CIII. ACETYLCHOLINE METABOLISM IN THE CENTRAL NERVOUS SYSTEM

THE EFFECTS OF POTASSIUM AND OTHER CATIONS ON ACETYLCHOLINE LIBERATION

BY P. J. G. MANN, M. TENNENBAUM AND J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

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THE physiological evidence for the existence of a relationship between potassium ions and acetylcholine liberation in nervous structures [Brown & Feldberg, 1936; Feldberg & Guimaraes, 1936; Beznak, 1934] led us to examine the effects of changes of potassium ion concentration on the synthesis and liberation of acetylcholine in brain tissue *in vitro*. The results have thrown new light on the nature of the dynamic equilibria, involving acetylcholine metabolism, which exist in the central nervous system. The present communication is concerned with a description of these results and of their consequences.

TECHNIQUE

Estimation of acetylcholine. The ester was estimated by measuring the contraction of the eserinizied 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. All results are given in terms of acetylcholine chloride per g. tissue. Details of the estimation are given by Quastel *et al.* [1936].

Preparation and examination of brain tissue. The brain was carefully removed from the animal (rat, guinea-pig) as soon as possible after death. It was then either minced or sliced, the slices not being confined entirely to the cortex of the brain, but being taken from the entire cerebrum. Known quantities of the tissue (100–200 mg. wet weight)¹ were placed in suitable media in Warburg manometric vessels. The slices, 0.2–0.3 mm. thick, were bathed in Locke medium, allowed to drain thoroughly and weighed quickly on a torsion balance before being immersed in the medium in the Warburg flask. About 100 mg. wet weight of the tissue were placed in each flask, care being taken that there was an approximately equal distribution of the slices between the flasks. Possible inaccuracies due to the presence of different proportions of grey matter to white matter in the various flasks were eliminated by frequent repetition of the experiment, those results being accepted which had been consistently obtained after a number of experiments.

¹ It is important not to employ too large quantities of tissue under our experimental conditions, since not only may it be difficult to remove metabolites from such large quantities of tissue but the rates of O₂-uptake may be so great as to be limited by the diffusion of oxygen into solution and not by the amount of tissue or by the concentration of metabolite. Under such conditions it is clearly impossible to carry out comparative experiments on the influence of metabolites, etc. on the rates of acetylcholine formation. We have found that amounts of the order given, e.g. 100 mg. wet weight, are the most convenient.

The vessels were filled with O_2 , or a mixture of 95 % O_2 and 5 % CO_2 , or with other gases as will be indicated. The experimental period varied from 1 to 3 hr. at 37° , but was usually 1 hr., excluding an initial period of 15 min. allowed to enable the contents of the vessels to arrive at the temperature of the bath. The rates of O_2 -uptake or of gas exchange by the tissue in each vessel were always noted, as these gave valuable indications of the metabolic activities of the tissues under the given experimental conditions. At the end of the experimental period, the acetylcholine activity of the contents of each manometric vessel was estimated.

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. The phosphate-Locke medium generally used consisted of NaCl, 0.13 *M*; KCl, 0.004 *M*; $CaCl_2$, 0.002 *M*; sodium phosphate buffer solution pH 7.4, 0.03 *M*. The bicarbonate-Locke medium, which was used in presence of an atmosphere of 95 % O_2 + 5 % CO_2 , consisted of NaCl, 0.13 *M*; KCl, 0.004 *M*; $CaCl_2$, 0.002 *M*; $NaHCO_3$, 0.025 *M*. To these media were added glucose (0.01 *M*), etc., as occasion demanded.

When minced brain tissue was used in a phosphate medium, the manometric vessels were filled with air.

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

Estimation of free acetylcholine. The free acetylcholine is the amount found in the medium surrounding the tissue at the termination of an experiment. The slices were removed, after careful draining, from the contents of the vessel and placed in an eserinated-Locke medium. The contents of the vessel were then tested directly on the eserinated leech preparation, and the acetylcholine activity was estimated. When minced tissue was used, the contents of the manometric vessel were centrifuged and the acetylcholine activity of the clear centrifugate was estimated.

Estimation of "combined" acetylcholine. The slices from the manometric vessels, after being well washed in an eserinated Locke medium, were broken up with a glass rod and suspended in a solution of 2.1 ml. eserine-Locke medium, 0.5 ml. sodium phosphate (0.2 *M*) buffer solution pH 7.4 and sufficient *N* HCl to bring its pH to 2.0–3.0. After 30 min. at room temperature, the solution was made neutral (pH 7.4) by the addition of a few drops of *N* NaOH, the suspension was centrifuged and the acetylcholine activity of the clear centrifugate was estimated. The same treatment was accorded to minced tissue after this had been centrifuged and well washed with eserinated Locke medium.

This method of estimating "bound" or "combined" acetylcholine has been found to give quantitatively similar results to those obtained by trichloroacetic acid, chloroform or heat treatments.

Estimation of "total" acetylcholine. When it was unnecessary to make separate estimates of free and "combined" acetylcholine, the "total" (i.e. free + "combined") acetylcholine was determined by adding *N* HCl to the suspensions in the manometric vessels until the pH was 2.0–3.0, keeping them at room temperature for 30 min. and then making the solution neutral with *N* NaOH. The slices were well broken up with a glass rod before the solution was made acid. The suspensions were finally centrifuged and the "total" acetylcholine was estimated in the clear centrifugate.

Preliminary remarks concerning acetylcholine synthesis in brain

Quastel *et al.* [1936] showed that the formation of free acetylcholine by brain cortex slices is greatly increased when they are allowed to respire in a physiological medium containing eserine and either glucose, lactate or pyruvate. Since no such increased rate of formation of acetylcholine occurs when the slices are incubated in this medium under anaerobic conditions or when they are incubated in a medium free of a suitable metabolite (even if oxygen is freely available) it is evident that synthesis of acetylcholine takes place in brain tissue only under the proper physiological conditions. In the absence of eserine no acetylcholine can be detected, this being clearly due to the activity of the choline esterase present in the brain tissue.

It was then found by Mann, Tennenbaum & Quastel [1938] that there exists in brain tissue a combined form of acetylcholine which is pharmacologically inactive and which breaks down to yield free acetylcholine under a variety of conditions. It is stable under neutral conditions at 0°, but is less so at higher temperatures. Treatment with acid, e.g. at pH 3, releases free acetylcholine from the bound form at room temperature, but breakdown takes place at a higher pH, e.g. 6.0 or 6.5, at 37°. The substance releases free acetylcholine after treatment with a denaturing agent, e.g. after shaking with chloroform. It is apparently non-dialysable or diffusible and it has the properties of a protein complex. There can be little doubt that this substance is identical with the complex in brain tissue described by Corteggiani [1937] which on heating to 70° for 3 min. gives rise to acetylcholine. The action of alcohol, or of acid alcohol, in liberating free acetylcholine, described by Loewi [1937], is probably due to a denaturing action on "bound" acetylcholine. It has been thought for some time [see Gaddum, 1935] that acetylcholine found in tissue extracts may not be derived entirely from the freely diffusible ester in the tissue but that it may be present in the tissue as a comparatively stable precursor.

"Bound" acetylcholine was found by Mann, Tennenbaum & Quastel [1938] to be synthesized in brain tissue when the latter is allowed to respire in presence of glucose, lactate or pyruvate, 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, but, of course, in this case no free acetylcholine can be detected. The effect of glucose, lactate or pyruvate in securing synthesis of the "bound" acetylcholine is much greater than that due to added acetylcholine. It became clear that the experimental results were not consistent with the view that free acetylcholine is first formed and that this is adsorbed by the cell constituents to form "bound" acetylcholine. It seemed more probable that the synthesis of acetylcholine takes place through the intermediate formation of "bound" acetylcholine which we have therefore referred to as acetylcholine precursor. The main reasons for this conclusion are as follows.

(1) The formation of the complex is not greatly higher in the presence of eserine, which prevents the destruction of free acetylcholine, than in its absence [Mann, Tennenbaum & Quastel, 1938]. It would be expected that if the complex were formed secondarily by adsorption of free acetylcholine on cell structures the presence of eserine would greatly increase the formation of the complex because of the presence of an increased concentration of free acetylcholine.

(2) The rate of formation of the complex in the brain is more rapid than that of free acetylcholine. Typical results illustrating this statement are shown in Table I.

Table I

A fresh rat brain was minced finely and suspended in 5 vol. Locke medium in the absence of eserine. The suspension was divided into several equal parts which were then placed in Warburg vessels containing a phosphate-glucose (0.01 *M*)-eserine medium. Incubation was allowed to proceed in an atmosphere of air at 37°. The contents of the first vessel were analysed at once for free and combined acetylcholine; this gave the initial figures (i.e. preformed ester). The contents of the second vessel were analysed after 30 min. incubation, those of the third after 60 min. and so on.

Time min.	Free	Combined	Total acetylcholine μg./g.
0	0	<2.5	<2.5
30	2.5	6.3	8.8
60	3.7	7.5	11.2
120	7.5	7.5	15.0

Evidently the amount of "combined" acetylcholine rapidly reaches a limiting value whilst that of free acetylcholine slowly increases with time. This could occur if the complex were formed first, with subsequent breakdown to free acetylcholine, or if free acetylcholine were formed first with subsequent rapid adsorption to form the complex. Experiments designed to test the latter possibility have failed to show a sufficiently rapid rate of formation of the complex from added acetylcholine in presence of brain tissue [Mann, Tennenbaum & Quastel, 1938] and the conclusion must therefore be drawn that the complex, and not the free, acetylcholine is first produced.

Trethewie [1938] argues that the complex is an association of acetylcholine with cell debris. Doubtless this is true, but since it is agreed that all but traces of acetylcholine cannot be diffusible in brain tissue and are therefore "bound", and since experiment [Mann, Tennenbaum & Quastel, 1938] has shown that the ester is not adsorbed by a variety of brain tissue components, it follows that the complex must be an association of acetylcholine with a specific constituent of the debris. It is reasonable to regard the complex as a definite chemical entity until evidence to the contrary is forthcoming.

That some formation of the complex takes place when acetylcholine is added to brain tissue initially devoid of it is apparent from the experiments of Mann, Tennenbaum & Quastel [1938] and of Corteggiani [1938]. It therefore seems that an equilibrium exists in the cell between the complex and the free acetylcholine. This is borne out by the fact that in presence of eserine there is a less rapid rate of disappearance of the complex than in its absence (see also Corteggiani [1938]). Typical results indicating this fact are shown in Table II. Eserine, by allowing accumulation of free acetylcholine to occur, enables also some formation of the bound form to take place and thus has the effect of stabilizing the complex.

Table II

A rat brain was minced and suspended in 5 vol. saline. This was divided into several equal parts and one part was examined immediately for "bound" acetylcholine. Each of the remaining parts was placed in an appropriate medium in an atmosphere of nitrogen and incubation was allowed to proceed for 2½ hr. at 37°. After this period the contents of the vessels were analysed for the bound ester.

Medium	(initial value)	"Combined" acetylcholine μg./g.
None		2.8
Phosphate-Locke		1.0
Phosphate-Locke-eserine		2.0

The following reaction schemes account for the results which have been obtained so far (see Mann, Tennenbaum & Quastel [1938]):

Reaction (1) is normally rapid compared with reaction (2). The quantity of free acetylcholine in the cell must normally be low owing to the activity of the choline esterase present, the amount present being determined by the conditions of the dynamic equilibrium set up.

The conclusion that glucose, or a breakdown product of glucose, is important for the synthesis of acetylcholine has received confirmation from MacIntosh's [1938] perfusion experiments carried out on the cat's superior cervical ganglion.

Effects of K^+ on the synthesis of acetylcholine in brain

Experiments with rat brain slices show that the addition of K^+ to the medium in which the tissue is respiring may bring about a very large increase in the rate of acetylcholine formation. Typical results are shown in Table III. The effect of the addition of 0.027 *M* K^+ may result in the formation of acetylcholine to the extent of over 40 γ /g.¹

Table III

Rat brain slices in glucose (0.01 *M*)-bicarbonate-Locke-eserine medium. KCl was added to the medium, with a corresponding decrease in NaCl to preserve isotonicity. 95% O_2 + 5% CO_2 . 1 hr. 37°.

Exp. ...	Total acetylcholine formed $\mu\text{g./g.}$				
	A	B	C	D	E
Medium with no extra KCl	12.7	10.4	12.0	20.0	21.3
+ 0.0135 <i>M</i> KCl	16.5	—	—	—	—
+ 0.027 <i>M</i> KCl	37.5	41.0	34.0	41.0	45.0
+ 0.054 <i>M</i> KCl	—	—	15.0	—	—
+ 0.11 <i>M</i> KCl	—	4.0	—	—	—
+ 0.13 <i>M</i> KCl	4.3	—	—	—	—

The presence of a much greater $[K^+]$ brings about, however, a fall in the rate of formation of acetylcholine. Concentrations of the order of 0.1 *M* K^+ inhibit the synthesis of the ester.

It is evident that the accelerating action of 0.027 *M* KCl on the rate of formation of acetylcholine is due to K^+ itself and not to a fall in the $[Na^+]$. A fall in the NaCl concentration to the extent of 0.027 *M* has little or no effect on the rate of formation of acetylcholine.

Analysis of the "total" acetylcholine formed in presence of excess K^+ shows that the increase of acetylcholine formation lies entirely with the free acetylcholine, the amount of "combined" ester being usually decreased. These results are shown in Table IV.

If eserine is added to the medium at the termination of an experiment, it is found that the action of added K^+ is to decrease the formation of acetylcholine.

¹ Control experiments have been carried out to show that the amount of K^+ introduced with the medium into the solution bathing the leech does not itself cause a contraction or affect that due to acetylcholine.

Table IV

Rat brain slices. Glucose-bicarbonate-Locke-eserine medium. 95% O₂ + 5% CO₂. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$			
	Exp. A		Exp. B	
	Free	Combined	Free	Combined
Medium + no added KCl	3.7	9.0	3.2	12.8
+ 0.027 M KCl	30.0	7.5	20.1	6.6

This is due to the fact that when eserine is added at the end of an experiment, the acetylcholine estimated is almost wholly "bound" acetylcholine. These facts are shown in Table V.

Table V

Rat brain slices. Glucose-bicarbonate-Locke medium. 95% O₂ + 5% CO₂. 1 hr. 37°.

	"Total" acetylcholine formed $\mu\text{g./g.}$	
	Eserine added at beginning of the exp.	Eserine added at the end of the exp.
Medium + no added KCl	20.0	17.0
+ 0.027 M KCl	41.0	11.0

The action of K⁺ in increasing the formation of free acetylcholine in brain slices takes place also in phosphate media, but the effect is less marked than in bicarbonate media (Table VI).

Table VI

Rat brain slices. Phosphate-glucose-Locke-eserine medium. O₂. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$	
	Free	Combined
Medium + no extra KCl	3.5	8.6
+ 0.027 M KCl	10.0	7.0

The stimulating action of K⁺ is confined to a "synthesizing" medium

The stimulating effect of K⁺ on acetylcholine formation is not confined to glucose. It takes place also in a pyruvate medium in which synthesis of acetyl-

Table VII

Rat brain slices. O₂ or 95% O₂ + 5% CO₂. 1 hr. 37°.

Exp. A	Medium	Acetylcholine formed $\mu\text{g./g.}$	
		Free	Combined
	Phosphate-Locke-eserine-pyruvate (0.01 M)	3.5	8.9
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.027 M KCl	9.6	6.8
	Bicarbonate-Locke-eserine-pyruvate (0.03 M)	6.8	13.7
	Bicarbonate-Locke-eserine-pyruvate (0.03 M) + 0.027 M KCl	30.7	9.2
		Total acetylcholine formed $\mu\text{g./g.}$	
Exp. B			
	Phosphate-Locke-eserine-pyruvate (0.01 M)		11.2
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.011 M KCl		12.3
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.021 M KCl		14.7
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.04 M KCl		15.7
	Phosphate-Locke-eserine-pyruvate (0.01 M) + 0.069 M KCl		12.2
Exp. C			
	Bicarbonate-Locke-eserine		3.1
	Bicarbonate-Locke-eserine + 0.027 M KCl		2.5

choline occurs. It does not take place, however, in the absence of a substrate, where no synthesis obtains. As in the case of glucose, the effect of K^+ on acetylcholine synthesis in a pyruvate medium is less in a phosphate medium than in bicarbonate, and the accelerating action lies entirely with free acetylcholine. These results are shown in Table VII.

The antagonistic actions of Ca^{++} and Mg^{++}

The addition of Ca^{++} to a glucose-bicarbonate-Locke medium decreases the rate of formation of acetylcholine by brain slices and neutralizes the accelerating action of added K^+ . Mg^{++} has a similar neutralizing action. Typical results are shown in Table VIII.

Table VIII

Rat brain slices. Bicarbonate-glucose-Locke-eserine media. 95% O_2 + 5% CO_2 . 1 hr. 37°.

	Exp. A	Total acetylcholine formed $\mu g./g.$
Medium		21.3
+ 0.027 M KCl		45.0
+ 0.02 M $CaCl_2$		10.4
+ 0.027 M KCl + 0.02 M $CaCl_2$		15.0
+ 0.027 M KCl + 0.015 M $CaCl_2$		15.0
	Exp. B	
Medium		18.2
+ 0.027 M KCl		40.5
+ 0.02 M $MgCl_2$		20.2
+ 0.027 M KCl + 0.02 M $MgCl_2$		18.8

Action of Rb^+ and Cs^+

The effects of K^+ and Ca^{++} on acetylcholine liberation in brain slices are in complete accord with the results of Brown & Feldberg [1936] on the action of these ions on the liberation of acetylcholine from the perfused superior cervical ganglion of the cat. These authors further showed that Rb^+ , and to a lesser degree Cs^+ , have effects similar to K^+ .

Rb^+ and Cs^+ have effects similar to K^+ on the liberation of acetylcholine by brain slices (see Table IX), Cs^+ being rather less active than K^+ or Rb^+ .

Table IX

Rat brain slices. Bicarbonate-glucose-Locke-eserine medium. 95% O_2 + 5% CO_2 . 1 hr. 37°.

	Acetylcholine formed $\mu g./g.$	
	Free	Combined
Medium		13.6
+ 0.027 M KCl	5.2	5.0
+ 0.027 M RbCl	27.8	5.0
+ 0.027 M CsCl	27.9	5.0
	23.0	4.7

Interpretation of the K^+ effect

The action of K^+ might be explained as being due to a catalysing action of these ions on the rate of breakdown of the combined acetylcholine into the free ester. It is difficult to understand, however, with this explanation how Ca^{++} brings about its antagonistic effects. It is equally difficult to understand why the addition of 0.027 M KCl to a medium should directly influence the breakdown of the complex into free acetylcholine when it is remembered that $[K^+]$ in the nerve cell may reach a concentration of 0.1 M.

It is reasonable to assume that Ca^{++} acts by neutralizing some effect of added K^+ on cell permeability, such an assumption being in accordance with the known physiological behaviour of these ions.

On this hypothesis the addition of K^+ brings about a change in nerve cell permeability so that free acetylcholine diffuses into the outer medium at a relatively rapid rate. Ca^{++} has an antagonistic effect.

Acetylcholine formation in Locke and saline media

It would be expected from the observation that the presence of Ca^{++} retards the liberation of acetylcholine, that an increased rate of formation of free acetylcholine will take place when respiration of brain slices occurs in a Ca -free medium.

This doubtless accounts, at any rate partly, for the fact that when brain slices respire in a Locke medium a smaller quantity of free acetylcholine is produced than when respiration occurs in a medium free from K^+ and Ca^{++} . The $[\text{K}^+]$ in the Locke medium is probably insufficient to neutralize the retarding action of the Ca^{++} present. Moreover, the effects of adding 0.027 *M* KCl to a saline medium (i.e. free from Ca^{++}) on the liberation of free acetylcholine by brain slices is far less marked than when this concentration of K^+ is added to a Locke medium.¹ These results are shown in Table X, where it will be observed that the differences found between the rates of free acetylcholine formation in Locke and saline media occur in both phosphate and bicarbonate media. The phenomenon occurs also in pyruvate media. It should be noted that the amount of "combined" acetylcholine is decreased under those conditions where an increase in the free acetylcholine takes place.

Table X

Rat brain slices. O_2 or 95% O_2 + 5% CO_2 . 1 hr. 37°.

Exp. A	Medium	Acetylcholine formed $\mu\text{g./g.}$	
		Free	Combined
	Glucose-eserine-Locke-bicarbonate	3	17
	Glucose-eserine- NaCl -bicarbonate	14.7	11
Exp. B			
	Glucose-eserine-Locke-phosphate	3.5	8.6
	Glucose-eserine-Locke-phosphate 0.027 <i>M</i> KCl	9.6	6.8
Exp. C			
	Glucose-eserine- NaCl -phosphate	6.5	8.0
	Glucose-eserine- NaCl -phosphate 0.027 <i>M</i> KCl	6.5	8.0
Exp. D			
	Pyruvate (0.01 <i>M</i>)-eserine-Locke-phosphate	3.5	8.9
	Pyruvate-eserine- NaCl -phosphate	6.5	8.0

Effect of K^+ on acetylcholine formation by minced brain tissue

It is an interesting fact that the marked increase in the rate of formation of "total" acetylcholine effected by K^+ on intact brain slices does not take place with minced brain tissue. The presence of K , however, brings about a fall in the "combined" acetylcholine with a corresponding increase in the amount of free acetylcholine. These results may be seen in Table XI.

¹ In fact this concentration of K^+ has been observed sometimes to inhibit the synthesis of total acetylcholine if Ca is absent from the medium.

Table XI

Exp. A. A rat brain was minced finely and suspended in 3 vol. saline and divided into several equal parts, each of which was added to the appropriate medium in a Warburg flask. Medium: glucose-bicarbonate-eserine-Locke. When KCl was present, the NaCl was decreased correspondingly to preserve isotonicity. 95% O₂ + 5% CO₂. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$		
	Free	Combined	Total
Medium + no added KCl	7.5	6.0	13.5
+ 0.027 <i>M</i> KCl	11.2	3.0	14.2
+ 0.053 <i>M</i> KCl	5.6	1.9	7.5
+ 0.08 <i>M</i> KCl	3.0	1.5	4.5

Exp. B. As in exp. A but with the following medium: phosphate-eserine-Locke. Air. 1 hr. 37°.

	Acetylcholine formed $\mu\text{g./g.}$		
	Free	Combined	Total
Medium	3.8	7.0	10.8
+ 0.07 <i>M</i> KCl	6.4	3.0	9.4
+ glucose	7.9	11.2	19.1
+ glucose + 0.07 <i>M</i> KCl	7.6	5.3	12.9

Action of K⁺ on "combined" acetylcholine

To perceive more clearly the effects of K⁺ experiments were carried out with brain slices and minced brain under conditions where synthesis of the ester was reduced to a minimum.

In these experiments minced brain tissue was allowed to respire in a phosphate-glucose-eserine medium in an atmosphere of air for 1½ hr. at 37° to build up a relatively high concentration of "combined" acetylcholine. The tissue was then centrifuged, the clear centrifugate containing the free acetylcholine was discarded and the tissue deposit containing the "combined" ester was immersed in a glucose-free Locke-eserine medium. The effect of added K⁺ on the rate of breakdown of the "combined" ester under such conditions, where little or no synthesis of further acetylcholine took place, was investigated. The results showed that the effect of added K⁺ was always to reduce the amount of "combined" ester and to increase the free acetylcholine. When the experiments with added K⁺ were carried out under anaerobic conditions, the increase in the free acetylcholine was equal to the decrease in the "combined" acetylcholine, the "total" acetylcholine being unaffected by K⁺. Under aerobic conditions it was usually found that the "total" acetylcholine was decreased by the addition of K⁺. This was doubtless due to the fact that some synthesis of acetylcholine usually takes place under aerobic conditions when minced tissue is used owing to the presence of small quantities of metabolites which cannot be washed out, and the addition of K⁺ inhibits this synthesis. The effect, however, of K⁺ in causing "combined" acetylcholine to break down to form free acetylcholine is as evident under aerobic conditions as it is under anaerobic conditions. Typical results are shown in Table XII. In this table, too, is shown the effect of added K⁺ on the "combined" (preformed) acetylcholine of brain slices incubated in an atmosphere of 95% O₂ and 5% CO₂ in a glucose-free medium at 25° when very little synthesis takes place. The usual action of K⁺ in causing breakdown of the "combined" ester will be observed.

Table XII

Exp. A. Preliminary synthesis of "combined" acetylcholine by minced brain in phosphate-glucose-Locke-eserine medium for $1\frac{1}{2}$ hr. at 37° . Tissue residue, after centrifuging, was divided into several parts and placed in Warburg vessels containing phosphate- or bicarbonate-NaCl-eserine media with different concentrations of KCl. Conditions either aerobic (air or 95% O_2 + 5% CO_2) or anaerobic (95% N_2 + 5% CO_2). 1 hr. 37° .

Medium. (Eserine always present)	Acetylcholine found $\mu\text{g./g.}$		
	Free	Combined	Total
Exp. 1			
N_2 -bicarbonate-NaCl	4.3	6.0	10.3
N_2 -bicarbonate-NaCl + 0.08 M KCl	6.4	3.7	10.1
N_2 -bicarbonate-NaCl + 0.12 M KCl	6.4	3.7	10.1
Exp. 2			
O_2 -bicarbonate-NaCl	3.8	10.5	14.3
O_2 -bicarbonate-NaCl + 0.04 M KCl	5.5	4.5	10.0
O_2 -bicarbonate-NaCl + 0.08 M KCl	6.4	3.3	9.7
O_2 -bicarbonate-NaCl + 0.12 M KCl	6.4	3.3	9.7
Exp. 3			
Air-phosphate-NaCl	3.0	8.1	11.1
Air-phosphate-NaCl + 0.12 M KCl	6.5	4.5	11.0
Exp. 4			
Air-phosphate-NaCl	3.0	13.5	16.5
Air-phosphate-NaCl + 0.08 M KCl	5.9	7.5	13.6
Air-phosphate-NaCl + 0.12 M KCl	6.9	6.7	13.6
Exp. 5			
N_2 -phosphate-NaCl	4.3	5.3	9.6
N_2 -phosphate-NaCl + 0.12 M KCl	6.0	3.7	9.7
Exp. B. Rat brain slices. Bicarbonate-eserine-Locke. 95% O_2 + 5% CO_2 . 1 hr. 25° .			
Medium	0.9	2.6	—
Medium + 0.027 M KCl	1.5	1.5	—

Permeability of the nerve cell to acetylcholine in presence of K^+

The effect of K^+ in causing breakdown of the "combined" acetylcholine may be due to a direct action on the complex, as stated earlier; but since such an explanation fails to account for the antagonistic action of Ca^{++} on the liberation of free acetylcholine, an effect on cell permeability is considered to afford a more reasonable explanation of the phenomenon.

If K^+ effects a liberation of free acetylcholine by causing a change in permeability of the nerve cell so that free acetylcholine diffuses out more rapidly than in the absence of K^+ , it follows that there must exist normally within the cell a certain amount of diffusible or free acetylcholine. In brain tissue, in the presence of eserine, the "combined" acetylcholine remains comparatively stable. Yet it is able to break down normally to form free acetylcholine—otherwise changes of permeability alone could effect no increased liberation of the ester. The most obvious manner to reconcile these facts is to assume that an equilibrium exists in the cell between the free and "combined" forms of the ester. A disturbance of this equilibrium due to diffusion of acetylcholine through the cell membrane, when the permeability is increased by added K^+ , will result in a decrease in the cell of the amount of "combined" acetylcholine. This, presumably, is what takes place in brain tissue when K^+ is added and when no synthesis is occurring. Under optimal respiratory conditions, however, the "combined" acetylcholine is being rapidly synthesized, its amount being determined by the amount of tissue constituent with which the ester is combined. The rate of its breakdown to free acetylcholine is determined by equilibrium

conditions within the cell and by the rate of diffusion of acetylcholine out of the cell. When the latter is greatly increased by the addition of K^+ to the tissue, the concentration of free acetylcholine in the cell will fall and the apparent rate of breakdown of the "combined" acetylcholine will become greater. The amount of the complex at any time, however, will not change until the rate of its breakdown approaches the rate of its synthesis. This picture of events in the cell enables us to understand how K^+ ions cause an apparent breakdown of "combined" to free acetylcholine in brain tissue under conditions where no synthesis is taking place, and how they may cause a very great increase in the rate of synthesis of free acetylcholine when they are added to brain tissue respiring under optimal conditions.

The view put forward depends upon the hypothesis of a dynamic equilibrium being set up within the cell according to the following scheme:

Some evidence for the existence of an equilibrium in the nerve cell between "combined" and free acetylcholine has already been given. It is possible to test the hypothesis rigidly and experiments will now be described which clearly indicate the existence of such an equilibrium.

Demonstration of the existence in brain tissue of an "equilibrium" concentration of free acetylcholine

When brain slices respire in a glucose-eserine-Locke medium containing 0.027 *M* KCl the total acetylcholine formed may reach a value varying between 30 and 50 $\mu\text{g./g.}$ of tissue. This high value is reached within the first hour of the experiment and is increased only to a small extent during the second hour. The small increase found during the second hour is not due to a decrease in metabolic activity of the tissue, for if the tissue is removed from the medium after the first hour and is placed in a fresh glucose medium for another hour, it evinces most of its former synthetic activity. Results illustrating this fact are shown in Table XIII.

Table XIII

Rat brain slices. Bicarbonate-glucose-eserine-Locke medium containing 0.027 *M* KCl. 95% O_2 + 5% CO_2 . 37°.

	$\mu\text{g./g.}$
Total acetylcholine found after 1 hr.	31.2
after 2 hr.	41.3
$\therefore$ Acetylcholine synthesized during 2nd hr.	10.1
Tissue slices removed from synthesizing medium after 1 hr. and placed in fresh glucose-eserine-bicarbonate-Locke-KCl medium	
Total acetylcholine found after 1 hr.	26.0

It seems most probable from these experiments that the synthesis of acetylcholine is retarded by the growing accumulation of acetylcholine itself, and that, in fact, when the acetylcholine concentration reaches a critical value no further increase in synthesis of "total" acetylcholine occurs—i.e. an equilibrium is established.

This can be proved by experiments in which acetylcholine is added to a medium, with and without added K^+ , in which brain tissue is respiring.

In these experiments a known quantity, e.g. 50 $\mu\text{g.}$, of acetylcholine was added to brain slices respiring in a bicarbonate-glucose-Locke-eserine medium, with and without the addition of 0.027 M KCl. It was found that in presence of this quantity of acetylcholine the presence of the added K^+ effected little or no increase in the "total" acetylcholine. It was also easy to show that the presence of added acetylcholine greatly decreased the synthesis of acetylcholine, this being most clearly observable in the presence of 0.027 M KCl. A concentration of acetylcholine of 10 $\mu\text{g./ml.}$ retarded the synthesis of acetylcholine by 40 % in the presence of 0.027 M KCl and had no effect on the synthesis in the absence of K^+ . Typical results are shown in Table XIV.

Table XIV

Rat brain slices. Bicarbonate-glucose-eserine-Locke medium. 95 % O_2 + 5 % CO_2 . 1 hr. 37°.

		"Total" acetylcholine found	"Total" acetylcholine synthesized
Exp. A			
Medium		20	20
	+ 50 $\mu\text{g.}$ acetylcholine	66	16
	+ 0.027 M KCl	33	33
	+ 0.027 M KCl + 50 $\mu\text{g.}$ acetylcholine	66	16
Exp. B			
Medium		20	20
	+ 30 $\mu\text{g.}$ acetylcholine	50	20
	+ 0.027 M KCl	40.5	40.5
	+ 0.027 M KCl + 30 $\mu\text{g.}$ acetylcholine	54	24
Exp. C			
Medium		23	23
	+ 50 $\mu\text{g.}$ acetylcholine	64	14
	+ 0.027 M KCl	50	50
	+ 0.027 M KCl + 50 $\mu\text{g.}$ acetylcholine	66	16

The inhibitory action of free acetylcholine on the synthesis of acetylcholine points to the existence of an equilibrium as expressed in the reaction scheme¹ (3). Moreover, the facts that this inhibition is observable in presence of excess of K^+ and that it occurs to a much less extent in the absence of K^+ are confirmatory of the conclusion that potassium greatly increases the permeability of the brain cell to acetylcholine.

Effects of NH_4^+

The addition of NH_4^+ to a medium in which brain slices are respiring has apparently little effect on the rate of synthesis of acetylcholine until the $[NH_4^+]$ reaches a value of approximately 0.05 M , when a decided inhibition takes place. Analysis, however, of the acetylcholine formed in the presence of NH_4^+ shows a considerable drop in the amount of "combined" acetylcholine and a corresponding increase in that of the free acetylcholine. It is evident that an effect similar to that of K^+ takes place with NH_4^+ , but that in contrast to K^+ , NH_4^+ ions at relatively low concentrations have highly inhibitory effects on the synthesis of acetylcholine by brain slices. This inhibitory action on acetylcholine synthesis is clearly shown when NH_4^+ is added together with K^+ to brain slices; the increased rate of synthesis found with K^+ is practically eliminated. Typical results are shown in Table XV.

It is most likely that NH_4^+ , like K^+ , causes an increased permeability of the brain cells for acetylcholine and would, like K^+ , have accelerating effects

¹ Conceivably acetylcholine may inhibit by competing with choline for a receptor group in the synthesizing enzyme.

Table XV

Rat brain slices. Bicarbonate-glucose-eserine-Locke medium. 95% O₂ + 5% CO₂.

		Acetylcholine formed, μ g.		
		Free	Combined	Total
Exp. A				
	Medium	4.9	10.1	15.0
	+ 0.027 <i>M</i> KCl	31.8	4.2	36.0
	+ 0.027 <i>M</i> NH ₄ Cl	10.6	3.1	13.7
	+ 0.027 <i>M</i> KCl + 0.027 <i>M</i> NH ₄ Cl	7.6	1.9	9.5
Exp. B				
	Medium	—	—	17.5
	+ 0.013 <i>M</i> NH ₄ Cl	—	—	20.0
	+ 0.027 <i>M</i> NH ₄ Cl	—	—	17.0
	+ 0.053 <i>M</i> NH ₄ Cl	—	—	7.0

on the formation of total acetylcholine were it not for its secondary inhibitory action on acetylcholine synthesis. It is very suggestive that the glutamic acid-glutamine system, which would act as an effective detoxicating process so far as NH₄⁺ in the brain is concerned, exists in relatively large amount in the central nervous system [Krebs, 1935]. The toxicity of NH₄⁺ may be due to its competition with choline for the enzyme synthesizing acetylcholine. It has already been shown by Mann, Woodward & Quastel [1938] that NH₄⁺ competes with choline for the choline oxidase system in liver.

It is worthy of note that NH₄⁺, like K⁺, greatly increases the aerobic glycolysis of brain tissue [Weil-Malherbe, 1938], an effect which has been ascribed to possible altered permeabilities of the cell membrane [Dickens & Greville, 1935].

A suggestion as to the chemical nature of "combined" acetylcholine

There is little evidence as yet which throws any light on the composition of the acetylcholine precursor or of "bound" acetylcholine, beyond the facts stated in our earlier paper [1938] which indicate that the substance is a protein complex. We would like, however, to make the suggestion that the complex is a compound of acetylcholine with its synthesizing enzyme. It is most probable that, when acetylcholine is first formed, its synthesis occurs at the surface of an enzyme, and it is not unreasonable to consider that a definite quantity of the compound between acetylcholine and the enzyme which synthesizes it exists normally in the cell. Such a view would involve the conclusion that the complex will only be found in tissues which are capable of synthesizing acetylcholine and so far as we know at present this is the case. The amount of "bound" acetylcholine present in the cell is also not irreconcilable with this view. The amount of complex present in brain under our experimental conditions, when synthesis is actively taking place and with eserine present, is of the order of 14 μ g./g., this being reckoned as acetylcholine chloride. Assuming that the complex is a union of one molecule of protein (say of mol. wt. 34,000) and of one molecule of acetylcholine, the amount of complex present in brain corresponding to 14 μ g./g. would be approximately 2.6 mg. per g. wet weight of tissue. Such a value would not be unexpected.

SUMMARY

1. The addition of potassium ions (0.027 *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. In a bicarbonate-glucose medium, or a

bicarbonate-pyruvate medium the total acetylcholine formed may reach a value of 40 μ g. per g. wet weight of tissue. High concentrations of potassium ions inhibit the synthesis of acetylcholine by brain tissue.

2. The accelerating effect of potassium ions does not take place in a substrate-free medium.

3. The increase in acetylcholine formation effected by potassium lies entirely with the free acetylcholine. The amount of "combined" acetylcholine is either unchanged or diminished.

4. The accelerating action of potassium ions is neutralized by the addition of calcium or magnesium ions.

5. Rubidium, and to a smaller extent caesium, ions have effects similar to those of potassium.

6. The addition of potassium ions to minced brain tissue does not have such a marked effect on acetylcholine synthesis as in the case of intact brain slices.

7. The addition of potassium ions to either minced brain tissue or intact brain slices, under conditions when synthesis of acetylcholine is not taking place, is to bring about a fall in the amount of "combined" acetylcholine with a corresponding (equal) increase in the amount of free acetylcholine. This occurs under anaerobic, as well as under aerobic, conditions.

8. The effect of potassium ions is less marked in a phosphate medium than in a bicarbonate medium.

9. The effect of potassium ions is to increase the permeability of the nerve cell, so that acetylcholine diffuses through the cell membrane at a greater rate than in the absence of potassium.

10. An equilibrium exists in the cell between free acetylcholine and "combined" acetylcholine. The addition of acetylcholine to a medium in which brain slices are respiring depresses the synthesis of acetylcholine by the tissue. This can only clearly be seen after the addition of potassium ions.

11. Ammonium ions act similarly to potassium ions in bringing about a breakdown of "combined" acetylcholine with a corresponding increase of free acetylcholine. They have a highly inhibitory action on acetylcholine synthesis by brain slices.

12. Reaction schemes accounting for these facts are described.

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