CLXXXV. ACETYLCHOLINE METABOLISM IN THE CENTRAL NERVOUS SYSTEM

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THE substance of this paper forms a continuation of our work [Mann *et al.* 1938, 1; 1939] on the conditions underlying acetylcholine formation in brain tissue *in vitro*. It demonstrates the influence of glucose at low concentrations, affords an explanation of results recently obtained on the acetylcholine contents of the brains of insulin-treated animals [MacIntosh, 1939] and indicates why Stedman & Stedman [1939] were unable to observe the accelerating effect of glucose on acetylcholine formation in brain.

Technique

The method of estimation of acetylcholine (free, "bound" and total), and the preparation and examination of brain tissue are described fully in previous papers [Mann *et al.* 1938, 1; 1939]. The reader is referred to these papers for full experimental details. All estimations are given in terms of acetylcholine chloride.

The effects of glucose at varying concentrations on acetylcholine synthesis by brain slices

It is now known [Quastel *et al.* 1936; Mann *et al.* 1938, 1; 1939] that in the presence of glucose and under aerobic conditions, brain tissue, whether sliced or minced, synthesizes acetylcholine. In the absence of glucose the synthesis is small or negligible. The importance of glucose for acetylcholine production has been confirmed by MacIntosh [1938] in perfusion experiments on intact ganglia. It is further known [Mann *et al.* 1939] that the addition of K ions (0.031 *M*) to a medium containing eserine, and in which intact brain slices are respiring, brings about a large increase in the rate of formation of acetylcholine. The total ester formed in 1 hr. at 37° may reach a value of over 40 μ g. (acetylcholine chloride) per g. wet weight of tissue.

Experiments have now been carried out on the effects of glucose at varying concentrations on the rate of acetylcholine formation by intact rat brain slices respiring in a medium containing eserine and 0.031 *M* KCl. The results are shown in Table I, where it will be seen that low concentrations of glucose are

Table I.

Rat brain slices in bicarbonate-Locke-eserine medium, containing KCl to give final concentration of 0.031 *M*. 95% O₂ + 5% CO₂. 1 hr. 37°.

Exp. ...	Total acetylcholine formed, μ g./g.				
	A	B	C	D	E
Medium with no added glucose	5.1	5.9	5.3	3.5	7.8
+ 5 mg. glucose/100 ml.	12.8	—	—	—	11.0
+ 10 mg. glucose/100 ml.	14.5	—	—	—	17.0
+ 20 mg. glucose/100 ml.	26.7	33.3	33.3	27.9	—
+ 192 mg. glucose/100 ml.	—	33.7	—	38.3	—

(1506)

effective in securing acetylcholine synthesis. In the absence of added glucose, or in the presence of the very small quantities still remaining in the washed brain slices, there is but little formation of acetylcholine. The addition of glucose at a concentration of only 5 mg./100 ml. brings about a definitely increased rate of synthesis. A glucose concentration of 20 mg./100 ml. brings about a rate of acetylcholine formation which may be nearly maximal.

This remarkable activity of glucose in securing acetylcholine synthesis in the central nervous system probably accounts for the finding of MacIntosh [1939] that the brains of insulin-convulsed rats contain as much acetylcholine as those of normal animals. Convulsions of the animal may take place at blood sugar concentrations which are still capable of giving rise to acetylcholine formation. The experiment described in Table II confirms those of MacIntosh and also shows that the brains of insulin-convulsed animals have retained their ability to synthesize acetylcholine in presence of glucose.

Table II

A rat brain was minced and suspended in ten times its volume of eserized Locke solution. 1 ml. of the suspension was added to 2 ml. phosphate-eserine-Locke medium (with glucose added) and shaken in air at 37° for 1 hr., after which estimations of acetylcholine were made. The same procedure was carried out with the brain of an insulin-convulsed rat. Acetylcholine estimations were also carried out on the suspension before the incubation at 37° was commenced; these are the initial estimations.

	Total acetylcholine, $\mu\text{g./g.}$	
	Normal rat brain	Insulin- convulsed rat brain
Initial estimations	5.9	5.1
Medium + glucose (0.01 M)	21.4	18.8

*The effects of fructose, mannose and galactose on acetylcholine formation
by brain slices*

Of the three sugars, fructose, mannose and galactose, only mannose approaches glucose in its activity in bringing about acetylcholine synthesis. These sugars were examined at a concentration of 20 mg./100 ml. A low concentration was employed so as to afford comparison with glucose which is very active at this concentration and also to avoid complications due to the possible presence of traces of glucose as an impurity in the sugar (especially the fructose¹) preparations. Typical results are quoted in Table III. These results show a

Table III

Rat brain slices. Bicarbonate-eserine-Locke medium $\text{K}^+ = 0.031 \text{ M}$. 95 % $\text{O}_2 + 5\%$ CO_2 . 1 hr. 37°.

	Total acetylcholine, $\mu\text{g./g.}$
Medium	3.5
+ 20 mg. glucose/100 ml.	27.9
+ 20 mg. fructose/100 ml.	7.2
+ 20 mg. galactose/100 ml.	5.4
+ 20 mg. mannose/100 ml.	20.0

small effect of fructose in increasing acetylcholine formation, but we doubt whether this finding is a real one, for in one or two experiments we have failed to observe even this small effect. It is also possible that traces of glucose in the

The fructose was a B.D.H. (glucose-free) preparation.

fructose preparation might yield an irregular small accelerating effect. The small effects of fructose and of galactose, as compared with that of glucose in securing acetylcholine synthesis, confirm the observations of Kahlson & MacIntosh [1939] on the effects of sugars on acetylcholine production in perfused ganglia. According to these authors galactose has 5 % of the activity of glucose whilst fructose is inert.

The failure of Stedman & Stedman to confirm the accelerating effect of glucose on acetylcholine synthesis by brain tissue

Stedman & Stedman [1939], in a recent paper, have failed to confirm the accelerating action of glucose on acetylcholine synthesis in isolated brain and state that their "results are in complete contradiction with those of Mann *et al.* [1938, 1]". Moreover they state that their "experiments were carried out under conditions as similar as possible to those of Mann *et al.*" It is necessary to consider their experimental method in some detail.

They ground 1 g. portions of minced rat brain tissue in 0.5 ml. saline-eserine solution, and washed these into flasks with 5 ml. phosphate-Ringer solution. To one flask was added 1 ml. saline and to the other was added 1 ml. saline containing 2 mg. glucose. The flasks, after filling with O_2 , were shaken at 37° for 2 hr. It was found that the yield of acetylcholine was identical in both cases—the yield being at least $18 \mu\text{g./g.}$, this being expressed in terms of acetylcholine bromide.¹ This yield is $14.4 \mu\text{g./g.}$ in terms of acetylcholine chloride.

It is easy to see that the failure of Stedman & Stedman to show an accelerating effect of glucose on acetylcholine production was due to their use of relatively large quantities of rat brain tissue. Such large quantities of tissues, which were not washed free of metabolites, introduced considerable concentrations of glucose and lactic acid into the phosphate-Ringer medium, the concentrations being sufficient to yield practically optimal synthesis under the experimental conditions employed. Moreover Stedman & Stedman made no observations of the rates of O_2 uptake by the suspensions in the two flasks. It is possible that under the conditions they employed the rates of O_2 uptake were so great as to be limited by the diffusion of O_2 into the medium and not by the concentration of metabolites present. Under such conditions the addition of a further quantity of metabolite (glucose) would not be expected to add materially to the effect of the glucose or other metabolites already present.

We have carried out experiments on the rates of formation of acetylcholine using varying quantities of minced brain tissue, in the presence and in the absence of added glucose. Typical results are noted in Table IV. It will be seen that the effect of added glucose is less when a large quantity of minced brain is taken than when a small quantity is employed. This would be expected in view of the quantity of glucose (or lactic acid) already present in the tissue. Moreover the yield of acetylcholine with small quantities of tissue in presence of glucose is higher than with large quantities of tissue in presence of glucose. This is probably due to the higher rates of O_2 consumption (per unit weight of tissue) which are secured when relatively small quantities of tissue are employed. The figures (obtained with small quantities of tissue) quoted in Table IV are similar to those quoted in our earlier papers. It will be noted that the yield of acetylcholine with small quantities of tissue in the absence of added glucose is relatively high. This is partly due to synthesis brought about by metabolites still

¹ Dr Stedman has kindly informed us that all his figures are expressed in terms of acetylcholine bromide.

Table IV

Exp. A. Two rat brains (3 g.) were chopped in presence of 0.6 ml. 1/100 eserine-sulphate-Locke solution. This was added to phosphate-Locke solution to give a total vol. of 9 ml. Appropriate volumes of the suspension were placed in Warburg flasks in which were placed solutions (saline, or phosphate-saline) which were either free from glucose or contained 0.01 *M* glucose. Total vol. in Warburg flasks = 3.2 ml. Air. 37°. 3 hr.

Medium	Amount of minced tissue present	Total acetylcholine $\mu\text{g./g.}$
Saline	1.0 g. + no glucose added	10.0
Saline	1.0 g. + glucose (0.01 <i>M</i>)	13.5
Phosphate-saline	0.1 g. + no glucose added	10.9
Phosphate-saline	0.1 g. + glucose (0.01 <i>M</i>)	16.5

Exp. B. Two rat brains were chopped and suspended in 2 vols. Locke medium. Appropriate quantities of the suspension were added to phosphate-eserine-saline media, with and without glucose, in Warburg flasks. Air. 37°. 2 hr.

Wt. of tissue taken g.	Total acetylcholine, $\mu\text{g./g.}$	
	No glucose added	Glucose (0.01 <i>M</i>) added
1.0	6.8	7.0
0.2	11.0	17.0
0.1	11.6	20.4

present and partly due to the acetylcholine present initially, or preformed, in the tissue before the commencement of the experiment.

We have carried out, also, experiments with relatively large quantities of tissue which have been washed with Locke solution. It is a difficult matter to remove metabolites from minced brain tissue by washing without removing at the same time factors necessary for the oxidation of glucose. Hence only a partial removal of metabolites is possible if the respiratory system is to be left intact. Results, in Table V, show that the glucose effect in increasing acetylcholine formation is greater with washed than with unwashed brain, and moreover that the acetylcholine yields by washed and unwashed brains, in presence of glucose, under the experimental conditions employed are the same.

Table V

Rat brains were minced and suspended in Locke solution. One part was washed with Locke solution by centrifuging. Appropriate volumes of unwashed and washed brain suspensions were placed in phosphate-saline medium with and without glucose. O_2 . 37°. 2 hr.

	Total acetylcholine, $\mu\text{g./g.}$
0.8 g. unwashed brain. No glucos	7.5
0.8 g. unwashed brain + glucose (0.01 <i>M</i>)	12.5
0.8 g. washed brain. No glucose	4.5
0.8 g. washed brain + glucose (0.01 <i>M</i>)	12.5

Finally, using unwashed minced brain tissue, we have shown that the acetylcholine production is less in an atmosphere of N_2 than in one of O_2 , whether glucose be added or not. Such a diminution in acetylcholine formation under anaerobic conditions when using relatively large quantities of unwashed brain tissue is to be expected, since we have shown in earlier work that the synthesis of acetylcholine due to the presence of glucose is suppressed in the absence of O_2 . These results are shown in Table VI.

Table VI

Rat brains were minced and suspended in 2 vols. Locke solution. Appropriate quantities were placed in Warburg vessels containing phosphate- eserine-Locke medium with or without glucose. Two vessels were filled with O_2 , and two with N_2 . Incubation for 2 hr. at 37° .

		Total acetylcholine, $\mu\text{g./g.}$
0.8 g. brain tissue. No glucose	O_2	9.0
0.8 g. brain tissue + glucose (0.01 M)	O_2	15.0
0.8 g. brain tissue. No glucose	N_2	3.0
0.8 g. brain tissue + glucose (0.01 M)	N_2	4.4

The observations which have just been recorded make it clear that if a relatively large quantity of minced brain tissue is employed under our experimental conditions, a relatively high rate of acetylcholine formation may take place, the rate being dependent on the amount of glucose present initially in the tissue and on the conditions of oxygenation. The addition of glucose may, or may not, materially increase this rate.

Hence the observation of Stedman & Stedman that the addition of glucose did not increase the acetylcholine formation in isolated brain tissue is one which might have been expected considering the experimental conditions employed and is certainly not a "complete contradiction" of our results.

It is clear that Stedman & Stedman agree that acetylcholine is produced when minced rat brain, suspended in a phosphate-Ringer-eserine medium, is incubated in O_2 at 37° . Yet earlier on in their paper they say: "Apparently water, like certain organic solvents, destroys the mechanism responsible for the production of acetylcholine in brain tissue." It is difficult to reconcile a statement such as this with their own observations on acetylcholine formation in rat brain tissue suspended in Ringer solution.

Yield of acetylcholine

Stedman & Stedman [1939] give the yield of acetylcholine in their experiment with rat brain tissue suspended in Ringer solution as $14.4 \mu\text{g./g.}$ (acetylcholine chloride). This amount, as they say, is of the same order as the amounts obtained by ourselves when using minced brain tissue in a phosphate medium. Usually small quantities of tissue in presence of glucose give higher figures, whilst larger quantities of tissue in absence of glucose give smaller figures—but the order of magnitude is the same. The reasons for this have already been given.

Our method of analysis of total acetylcholine depends on acidification of the suspension of the brain tissue with subsequent neutralization, the method being relatively rapid and simple [for details see Mann *et al.* 1939]. Stedman & Stedman use a method which they admit to be long and tedious and which obviously gives results of the same order as our own. For this reason we have adhered to the method we have usually used and have not carried out routine analyses by the method of Stedman & Stedman. We have, however, carried out numerous control experiments to show that estimations of acetylcholine based on extraction of the tissue with 10% trichloroacetic acid do not give results markedly higher than those obtained by our method.

It is clear that the yield of acetylcholine obtained by minced rat brain tissue suspended in a phosphate-Ringer medium after incubation at 37° for 2 hr. is small compared with that obtained by rat brain slices incubated in a bicarbonate-glucose-Locke-0.03 M K^+ medium at 37° for 1 hr. In the latter case yields varying from 30 to $50 \mu\text{g./g.}$ have been found [Mann *et al.* 1939; see also Table I].

The yields of acetylcholine from ether-soaked or chloroform-soaked brains as used by Stedman & Stedman are only a small fraction (one-third to one-quarter) of the yields obtained when rat brain slices are allowed to respire in a bicarbonate-glucose-potassium medium.

"Bound" acetylcholine

We [1938, 1] have shown that repeated washing of minced brain or of brain slices fails to extract all the acetylcholine and that a form of acetylcholine is left which is not attacked by choline esterase, but which is rapidly broken down at room temperature on treatment with acid (*pH* 2–3). This form of acetylcholine is referred to by us [1939] as "combined" or "bound" acetylcholine. The fact that such a combined ester, easily split by acid, exists in brain had been shown practically at the same time, and independently, by Loewi *et al.* [1938]. We [1938, 1; 1939] have already stated that this "bound" acetylcholine is probably identical with that described by Loewi and with that which Corteggiani [1937] has shown to give rise to free acetylcholine on heating at 70° for 3 min. We found [1938, 1] that "bound" acetylcholine is synthesized in brain tissue when the latter is allowed to respire in presence of glucose, lactate or pyruvate, in an eserine medium, but under anaerobic conditions or in the absence of a suitable metabolite no synthesis takes place. "Bound" acetylcholine is also synthesized by brain tissue under the proper physiological conditions in the *absence* of eserine, although no free acetylcholine can be detected. It was also shown [1938, 1; 1939] that the formation of free acetylcholine most probably takes place through the intermediate formation of "bound" acetylcholine which was therefore referred to as acetylcholine-precursor. The reader is referred to the papers in question for further details concerning the formation and properties of "bound" acetylcholine.

"Bound" acetylcholine is not only broken down to produce free acetylcholine after acid treatment but it is also broken down rapidly on shaking in aqueous media with chloroform, the amounts of free acetylcholine liberated being approximately the same. Details are given in our earlier paper [1938, 1]. It has been our custom when treating brain tissue suspension with chloroform (0.2 ml. chloroform to 5 ml. suspension) to leave the mixture at room temperature for 1 hr. But it may easily be shown that the "bound" acetylcholine is broken down in 10 min. at room temperature after the tissue is shaken with chloroform and that longer contact with chloroform leads to no appreciable increase in free acetylcholine. A typical result is shown in Table VII.

Table VII

Rat brain slices incubated aerobically in bicarbonate-Locke-glucose-eserine medium. 1 hr. 37°. Then washed in Locke solution to remove free acetylcholine. Divided into two equal lots, one shaken with chloroform and left at room temperature for 10 min. and the other shaken with chloroform and left at room temperature for 30 min. Chloroform removed *in vacuo* and the liberated free acetylcholine estimated.

	Acetylcholine liberated, $\mu\text{g./g.}$
After shaking with chloroform and standing for 10 min. at room temperature	12.0
After shaking with chloroform and standing for 30 min. at room temperature	12.0

The same phenomenon occurs with the "bound" acetylcholine which is present in brain tissue freshly obtained from the animal. It can scarcely be

argued that the free acetylcholine rapidly formed after treatment with acid or with chloroform is due to synthesis brought about by these reagents under our experimental conditions. Hence the "bound" acetylcholine in fresh brain tissue, prior to incubation, is preformed and has been referred to by us as the preformed acetylcholine precursor. Stedman & Stedman [1939], however, dispute the existence of the preformed combined form of acetylcholine or of the preformed precursor. These authors seem to be confused over the definition of the preformed complex. They appear to think that we state that the increase of acetylcholine production obtained after acidification of a brain suspension which has been incubated in O_2 at 37° for some time is a demonstration of the existence of, and a measure of the amount of, the "preformed precursor" in the tissue after incubation. This, of course, is not true. The measure of preformed complex cannot be obtained *after* incubation of the tissue, only *before* incubation. The amount of complex found after incubation is referred to as the "bound" or combined acetylcholine formed, and usually experiments are carried out to ascertain the initial quantities of both "bound" and free acetylcholine. Moreover, Stedman & Stedman state that we "only measure the acetylcholine content of the suspension medium" but it must be obvious that measurements of total and "bound" acetylcholine are only made on the suspension medium when the tissue suspended in it has been treated with acid or chloroform etc. to accomplish the breakdown of the complex.

Apparently Stedman & Stedman [1939] admit the existence of Loewi's "bound" acetylcholine which is broken down by acids as well as by alcoholic HCl. They do not admit the existence of the preformed acetylcholine complex we have described because (a) we state that it is broken down by chloroform as well as by acid treatment and (b) they [1939] find that incubation of chloroform-treated brain leads to greater acetylcholine production than is found when brain tissue is treated at once with trichloroacetic acid or acid alcohol.

A feature, however, of the work of Stedman & Stedman is their use of large quantities of brain tissue in the absence of added aqueous media. In their experiments with chloroform-treated ox brain they use 10 g. brain tissue at a time, this amount being macerated with 5 ml. of a solution of eserine in chloroform. They find that an initial figure of $1.3\text{--}1.4\mu\text{g./g.}$ acetylcholine bromide is increased by incubation for 2 hr. at room temperature in presence of chloroform to $6\mu\text{g./g.}$ acetylcholine bromide. It is possible—and indeed probable as suggested by us earlier—that some synthesis, due to the glucose or lactic acid present in the brain tissue employed, is taking place at room temperature, in spite of the known deleterious action of chloroform on acetylcholine synthesis at 37° [Mann *et al.* 1938, 1].¹ Whether chloroform—or ether—under certain conditions can accelerate this synthesis, is a question which will be discussed shortly.

It is apparent from what has been said, that the increased formation of acetylcholine in chloroform-treated brain incubated at room temperature is not evidence against the existence of the preformed "bound" acetylcholine which we have described. The evidence submitted in our earlier work [1938, 1] and confirmed here shows that the complex in question exists, that "bound" acetylcholine is broken down on shaking with chloroform (see Table VII, as well as earlier work), and that it is probably identical with the complex described by other workers [Corteggiani, Loewi].

It should be emphasized that "bound" acetylcholine is synthesized by

¹ It is obvious, since the brain tissue is not diluted by the presence of aqueous media the active substrates (e.g. glucose or its equivalent of lactic acid) must still be present at approximately the same high concentrations as found in brain *in vivo*.

isolated brain examined under optimal physiological conditions in the absence of eserine. The free ester is not detectable owing to its destruction by the choline esterase present. Clearly such synthesis must be taking place *in vivo* and accounts for the fact that acetylcholine can be found in brain when taken freshly from the animal. This ester is mostly in the "bound" condition, for only traces of the free ester can exist in presence of the choline esterase of the tissue. The complex is the preformed precursor or preformed "bound" acetylcholine, whose existence Stedman & Stedman apparently dispute.

We showed [1938, 2] that acetylcholine formation by chloroform-treated ox brain is less at 37° than at 18° and this fact Stedman & Stedman [1939] have confirmed. They could not confirm our statement that the synthesis is no less at 0 than at 37°. Conceivably some difference in technique is responsible for the divergence of opinion here, but the significant point that the formation of acetylcholine in chloroform-treated brain is greater at room temperature than at 37° is conceded. Stedman & Stedman explain this by stating that chloroform has a dual action in brain tissue, a facilitating action on acetylcholine formation accompanied by a destruction of the mechanism responsible for its formation. We [1938, 1] had already pointed out that chloroform exerts a dual action, for it breaks down "bound" acetylcholine into the free ester and inhibits the synthesis of the ester at 37°.

The effects of ether

Stedman & Stedman [1939] turn from a consideration of chloroform to that of ether, "the effect of which" they say "must clearly be similar to that of chloroform". With ether-treated brain they find that the yield of acetylcholine is greater than when the tissue is treated with chloroform and moreover that the effect of ether at 37° is greater than at room temperature. They find as large effects with rat brain as with ox brain.

In repeating this work we have confined ourselves entirely to rat brain since this can be obtained quite fresh.

We have confirmed the fact that brain tissue macerated with half its volume of a solution of eserine in ether produces more acetylcholine than a similar brain ground with a solution of eserine in saline after incubation at 37° under aerobic conditions. Typical results are given in Table VIII. They are of the same order

Table VIII

Two rat brains were chopped and weighed out into 1 g. lots. One lot was ground with 0.5 ml. saline-eserine sulphate (1/800). Another lot was ground with 0.5 ml. ether-eserine base (1/1000). Both were placed in stoppered flasks and incubated in air for 2 hr. at 37°. Total acetylcholine was estimated as the chloride at the end of this period by acid treatment, ether being finally removed by evaporation at room temperature *in vacuo*.

	Total acetylcholine, $\mu\text{g./g.}$	
	Exp. A	Exp. B
Brain incubated with saline eserine	5.0	6.2
Brain incubated with ether eserine	8.0	10.0

of magnitude as those given by Stedman & Stedman who cite as their presumably highest yield with rat brain treated with ether 16 $\mu\text{g./g.}$ (as acetylcholine bromide, i.e. 12.8 $\mu\text{g./g.}$ acetylcholine chloride). It may be emphasized again that this value is small compared with the yields of acetylcholine produced by rat brain slices incubated in bicarbonate-glucose- K^+ (0.031 *M*) medium (see Table I).

The fact that the presence of ether in minced brain should increase the acetylcholine formation is of interest in view of the fact that the addition of ether

to washed minced whole brain suspended in a glucose-saline or Ringer medium and incubated at 37° aerobically prevents acetylcholine synthesis. This result is shown in Table IX. Evidently when ether is shaken with a brain suspension

Table IX

Three rat brains were chopped, weighed into four 0.5 g. lots each of which was transferred to a centrifuge tube and each washed by centrifuging with 5 ml. eserized Locke solution. The washed tissues were mixed with 0.2 ml. 1/400 eserine sulphate solution. To two of the tubes were added 0.25 ml. ether and the contents were transferred to Warburg flasks containing media mentioned below. Incubation for 1 hr. in air at 37°.

Medium	Total acetylcholine formed, $\mu\text{g./g.}$
Phosphate-Locke	5.0
Phosphate-Locke-glucose (0.01 M)	10.0
Phosphate-Locke-ether	4.0
Phosphate-Locke-ether-glucose (0.01 M)	4.0

in an eserine-phosphate medium, the ability of glucose to induce synthesis of acetylcholine is inhibited. Ether is therefore more toxic to the synthetic processes in brain when it is shaken in an aqueous medium, than when it is macerated with minced brain and this is incubated aerobically in the absence of an aqueous medium. The inhibitory effect of ether on the respiration of brain tissue incubated in a phosphate-glucose-Ringer medium has been shown by Jowett & Quastel [1937].

It is to be noted that the two methods of administering ether to brain tissue differ greatly. When brain tissue is ground in a mortar with half its volume of ether, most of the latter disappears by evaporation, and but a fraction is left adhering to the brain substance. It is questionable whether the ether has penetrated all cells of the tissue. When it is shaken in an aqueous suspension with ether as in the Warburg manometric technique the brain cells are constantly exposed to a solution saturated with ether. Possibly in this case the penetration of ether into the cells, and therefore its destructive effects, are more marked than in the previous method which is that adopted by Stedman & Stedman.

It may be shown that ether, like chloroform or acid, when shaken with a suspension of broken up washed brain slices releases free acetylcholine from the "bound" ester. The breakdown of the "complex" into free acetylcholine takes place in 5 min. at room temperature. This result is shown in Table X.

Table X

Rat brain slices incubated in bicarbonate-glucose-eserine-Locke medium. 1 hr. 37°. 95% O₂ + 5% CO₂. Slices then well washed with saline eserine to remove free acetylcholine. Slices broken up and treated with (a) HCl to pH 2-3, for 5 min., then neutralized and centrifugate examined for acetylcholine, (b) ether (0.5 ml.) for 5 min., centrifuged, ether removed from centrifugate by evaporation *in vacuo* at room temperature and centrifugate then examined for acetylcholine.

	Acetylcholine found, $\mu\text{g./g.}$
(a) Acid treatment (5 min.)	14.2
(b) Ether treatment (5 min.)	15.8

Since it is evident that ether, like chloroform, is inhibitory to the synthesis of acetylcholine in brain when this tissue is examined in bicarbonate- or phosphate-Locke media, we have confined our experiments to the technique adopted by Stedman & Stedman to study the mechanism whereby ether accelerates acetylcholine synthesis in minced brain.

Action of ether on formation of free and "bound" acetylcholine

The accelerating action of ether on acetylcholine formation in minced brain incubated in air at 37° in the absence of the addition of an aqueous medium appears to lie entirely with the free acetylcholine, the "bound" ester being diminished in quantity. This is shown by the results given in Table XI. Thus,

Table XI

Three rat brains were minced and weighed out into portions of 0.8 g. tissue. One portion was ground in a mortar with 0.4 ml. saline-eserine and the other ground in a mortar with 0.4 ml. eserine ether. The two portions were placed in stoppered flasks and incubated aerobically at 37° for 2 hr. Then they were washed twice with saline-eserine, the centrifugates being examined for free acetylcholine. The washed residues were treated with acid to break down "bound" acetylcholine into the free ester which was estimated in the final centrifugate.

	Acetylcholine found, $\mu\text{g./g.}$		
	Free	"Bound"	Total
* Brain treated with saline eserine	4.2	1.5	5.7
Brain treated with ether eserine	7.4	0.5	7.9

although the total acetylcholine is increased by the presence of the ether, it is the free acetylcholine in the tissue whose amount is actually increased. This conclusion is confirmed by the following experiment. Minced rat brain tissue was incubated aerobically at 37°, after maceration with saline and with ether, no eserine being added until the *end* of the incubation time. After this late addition of eserine it was found that the total acetylcholine was definitely *less* in the ether-treated brain than in the saline-treated tissue. This result is shown in Table XII,

Table XII

Four rat brains were minced and weighed out into portions of 0.8 g. tissue. One portion was ground with 0.4 ml. saline, another with 0.4 ml. ether, a third with saline-eserine and a fourth with ether-eserine. These were transferred to Warburg flasks, in two of which 0.2 ml. eserine saline were present in the side tube. Incubated at 37° for 2 hr. in O₂. Eserine tipped in at the end of 2 hr.

		Total acetylcholine, $\mu\text{g./g.}$
Brain + saline	Eserine added after 2 hr.	3.0
Brain + ether	Eserine added after 2 hr.	1.0
Brain + saline	Eserine added initially	6.2
Brain + ether	Eserine added initially	11.7

where results when eserine was added initially are given for comparison. This finding, which apparently contradicts the conclusion that ether brings about an acceleration of acetylcholine formation, is easily understood when it is realized that the effect of ether is to increase the rate of formation of free acetylcholine. In the absence of eserine the latter is destroyed and only the bound acetylcholine is estimated when eserine is added at the termination of the experiment.

These effects of ether in presence and absence of eserine are remarkably similar to those of K ions noted in our last paper [1939], but they are on a much smaller scale.

We then decided to investigate the action of Ca ions on the ether acceleration of acetylcholine synthesis since we know [1939] that Ca ions neutralize the effects of K ions on the synthesis of the ester.

Action of Ca^{++} on acetylcholine formation in ether-treated brain

The addition of 0.02 *M* CaCl_2 to rat brain macerated with half its volume of ether eserine has the effect of greatly diminishing the accelerating action of ether on acetylcholine formation. Typical results are shown in Table XIII. Such a

Table XIII

Rat brains were minced and weighed out into lots of 0.8 g. tissue. Portions were ground in a mortar with (1) 0.4 ml. saline-eserine + 0.4 ml. saline, (2) 0.4 ml. saline-eserine + 0.4 ml. 0.08 *M* CaCl_2 , (3) 0.4 ml. ether-eserine + 0.4 ml. saline, (4) 0.4 ml. ether-eserine + 0.4 ml. 0.08 *M* CaCl_2 . These were transferred to stoppered flasks and incubated aerobically at 37° for 2 hr.

	Total acetylcholine formed, $\mu\text{g./g.}$		
	Exp. A	Exp. B	Exp. C
Brain + saline eserine	5.9	3.5	5.7
Brain + saline eserine + 0.02 <i>M</i> CaCl_2	5.9	—	—
Brain + ether eserine	7.1	6.7	7.9
Brain + ether eserine + 0.02 <i>M</i> CaCl_2	3.9	1.4	5.2*

* In this experiment the concentration of CaCl_2 was 0.01 *M*.

large diminishing effect on acetylcholine formation does not take place in the absence of ether. The further resemblance of the effect of ether to that which occurs with K ions is again obvious.

It must be noted, however, that the action of K ions on minced brain is much less pronounced than on brain slices [Mann *et al.* 1939] and it is not to be supposed on present evidence that K ions and ether are interchangeable in their effects on acetylcholine formation in minced brain.

The results suggest, however, that ether is acting on minced brain (in the absence of an aqueous medium) in a manner analogous to the action of potassium on brain slices. It was concluded by us [1939] that the latter depended on a change of cell permeability induced by the increased concentration of K^+ in the medium bathing the slices. In like manner we may imagine that ether exercises its effect by causing a changed permeability of the cells with which it comes into contact.¹ If this view is true then we may expect that the ether effect, like that of potassium, will only take place in the presence of a "synthesizing substrate" such as glucose. Unfortunately this cannot be tested by the technique adopted by Stedman & Stedman since it is difficult to wash away metabolites satisfactorily from large quantities of minced brain tissue without affecting the respiratory mechanisms of the tissue. Moreover such washing introduces aqueous media into the tissue which renders it more susceptible to the toxic action of the ether. The problem can, however, be attacked from a different point of view. Since it is known that acetylcholine synthesis due to the presence of glucose depends on aerobic conditions, it follows that if the view we have suggested is correct then the accelerating effect of ether will be considerably lower in N_2 than in O_2 .

Effects of anaerobic conditions on the ether acceleration of acetylcholine formation

The effect of substituting N_2 for O_2 in the atmosphere surrounding the ether treated brain greatly reduces the acetylcholine formation. Typical results are

¹ Similarly the relatively small enhancement of the rate of acetylcholine formation found with chloroform-treated brain when incubated, in the absence of aqueous media, at room temperature may be attributed to changes of cell permeability effected by the chloroform. Much of the argument applying to ether probably applies also to chloroform; the latter, however, is much more damaging than ether towards the synthesizing enzyme.

shown in Table XIV. Probably the yields found in N_2 would be still lower if all the O_2 still remaining in the tissue could be removed. It is experimentally difficult, however, to accomplish this.

Table XIV

Rat brains were minced and weighed out into lots of 0.5 g. Portions were ground with 0.25 ml. ether eserine, transferred to Warburg flasks one of which was filled with N_2 and the other with O_2 . Incubated for 2 hr. at 37° .

			Total acetylcholine formed, $\mu\text{g./g.}$	
			Exp. A	Exp. B
Brain.	Ether eserine.	O_2	11.7	7.5
Brain.	Ether eserine.	N_2	6.0	4.5

The observations recorded above indicate that the synthesis of acetylcholine in ether-treated minced brain depends on the presence of a metabolite such as glucose which is known to require aerobic conditions for synthesis of the ester. The action of ether in accelerating acetylcholine formation seems to us to be most easily explained on the same lines by which the K effect was interpreted, i.e. by a change in permeability of the cell. The reader is referred to our recent paper [1939] for full details of this.

Stedman & Stedman [1939] give no explanation of the ether effect beyond stating that one of the functions of the solvent is to act as vehicle for conveying the eserine into the tissue.

Acetoacetate as a possible precursor of acetylcholine in brain tissue

We have already shown [1938, 1, 2] that acetoacetate added to brain tissue respiring in a phosphate-Locke-eserine medium in O_2 at 37° does not yield acetylcholine at a rate which even approaches that brought about by the presence of glucose. Consequently we are loath to believe that acetoacetate is a precursor of acetylcholine¹. Stedman & Stedman [1939], however, find using their ether-treated minced brain technique that a yield of $17 \mu\text{g./g.}$ (acetylcholine bromide) is increased to $23 \mu\text{g./g.}$ by the addition to the tissue of 20 mg. sodium acetoacetate. In another experiment with ox brain a value of $8.7 \mu\text{g./g.}$ acetylcholine bromide was increased to $14.5 \mu\text{g./g.}$ by the addition of the same quantity of acetoacetate. They consider these results to be substantial evidence that acetoacetic acid is in fact a precursor of acetylcholine but they state they have failed to show a synthesis of acetylcholine from acetoacetate in aqueous media. Our own position is that we must regard accelerations of syntheses which take place only in ether- or chloroform-treated brains and which do not seem to occur in brain tissue bathed in a Ringer medium, as of questionable physiological significance so far as the mechanism of the biological formation of acetylcholine is concerned. It has not been proved, because acetoacetate enhances the effect of chloroform and of ether in increasing the rate of formation of acetylcholine in brain, that acetoacetate itself is converted into acetylcholine. Many factors appear to influence the action of chloroform and of ether on brain as the observations given above have shown and it may well be that acetoacetate is reacting in a physicochemical, rather than in a strictly chemical, sense. It is clear that further information is required before the conclusion can be accepted that acetoacetate is a direct precursor of acetylcholine.

¹ See also Kahlson & MacIntosh [1939] who have found acetoacetate to be ineffective as a promoter of acetylcholine synthesis in perfused ganglia.

SUMMARY

1. Glucose at low concentrations, e.g. below 20 mg. per 100 g. will bring about the synthesis of acetylcholine in intact rat brain slices.

2. Fructose and galactose have relatively small effects (that of fructose being doubtful) in securing acetylcholine synthesis, but mannose has an activity approaching that of glucose.

3. The failure of Stedman & Stedman to confirm the effect of glucose in enhancing acetylcholine production by brain is shown to be due to the limitations of their experimental technique.

4. The rate of formation of acetylcholine in brain tissue using the technique of Stedman & Stedman is much less than that obtained when intact rat brain slices respire in a bicarbonate-glucose medium containing 0.03 *M* K⁺.

5. Preformed "bound" acetylcholine exists in brain tissue freshly obtained from the animal and the suggestion of Stedman & Stedman that it does not exist is based upon insufficient experimental evidence.

6. The effect of ether in enhancing acetylcholine formation in minced brain tissue when incubated at 37° in the absence of an aqueous medium is confirmed.

7. Ether is highly inhibitory to acetylcholine formation in brain tissue examined in a phosphate- or bicarbonate-Locke medium. It is less toxic when small quantities are macerated with brain in the absence of an aqueous medium.

8. In brain suspensions in aqueous media both ether and chloroform break down "bound" into free acetylcholine, the reaction taking place quickly (5 min.) at room temperature.

9. The accelerating action of ether on acetylcholine formation in minced brain, in the absence of an aqueous medium, lies entirely with the production of free acetylcholine, the amount of "bound" ester formed being diminished in quantity. The accelerating action of ether is diminished or removed by the addition to the tissue of 0.02 *M* Ca⁺⁺. The accelerating action is also greatly diminished by carrying out the experiment under anaerobic conditions. These effects closely resemble, though on a smaller scale, the action of K⁺ on acetylcholine formation in brain slices. It is suggested that the explanation of the ether effect is similar to that advanced by us [Mann *et al.* 1939] for the accelerating action of K ions. The fact that the presence of O₂ greatly increases the formation of acetylcholine in ether-treated brain suggests that the same metabolites are responsible for the synthesis as those in brain examined under more physiological conditions, e.g. glucose or lactic acid.

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