little or no synthesis of acetylcholine or its precursor takes place with washed minced brain in presence of the following sodium salts: acetate, succinate, acetoacetate, α -glycerophosphate, hexosediphosphate, α -ketoglutarate under conditions where glucose produces a good synthesis. The presence of *dl*-glyceraldehyde greatly diminishes the acetylcholine synthesis effected by glucose (Exp. 4, Table XI).

With fresh brain tissue which has not been washed to free it of the metabolites still present the addition of glucose still brings about considerable synthesis of acetylcholine-precursor (Exp. 3, Table XI), but even under these optimal conditions succinate, acetate, acetoacetate, α -glycerophosphate, α -ketoglutarate effect no synthesis within the experimental error of estimation. Hexosediphosphate has been observed under such conditions to bring about a synthesis and it is probably the fact that, in the intact unwashed tissue, factors are present which will convert hexosediphosphate into lactate which accounts for this synthesis. Using the slice technique Quastel *et al.* [1936] found that α -glycerophosphate brought about an increase in acetylcholine formation; this has not been observed with minced tissue. Possibly this, again, is due to the presence of factors in the intact slices which will convert α -glycerophosphate into lactate or pyruvate; such factors may become ineffective on mincing either by dilution or destruction.

The main fact, however, is clear from the results recorded in Table XI that glucose, lactate and pyruvate give rise to a product or to conditions enabling synthesis of acetylcholine-precursor to take place.¹

As found with the slice technique aerobic conditions are essential for synthesis to take place (Exp. 1, Table XI) with minced brain. Possibly a slight synthesis occurs in the presence of glucose under anaerobic conditions, but the effect, if any, is small compared with that taking place in the presence of oxygen.

Respiration of brain and synthesis of acetylcholine-precursor. Synthesis of acetylcholine-precursor cannot depend merely on the presence of O_2 or on the

¹ It is conceivable that pyruvate itself takes part directly in the synthesis of the precursor; this possibility is being investigated.

Table XI

Exp. 1. A fresh rat brain was chopped finely and washed once with 20 ml. saline to remove diffusible metabolites. The centrifuged deposit was mixed with saline (five times the original volume of the rat brain) and divided into several equal parts. Each part was placed in a Warburg manometer vessel in presence of a phosphate-Locke-substrate- eserine medium and allowed to incubate for 2 hr. at 37°. Experiments were carried out in an atmosphere of O₂ and in an atmosphere of N₂. At the end of the experiment "total" acetylcholine was determined by acid treatment, as described in the text. The following results were obtained.

Substrate	Total acetylcholine chloride (γ/g.)			
	O ₂ present	O ₂ absorbed μl.	N ₂ present (anaerobic)	
			Exp. A	Exp. B
No substrate added	2.7	98	1.9	2.9
Glucose 0.01 <i>M</i>	8.9	391	3.2	3.7
Sodium pyruvate 0.01 <i>M</i>	5.3	409	2.1	2.9
Sodium α-glycerophosphate 0.013 <i>M</i>	2.7	189	1.3	—
Sodium acetate 0.01 <i>M</i>	2.7	95	1.3	—
Sodium succinate 0.01 <i>M</i>	2.1	214	1.9	—

Exp. 2. Similar to Exp. 1, using washed rat brain and aerobic conditions.

Substrate	O ₂ absorbed μl.	Total acetyl- choline chloride γ/g.
No addition	71	2.8
Sodium <i>dl</i> -lactate 0.01 <i>M</i>	315	9.5
Sodium pyruvate 0.01 <i>M</i>	335	11.4
Sodium hexosediphosphate 0.01 <i>M</i>	77	2.8

Exp. 3. Similar to Exp. 1, but using unwashed rat brain. Aerobic conditions.

Substrate	Total acetyl- choline chloride γ/g.
No addition	6.3
Glucose 0.01 <i>M</i>	14.8
Sodium succinate 0.01 <i>M</i>	6.3
Sodium α-ketoglutarate 0.01 <i>M</i>	6.3
Sodium acetate 0.01 <i>M</i>	6.3
Sodium α-glycerophosphate 0.01 <i>M</i>	6.3
Sodium acetoacetate 0.01 <i>M</i>	6.3

Exp. 4. Similar to Exp. 1, using washed rat brain. Aerobic conditions.

Substrate	O ₂ absorbed μl.	Total acetyl- choline chloride γ/g.
No addition	109	3.5
Glucose 0.01 <i>M</i>	503	15.6
<i>dl</i> -Glyceraldehyde 0.01 <i>M</i>	112	1.6
Glucose 0.01 <i>M</i> + <i>dl</i> -glyceraldehyde 0.01 <i>M</i>	158	4.7
Sodium acetoacetate 0.01 <i>M</i>	209	4.7
Sodium α-glycerophosphate 0.01 <i>M</i>	215	2.9
Sodium hexosediphosphate 0.01 <i>M</i>	153	3.5

uptake of O₂ by the brain tissue. Thus no synthesis takes place when brain tissue is allowed to respire in presence of sodium succinate although there is a definite O₂ uptake (Exp. 1, Table XI). Yet it is clear that when synthesis does occur it takes place in presence of those substances which secure optimal respiratory rates for brain tissue (glucose, lactate, pyruvate). This may mean that synthesis depends on the absorption of energy supplied by oxidative reactions taking place during the combustion of glucose, lactate or pyruvate, or it may mean

that these substances supply a specific metabolite necessary for synthesis to occur. Our data are as yet insufficient to decide between the two possibilities and the question must therefore be left open at present.

It is, however, worth considering that, anaerobically, in presence of glucose when extensive breakdown of glucose to lactic acid takes place with considerable energy yield little or no synthesis of acetylcholine takes place either with brain slices [Quastel *et al.* 1936] or with minced tissue. It seems more likely that some specific product of oxidation of glucose, lactate or pyruvate is directly concerned with the synthesis of the acetylcholine-precursor.

The presence of *dl*-glyceraldehyde greatly diminishes the synthesis of acetylcholine-precursor by brain tissue in a glucose medium and this must be correlated with the fact that *dl*-glyceraldehyde diminishes the respiration of brain in presence of glucose (Exp. 4, Table XI). Incubation of brain tissue with *dl*-glyceraldehyde in the absence of glucose does not result in any extra production of acetylcholine (Exp. 4, Table XI).

The presence of sodium fluoride (0.02 *M*) also diminishes the synthesis of acetylcholine-precursor by minced rat brain tissue in a glucose medium, a normal value of "total" acetylcholine of 11 γ /g. being reduced in presence of the fluoride to 5 γ /g. It was previously reported [Quastel *et al.* 1936] that fluoride (0.01 *M*) does not diminish the free acetylcholine formation by rat brain cortex slices in a glucose medium. The latter fact has been confirmed and the discrepancy between the results obtained with minced tissue and with brain slices is now under investigation.

Dependence of acetylcholine-precursor synthesis on the condition of brain tissue. Generally speaking it has been found that any treatment of brain tissue which results in a greatly lowered respiration of the tissue in presence of glucose also results in a diminished rate of synthesis of acetylcholine-precursor. Thus it will be seen from Table XII that the effect of freezing brain tissue reduces greatly

Table XII

A fresh rat brain was divided into two equal parts. One part was ground at 0° with a phosphate buffer solution, the other part was frozen in an ice-salt mixture and ground with phosphate buffer solution whilst frozen. Each part was then examined in the Warburg apparatus, respiration with and without glucose being allowed to take place for 2 hr. at 37°. Eserine was then added and the contents of the vessel examined for free and total acetylcholine.

	O ₂ uptake μ l.	Acetylcholine chloride (γ /g.)	
		Total	Free
(A) Frozen tissue	33	1.2	<1.0
Frozen tissue + glucose 0.02 <i>M</i>	28	<1.0	<1.0
(B) Tissue ground at 0°	470	3.5	2.5
Tissue ground at 0° and glucose 0.02 <i>M</i>	645	8.0	4.5

its respiratory activity in presence of glucose and at the same time causes cessation of acetylcholine synthesis. The same phenomenon will be seen on comparing Exps. 1 and 2, Table II.

Vigorous washing of brain tissue not only removes metabolites such as glucose or lactate, but removes factors necessary for the respiratory activity of the tissue [Quastel & Wheatley, 1934]. At the same time it is found that the ability to synthesize the acetylcholine-precursor is also lessened. Thus a fresh unwashed minced rat brain gave a total yield of 20.7 γ /g. acetylcholine chloride

when incubated in a phosphate-Locke-glucose-eserine medium, whereas a fresh minced rat brain washed (by centrifuging) with four lots of 20 ml. saline gave a total yield of 3 γ /g. when examined under the same conditions. Hence, save when required for exceptional purposes, brain tissue should not be washed more than once with a small quantity of saline in order to obtain a good respiratory rate and a good synthesis of acetylcholine. Experiments have not been carried out so far in this work with the slice technique. On comparing, however, the yields of acetylcholine found by Quastel *et al.* [1936] by rat brain cortex slices under the most favourable conditions, it will be seen that they compare well with the yields of "total" acetylcholine found by the present technique. Thus it was found that a typical rate of acetylcholine chloride formation in a phosphate-glucose-eserine medium was 4.0 γ per 100 mg. dry weight of tissue per hour. This is equivalent to 16.0 γ acetylcholine chloride per g. wet wt. per 2 hr. This, however, is only the "free" acetylcholine found in the medium after removal of tissue slices and does not include the amount in the acetylcholine-precursor still contained in the tissue slices. Hence the yields of "total" acetylcholine by brain cortex slices when examined under optimal conditions must exceed those formed with minced whole brain tissue examined under similar conditions.

Reference may be made again, at this juncture, to the effects of chloroform. This substance in common with other narcotics depresses the respiration of brain. It is found also that incubation of minced brain in a phosphate-glucose-eserine medium in presence of chloroform diminishes the acetylcholine-precursor synthesis (see Table XIII).

Table XIII

4 g. fresh ox brain were minced, mixed with 3 vol. saline containing eserine (1/4000) and the suspension was divided into several equal parts.

	Total acetylcholine chloride γ /g.
(A) One part incubated in a phosphate-glucose-Locke medium (3 ml.) for 2 hr. at 37°	4.5
(B) One part incubated in a phosphate-glucose-Locke medium (3 ml.) containing 0.2 ml. chloroform for 2 hr. at 37°	1.6

Effects of addition of choline¹ or acetylcholine¹ on the synthesis of acetylcholine-precursor. The presence of choline increases the rate of formation of acetylcholine-precursor. This is specially marked with brain tissue which has been washed vigorously to free it as far as possible of choline originally present. The effect is small in the absence of glucose from the medium but is markedly increased when glucose is present. These results are to be seen in the typical Exps. 1, 3 and 8, Table XIV. Moreover, the accelerating action of choline is to be observed both in the absence and in the presence of eserine, Exp. 2, Table XIV. When using choline, control experiments were at first carried out to determine the effect of the choline present on the leech preparation, but later the suspensions of tissue, at the end of the experiments were centrifuged, the centrifugates containing the free choline discarded and analyses made on the deposits. The latter were suspended in eserine saline and "total" and free acetylcholine estimated. The fact that such deposits show the presence of "free" acetylcholine indicates that the acetylcholine-precursor breaks down partly to acetylcholine at room temperature in the presence of eserine (see also Table VI).

The addition of choline alone never secures a synthesis of acetylcholine-precursor greater than that found with fresh brain examined under optimal respiratory

¹ These substances were added to the tissue as their chlorides.

conditions. Considering that choline is a normal constituent of brain tissue it follows from these experiments that choline must be at least one factor in the building up of the acetylcholine-precursor.

When acetylcholine is added to minced brain tissue in absence of eserine an increase in acetylcholine-precursor may be observed (Exps. 5, 7 and 8, Table XIV) but its effects can be interpreted as due to the choline produced by hydrolysis of the ester by choline esterase. Its effect is not greater than that of an equivalent quantity of choline.

Table XIV

Exp. 1. Rat brain was minced and washed several times to remove metabolites including choline as far as possible. The suspension was divided into several equal parts, which were placed in Warburg manometer vessels in an appropriate medium containing eserine and respiration was allowed to take place for 2 hr. at 37°. They were finally examined for free and "total" acetylcholine. The volume in each vessel was 3 ml.

Medium:	Acetylcholine chloride γ/g.	
	Total	Free
(A) Phosphate-Locke	<1.0	—
(B) Phosphate-Locke-choline (1 mg.)	2.6	—
(C) Phosphate-Locke-glucose 0.01 <i>M</i>	7.3	3.1
(D) Phosphate-Locke-glucose 0.01 <i>M</i> choline (1 mg.)	13.5	6.3
(E) Control: choline (1 mg.). No tissue added	Not measurable	

Exp. 2. As in Exp. 1, but eserine added at different times and respiration allowed to proceed for 3 hr. In this experiment the suspensions were centrifuged at the end of the experiment and acetylcholine estimations were made on the deposits.

Medium:		Total acetyl- choline chloride γ/g.
(A) Phosphate-Locke-glucose 0.01 <i>M</i>	} Eserine added after 3 hr.	4.1
(B) Phosphate-Locke-glucose + choline (1 mg.)		6.9
(C) Phosphate-Locke-glucose	} Eserine added at com- mencement of exp.	4.8
(D) Phosphate-Locke-glucose + choline (1 mg.)		9.0

Exp. 3. Rat brain was chopped finely, mixed with 2 vol. saline and divided into several parts. After allowing respiration to take place in the appropriate medium, eserine being present from the beginning, for 2 hr. at 37°, the suspensions were centrifuged and estimations made on the deposits.

Medium:	Acetylcholine chloride γ/g.	
	Total	Free
Phosphate-glucose 0.01 <i>M</i> -Locke	11.1	3.7
Phosphate-glucose 0.01 <i>M</i> -Locke + choline (1 mg.)	18.5	4.6

Exp. 4. Rat brain was chopped finely, washed and mixed with 3 vol. saline. Subsequent treatment as in Exp. 3 but the final centrifuged deposit was washed three times with 10 ml. saline to remove free acetylcholine. Eserine was present from the commencement of the experiment.

Medium:	Acetylcholine chloride γ/g.	
	Total	Free
Phosphate-Locke	1.7	0.4
Phosphate-Locke + 50 γ acetylcholine	1.9	1.0
Phosphate-Locke + glucose 0.01 <i>M</i>	5.8	1.0
Phosphate-Locke + glucose 0.01 <i>M</i> and 50 γ acetylcholine	5.8	1.9

Exp. 5. As in Exp. 4.

Medium:		
(A) Phosphate-Locke	<1.0	—
(B) Phosphate-Locke and 100 γ acetylcholine	2.0	<1.0

Table XIV (*cont.*)

Exp. 6. Rat brain was minced, and mixed with 4 vol. saline and divided into several equal parts. Each part was placed in an appropriate medium and respiration allowed to take place for 2 hr. at 37°. After this period eserine (1/4000) was added to the vessels and their contents analysed for acetylcholine.

Medium:	Acetylcholine chloride γ/g.	
	Total	Free
(A) Phosphate-Locke	2.7	<1.0
(B) Phosphate-Locke + 300 γ acetylcholine	2.9	<1.0
(C) Phosphate-Locke + glucose 0.01 M	9.4	5.1
(D) Phosphate-Locke + glucose + 300 γ acetylcholine	11.8	3.6

Exp. 7. As in Exp. 6 but the minced brain was well washed before being placed in the manometer vessels. Eserine was added at the end of the experiment (2 hr.).

Medium:		
(A) Phosphate-Locke	0.9	<0.4
(B) Phosphate-Locke + 500 γ acetylcholine	3.1	1.1

Exp. 8. As in Exp. 7 but respiration was allowed to take place for 3 hr. after which eserine was added. The suspensions were centrifuged and the deposits analysed.

Medium:	Acetylcholine chloride γ/g.	
	Total	Free
(A) Phosphate-Locke-glucose	3.9	0.7
(B) Phosphate-Locke-glucose + 1 mg. choline	9.5	0.7
(C) Phosphate-Locke-glucose + 1 mg. acetylcholine	6.9	2.2

There is apparently a small effect of acetylcholine in increasing the quantity of precursor when eserine is present with the brain tissue from the beginning of the experiment. This is seen in Exp. 5. The effect is small and sometimes not to be detected (Exp. 4, Table IV). Conceivably there is a small adsorption of acetylcholine on the brain tissue which is liberated subsequently by acid treatment. It is clear, however, in view of the fact that acetylcholine cannot accumulate in large quantity owing to the presence of choline esterase that the adsorptive effect is too small to be of physiological significance. It is to be noted (Exps. 4 and 6, Table XIV) that the presence of glucose secures a greater synthesis of acetylcholine-precursor than is achieved by the addition of large quantities (up to at least 500 γ) of acetylcholine in the absence of glucose. It follows from this fact that the synthetic action of glucose cannot be due in the first place to the synthesis of free acetylcholine which then becomes adsorbed on the tissue proteins.

It follows from these experiments that choline is one of the constituents which lead to the formation of acetylcholine-precursor and that acetylcholine is not an intermediate between choline and the precursor.

Rate of formation of acetylcholine-precursor. Acetylcholine-precursor is produced rapidly during the early part of an experiment in which minced brain tissue is incubated in a phosphate-glucose-eserine medium and after reaching a limiting value it no longer accumulates. This is seen in Exp. 1, Table XV, where the "total" acetylcholine found after respiration had been allowed to take place in a glucose-eserine medium for 2 hr. was 11.1 γ/g. and this value only increased to 11.9 γ/g. when the respiration was prolonged for a further 3 hr. The result is

seen too in Exp. 2, Table XV, where the "total" acetylcholine found after an experimental period of 3 hr., using both glucose and lactate media, is little greater than that found in an experiment lasting only 1.5 hr.

Table XV

Exp. 1. Rat brain was chopped finely, mixed with 2 vol. saline and divided into several parts.

	Acetylcholine chloride γ/g.	
	Total	Free
(A) One part allowed to respire in a phosphate-Locke-glucose-eserine medium for 2 hr. at 37°	11.1	3.7
(B) Another part allowed to respire under similar conditions for 5 hr. at 37°	11.9	4.6

Exp. 2. As in Exp. 1.

(A) One part allowed to respire in a phosphate-Locke-glucose medium for 90 min. Eserine then added ...	8.0	2.7
(B) One part allowed to respire in phosphate-Locke-glucose medium for 90 min. Eserine added and respiration allowed to continue for a further 90 min.	8.0	8.0
(C) One part allowed to respire in a phosphate-Locke-lactate medium for 90 min. Eserine then added ...	8.0	1.6
(D) One part allowed to respire in a phosphate-Locke-lactate medium for 90 min. Eserine then added and respiration allowed to continue for a further 90 min.	8.25	6.0

Glucose = 0.01 M; lactate = 0.01 M.

The "free" acetylcholine does, however, tend to accumulate as time goes on so long as eserine is present in the medium. The rate of such accumulation has been found to depend greatly on the development of acid conditions in the medium as metabolism proceeds. This point will be dealt with shortly.

The fact that the amount of acetylcholine-precursor obtained in brain tissue examined under optimal respiratory conditions quickly reaches a limiting value indicates either that some component of the synthetic system is completely utilized or that an equilibrium value is reached. A limiting factor cannot be the choline concentration since experiments with added choline do not show any appreciable increase of the limiting values of acetylcholine-precursor found under optimal conditions. Nor can a limiting factor be the supply of energy, or of a specific product, produced during the respiration of brain tissue in presence of glucose, lactate or pyruvate, for respiration proceeds at a steady rate for a considerable time after the amount of acetylcholine-precursor has reached its limiting value. It follows that the limiting factor is a tissue constituent other than choline or a metabolic product from glucose. It seems to us a reasonable hypothesis that this limiting factor is a specific protein with which combination takes place to form the precursor. When the protein, like an enzyme, becomes saturated, a limit is reached to the amount of precursor which can be formed. When the precursor breaks down normally to form acetylcholine the specific protein is regenerated and under optimal conditions will again become saturated.

Effect on the rate of synthesis of acetylcholine-precursor, of the time at which eserine is added to the tissue. On the addition of eserine to a medium, in which brain tissue has been respiring, 2 hr. after the commencement of an experiment the "total" acetylcholine found is usually less than that found when eserine is added either at the commencement of the experiment or after respiration has

proceeded for a relatively short time, e.g. 30 min. Typical results will be found in Exps. 1 and 2, Table XVI, and in Exp. 2, Table XIV.

This phenomenon is doubtless due to the fact that when eserine is present in the medium the acetylcholine formed by the slow breakdown of the precursor accumulates and more precursor is formed from choline still present in the tissue until the limiting conditions are obtained. In the absence of eserine, however, the acetylcholine formed by breakdown of the precursor is hydrolysed by the choline esterase present, choline is formed and this helps to regenerate the precursor.

Table XVI

	Acetylcholine chloride γ/g.	
	Total	Free
Exp. 1:		
(A) Minced and washed rat brain incubated aerobically in a phosphate-Locke-glucose medium for 2 hr. at 37°. Eserine added at commencement of the experiment	8.6	6.0
(B) As in (A) but eserine added at the termination of the experiment	6.5	3.2
Exp. 2:		
(A) As in Exp. 1 (A) but eserine added after 30 min. ...	7.2	5.0
(B) As in Exp. 1 (B), i.e. eserine added after 2 hr. ...	3.6	1.2

Velocity of breakdown of acetylcholine-precursor to acetylcholine. Under optimal respiratory conditions in brain tissue at pH 7.4 the velocity of acetylcholine-formation from the precursor is slow. This is seen, for example, in Exp. 1, Table XV. It has been shown that eserine does not interfere, measurably, with this breakdown (Table X) and that acetylcholine does not disappear from the system when eserine is present (Table IX). The precursor must therefore have considerable stability under the conditions stated.

The velocity of breakdown, however, is much accelerated when the pH is lowered. It will be seen from the results quoted in Table XVII that a shift of pH from 7.0 to 6.5 or 6.0 will increase at 37° the rate of formation of free acetylcholine from the precursor. Such a change of pH has but little effect at 0° or at 18°, but it clearly has a much larger effect at 37°.

Table XVII

Rat brain was chopped finely, mixed with 3 vol. saline and divided into several equal parts. Each part was allowed to respire in a phosphate-Locke-lactate medium for 90 min. at 37° after which eserine was added to the media. The suspensions were then removed from the vessels, centrifuged and the deposits replaced in Warburg vessels containing lactate-Locke-phosphate media of different pH and respiration allowed to proceed for a further 90 min. The contents of the vessels were then analysed for free acetylcholine.

pH	Free acetylcholine found (γ/g.)
7.0	5.0
6.5	6.25
6.0	8.75

From a physiological standpoint the rate of breakdown of acetylcholine-precursor to acetylcholine is of as great importance as the rate of breakdown of acetylcholine itself. These experiments indicate that a local decrease of pH may play an important part in determining the rate of free acetylcholine-formation.

Factors controlling this rate of breakdown of the precursor will form the subject of another investigation.

Can acetylcholine-precursor be an adsorption complex of acetylcholine itself? Results have already been given which show that, in the synthesis of the precursor, acetylcholine is not first formed with subsequent attachment to a tissue "carrier", from which, by elution or denaturation, free acetylcholine may be liberated. To test this point more definitely, numerous experiments were carried out to ascertain whether adsorption of acetylcholine takes place on various brain tissue constituents; all, however, gave negative results. Brain tissue which had been washed free of acetylcholine and in which the amount of precursor had been very considerably reduced failed to take up acetylcholine to any measurable extent. It is true that a small quantity of precursor can be produced from acetylcholine under the conditions cited in Exp. 5, Table XIV. It would have been expected, however, that if adsorption of free acetylcholine was of real biological importance, such an adsorption would have been considerable with the large quantities of brain tissue and freshly prepared brain constituents (sterols, phospholipoids, proteins) which were used. Tissues other than brain, e.g. blood, liver, also failed to take up free acetylcholine. It appears to us, therefore, that acetylcholine-precursor cannot be a true adsorption compound of acetylcholine and a tissue constituent.

It has already been suggested that the tissue constituent which forms part of the acetylcholine-precursor is a specific protein. This suggestion is of course at present purely provisional. It appears to us most likely to be a protein, since liberation of acetylcholine takes place (a) on denaturation, (b) on acid treatment, (c) on heat treatment. It appears to be specific since acetylcholine synthesis does not take place in such organs as liver, kidney, or testis [Quastel *et al.* 1936] and appears to be confined to the nervous system. Muscle, however, has not yet been fully investigated.

Provisional scheme of the mechanism of acetylcholine-formation in brain in vitro. A provisional scheme of the manner of formation of acetylcholine in brain is put forward in the diagram below:

This scheme incorporates the results which have been found so far experimentally. It will be observed that the "tissue constituent" behaves as a catalyst and resembles an enzyme, since it brings about the synthesis of acetylcholine from choline and is itself regenerated. The reversible production of acetylcholine-precursor will explain why the precursor may disappear in presence of eserine at 37° in the absence of glucose, lactate or pyruvate (see p. 249) without giving rise to acetylcholine. It will be seen that a choline "cycle" is established. The possible formation of the precursor by a small adsorption of free acetylcholine on the tissue constituent is indicated by the dotted arrow. The scheme accounts for the results obtained when eserine is added to the brain tissue at different stages of the experiment, for the effects of choline and acetylcholine and for the rates of formation of the precursor. It is at present doubtful whether glucose, lactate or pyruvate gives rise to a specific product combining

with the choline or whether by a series of oxidative reactions they secure conditions suitable for combination to take place with another metabolite.

SUMMARY

1. There exists in fresh brain tissue a combined form of acetylcholine, termed acetylcholine-precursor, which is pharmacologically inactive and which breaks down to yield free acetylcholine. A method for the estimation of the precursor is given.

2. The breakdown of acetylcholine-precursor may be effected by denaturing agents, e.g. shaking with excess chloroform, or by treatment with acid. At pH 3.0 free acetylcholine is liberated from the precursor at room temperature. Breakdown of the precursor to free acetylcholine takes place at a higher pH , e.g. 6.0 or 6.5 at 37° . The precursor is non-dialysable or diffusible and has the properties of a protein complex.

3. Acetylcholine-precursor is stable under neutral conditions at 0° but less so at higher temperatures.

4. Acetylcholine-precursor is synthesized in brain tissue when the latter is allowed to respire in presence of glucose, sodium lactate or sodium pyruvate. Under anaerobic conditions little or no synthesis takes place. The sodium salts of the following acids bring about little or no synthesis of acetylcholine precursor under conditions when glucose, lactate or pyruvate are effective: acetic, succinic, acetoacetic, α -ketoglutaric, α -glycerophosphoric, hexosediphosphoric; *dl*-glycer-aldehyde is also inert and inhibits the synthesis of acetylcholine-precursor in presence of glucose.

5. The presence of eserine has no measurable effect on the synthesis or breakdown of acetylcholine-precursor.

6. The presence of choline or acetylcholine increases the rate of synthesis of acetylcholine-precursor when brain is respiring under optimal conditions. The effect of acetylcholine is not greater than that of choline and it is concluded that its effect is due to choline liberated by choline esterase.

7. The effect of glucose, lactate or pyruvate in securing synthesis of acetylcholine precursor is much greater than that due to acetylcholine. It is unlikely that the latter is first formed and that this produces "acetylcholine-precursor" by an adsorption process.

8. A provisional scheme of acetylcholine synthesis is put forward to account for the experimental results.

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