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FORMATION OF ACETYLCHOLINE IN CELL-FREE EXTRACTS FROM BRAIN

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Synthesis of acetylcholine in brain slices and pulp was described by Quastel, Tennenbaum & Wheatley (1936) and by Stedman & Stedman (1937). Their main results have been confirmed by several workers. Synthesis of acetylcholine was shown to occur in brain which had been dried in a desiccator and powdered before use (Feldberg, 1945). In all these instances the synthesis took place aerobically and depended on the presence of some particulate matter, though not necessarily on that of intact cells.

Recently, Nachmansohn & Machado (1943) have shown that acetylcholine is formed *anaerobically* in homogenized brain tissue, provided that adenosinetriphosphate (ATP), fluoride and choline are added. They expressed the view that the acetylation of choline is catalysed by an enzyme, the 'choline acetylase'. They claim to have brought this enzyme into solution, although the evidence presented in their paper does not exclude the possibility that the enzyme might have been associated with the cell granules which abound in homogenized brain tissue.

The observations described in this paper show that the enzymic system which catalyses the formation of acetylcholine can be completely separated from the particulate matter of the brain, and that it can be obtained in the form of cell-free solutions prepared by saline extraction of the acetone-dried tissue. The use of such extracts enabled us to follow up the metabolism of ATP in the course of the synthesis of acetylcholine.

In this paper evidence is offered which shows that the connexion between the presence of ATP and the acetylation of choline is not merely restricted to anaerobic conditions, but that it also exists under aerobic conditions which resemble more closely the circumstances under which acetylcholine is formed *in vivo*.

The use of enzyme extracts prepared from acetone-dried brain powder made it possible to study the finer mechanism of the action of glucose and of Ca-

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and K-ions on the synthesis proper, without interference from any reactions associated with the release of acetylcholine. In previous studies with these substances such a distinction was hardly possible.

METHODS

Rats were killed by a blow on the neck, and the brains were removed by transverse section in front of the cerebellum. The brains were either homogenized or converted into acetone-dried powder.

The tissue was homogenized with Ca-free Ringer-bicarbonate solution and diluted so that 3 ml. were equivalent to 250 mg. brain, fresh weight. To prepare the acetone powder, the brains were ground in a mortar with ice-cold acetone; the powder was collected on a small Buchner funnel and dried in air. One g. dry powder, which corresponded to between 5·5 and 6 g. fresh tissue, was ground with 50 ml. solution and centrifuged. The extracts were made with the Ca-free Ringer-bicarbonate solution or with a 'Mg-saline solution', composed of 0·9 % NaCl and 0·029 % $MgSO_4$. In some experiments Mg was omitted, in others phosphate was added to the saline solutions. These changes did not affect the main results.

The incubation was carried out at 37° C. in Thunberg tubes. The main compartment of each tube contained 3 ml. of homogenized brain or 2·5 ml. of the centrifuged extract from acetone powder. It also received the following substances (each in 0·1 ml.): 6 mg. KCl, 2–3 mg. NaF, 0·5 mg. eserine sulphate, 3 mg. choline and 1·5 mg. Na-acetate. ATP was introduced into the side bulb of the Thunberg tube. The amounts of ATP used in the experiments with homogenized brain contained from 1·2 to 1·5 mg. of the easily hydrolysable Pyro-P_7, and, in the experiments with acetone powder, from 0·2 to 0·7 mg. P_7.

The Thunberg tubes were evacuated and refilled three times with either N_2 or a gas mixture containing 5 % CO_2 and 95 % N_2 or 95 % O_2 respectively. The N_2 and the $N_2 + CO_2$ mixture were passed over a heated copper wire to remove all traces of oxygen. The contents of the main compartment and the side bulb were then mixed, and the mixtures incubated for 1 hr.

Some of the incubated samples were used for the estimation of acetylcholine; in others phosphorus was determined. For the estimation of acetylcholine the samples were treated with 1 ml. 0·33 *N* HCl, boiled for a minute or two and cooled. They were then carefully neutralized with 0·33 *N* NaOH, made up to a definite volume, and assayed on the frog's rectus abdominis muscle against acetylcholine solutions of known strength to which equivalent amounts of the same extract were added, the acetylcholine of which, however, had previously been destroyed by a brief boiling in alkaline solution. The detailed procedure has been described elsewhere (Feldberg, 1945). Usually, concentrations equivalent to 1 mg. homogenized brain or 0·1 to 0·2 mg. acetone powder per 1 ml. solution were used for the assay.

The phosphorus estimations were carried out in samples deproteinized with trichloroacetic acid. Phosphorus was estimated in the protein free filtrate by the method of Fiske & Subbarow, as modified by Lohmann & Jendrassik (1926). Two fractions were determined, the inorganic phosphate (P_0) and the fraction hydrolyzable to inorganic phosphate by 7 min. hydrolysis with *N* HCl (P_7).

Substrates used. Adenosinetriphosphate (ATP) was prepared as Ba-salt by a modified method of Lohmann (1931). Before use, the Ba-salt was converted to the Na-salt and the pH of the solution adjusted to 7. Adenosinediphosphate (ADP) was prepared from ATP by the enzymic action of myosin (Bailey, 1942). A sample of inosinetriphosphate (ITP) was kindly given to us by Dr A. Kleinzeller.

RESULTS

Homogenized brain

The homogenized tissue had the appearance of a milky fluid. Cell debris was present, but could be removed by centrifugation. Although the supernatant fluid was free from acetylcholine, the debris contained preformed acetylcholine in an amount equivalent to 1–2μg./g. brain.

In order to examine the synthesis of acetylcholine, samples were set up containing the cell debris, the centrifuged supernatant fluid or the whole homogenized tissue, and incubated anaerobically in the presence of eserine, ATP, NaF, choline, Na-acetate and KCl. We found that the debris was unable to synthesize acetylcholine. On the other hand, both the supernatant fluid and the whole homogenized brain tissue were able to promote the synthesis. The supernatant solution was, however, more active than the whole homogenized tissue. For instance, the figure of 17·5μg./g. recorded in Exp. 1 of Table 1, was obtained with the centrifuged homogenized tissue, whereas only

TABLE 1. Synthesis of acetylcholine in centrifuged homogenized brain under anaerobic and aerobic conditions

One hr. incubation in presence of eserine, ATP, NaF, KCl, choline and Na-acetate

	μg. acetylcholine/g. brain (fresh weight)	
Exp. no.	95 % N_2+5 % CO_2	95 % O_2+5 % CO_2
1	17·5 (29·5)	3·3
2	18	1·8
3	15·5 (21)	0·5
4	9·3	2·0

Figures in brackets relate to values obtained by the method of testing as used by Nachmansohn & Machado (see text).

10·5μg./g. were synthesized by the equivalent amount of the non-centrifuged homogenized tissue. In the presence of ATP, but without the addition of either NaF or choline, the amounts of acetylcholine synthesized were much smaller. In the absence of ATP alone there was no synthesis at all. These facts are in agreement with the findings of Nachmansohn & Machado (1943). On the whole, however, our values (Table 1) were much lower than those of these authors (35–100μg./g.). This discrepancy may have been due to the following causes.

(*a*) Our procedure for homogenizing the brain tissue may not have been as effective as that of Nachmansohn and Machado.

(*b*) The enzyme connected with the synthesis of acetylcholine may have been partly inactivated in the course of the homogenizing process which was carried out in air. It will be shown later that the enzyme system under investigation is adversely affected by aerobic conditions.

(*c*) Our method of assaying the acetylcholine content of the extracts differed from that used by Nachmansohn & Machado. We determined the acetylcholine content by comparing the effect of extracts on the frog's rectus muscle with that of inactivated extracts to which a known amount of acetylcholine had been added. In this way, proper account was taken of the fact that tissue extracts, as well as choline in subthreshold doses, make the rectus muscle more sensitive to the action of acetylcholine. Nachmansohn & Machado assayed their extracts against pure acetylcholine solutions. Exps. 1 and 3 of

Table 1 show that the values obtained by our method of testing are lower than those obtained by the method of Nachmansohn & Machado.

Breakdown of ATP. We found that, even in the presence of NaF, ATP is rapidly dephosphorylated by the homogenized brain tissue, with the liberation of inorganic phosphate. The supernatant fluid from the homogenized tissue was less active, so that 15–20% ATP were left intact after 1 hr. incubation. This may explain the higher yield of acetylcholine in experiments with the supernatant fluid, as compared with those in which whole homogenized tissue was used. The breakdown of ATP was the same in the presence and in the absence of choline. ATP could not be replaced by Na-pyrophosphate in the synthesis of acetylcholine. It was also found that the pyrophosphate is stable in the homogenized brain tissue, both in the presence and in the absence of NaF.

Azide was without any inhibiting effect on the synthesis of acetylcholine.

Glucose was found greatly to inhibit the synthesis. This action of glucose coincided with a marked inhibitory effect on the rate of dephosphorylation of ATP.

If no KCl was added to the incubated samples, the yield of acetylcholine was decreased by about 50 %.

The rate of acetylcholine formation was considerably reduced under aerobic conditions (Table 1).

Acetone powder from brain

The centrifuged extract obtained from the acetone powder synthesized anaerobically large amounts of acetylcholine in the presence of eserine, ATP, NaF, KCl, choline and Na-acetate. The amounts synthesized in 1 hr. by the extracts, obtained from several preparations of the acetone powder, varied from 140 to 400μg. acetylcholine per g. acetone powder, i.e. from 500 to 1430μg./g. dry material contained in the centrifuged extract. The high values obtained with extracts suggest that the enzyme system responsible for the synthesis of acetylcholine is not affected by the treatment of the brain tissue with acetone. An acetone powder kept in a desiccator in the ice-box retained the full activity for 1 month.

As a rule, the experiments were carried out at 37° C. At 20° C. the synthesis was much smaller. Thus, for instance, in a sample kept for 1 hr. at 20° C. only 65μg./g. were formed, as compared with 253μg. acetylcholine produced at 37° C.

In Fig. 1 the amounts of synthesized acetylcholine are plotted against the time of incubation. It can be seen that the rate of synthesis decreases progressively, but is still well pronounced after 2 hr. of incubation.

We have tested the effect of various concentrations of ATP on the rate of synthesis of acetylcholine, and we found that the optimal concentration was that of 0·2 mg. ATP-P_7 in a 3·5 ml. sample, containing the extract from

50 mg. acetone powder. Thus, in one series of experiments, the amounts of acetylcholine synthesized in the presence of 0, 0·01, 0·05, 0·1 and 0·2 mg. ATP-P_7 were 6, 7·5, 113, 155 and 212μg./g. respectively. Higher concentrations of ATP occasionally enhanced, but sometimes decreased the yield of acetylcholine. In all these experiments the synthesis took place in the presence of NaF. In the absence of NaF the amounts of acetylcholine formed were 25–60 % less.

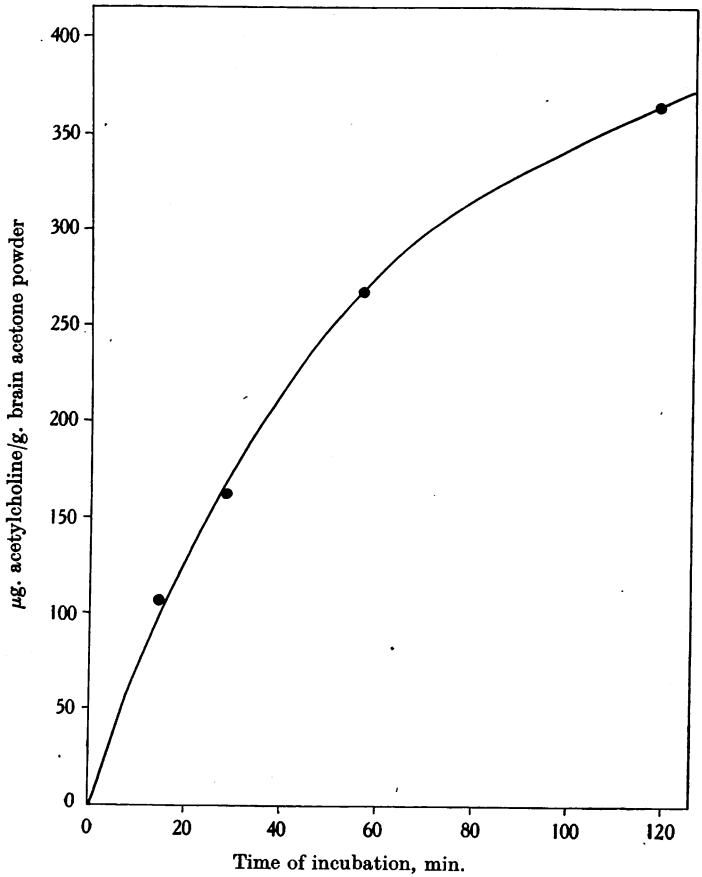

Fig. 1. Synthesis of acetylcholine in extracts made from acetone-dried brain powder.

The addition of Na-acetate was not essential for the synthesis. In the absence of choline, however, the synthesis was considerably reduced. Thus, for instance, 253μg./g. were formed in presence of both acetate and choline as compared with 250μg. of acetylcholine, formed in absence of acetate, and 41μg., formed in absence of choline.

With regard to eserine, we found that its presence was not essential, provided that the samples contained NaF. The concentration of NaF was sufficient

to abolish the activity of cholinesterase in our samples. Nevertheless, eserine was added to all samples as a routine measure.

Synthesis of acetylcholine in extracts made from the brain acetone powder was lower under aerobic than under anaerobic conditions (Table 2), although the difference was not as pronounced as in homogenized brain (Table 1).

TABLE 2. Synthesis of acetylcholine under anaerobic and aerobic conditions

One hr. incubation in presence of eserine, ATP, NaF, KCl, choline and Na-acetate

μg. acetylcholine/g. acetone powder	
Anaerobically	Aerobically
140	60
150	80
190	107
200	120
240	177
267	130

In their paper Nachmansohn & Machado expressed the opinion that the 'choline acetylase' may belong to the class of enzymes in which the active groups are the sulphydryl groups. Their view was based on the observation that iodoacetic acid, Cu-ions and iodine inhibit the synthesis of acetylcholine in the homogenized brain. Using the extracts prepared from the acetone powder, we found that 4×10^{-4} *M* Na-iodoacetate inhibited the synthesis by 80 %, whereas $2{\cdot}5 \times 10^{-6}$ *M* $CuSO_4$ inhibited the synthesis by 30 %. We have established an additional fact which supports the conception that SH— groups are essential for the synthesis of acetylcholine.

TABLE 3. The effect of sulphydryl compounds on the synthesis of acetylcholine

One hr. incubation in presence of eserine, ATP, NaF, KCl, choline and Na-acetate

Compounds added to the saline extract from 50 mg. acetone powder	μg. acetylcholine/g. acetone powder	
	Anaerobically	Aerobically
—	195	114
2 mg. reduced glutathione	230	250
2 mg. oxidized glutathione	28	35
2 mg. cysteine	340	230
4 mg. *d*, *l*-cystine	50	36

The experiments recorded in Table 3 show that reduced glutathione and cysteine have a powerful activating action on the aerobic synthesis of acetylcholine. Under the influence of the two SH— compounds, the synthesis of acetylcholine was brought to the same or even to a higher level than that observed anaerobically. This may be due to the fact that the SH— compounds prevent the oxidation of the SH— groups in the enzyme to inactive —SS— groups. The SH— compounds had some activating effect also on the anaerobic synthesis which suggests that some SH— groups of the enzyme might have undergone an oxidation in the course of the preparation or extraction of the acetone powder.

In contrast to the SH— compounds, the corresponding —SS— compounds, oxidized glutathione and cystine, inhibited the synthesis of acetylcholine both under aerobic and anaerobic conditions (Table 3).

The SH— compounds could not be replaced by hydroquinone nor by ascorbic acid. In fact, these two reducing agents inhibited the synthesis of acetylcholine.

TABLE 4. Liberation of inorganic phosphate from ATP

Saline extracts from 50 mg. acetone powder incubated anaerobically in presence of eserine, ATP, KCl, choline and Na-acetate

Exp. no.	Incubation hr.	NaF	μg. P_0	μg. P_7
1	0	0	290	660
	1	10^{-2} N	310	
	1	0	400	
2	0	10^{-2} N	184	468
	1	10^{-2} N	244	
	1	0	404	

Breakdown of ATP. Unlike the homogenized brain tissue, the extracts prepared from the active powder only very slowly liberated ortho-phosphate from ATP, particularly in the presence of NaF (Table 4). The rate with which free phosphate was split off remained the same in the presence and in the absence of oxygen, and was not affected by the addition of choline. Altogether no evidence was found for the existence of a relation between the synthesis of acetylcholine and the liberation of inorganic phosphate from ATP.

The role of ATP in the glycolysis of tissues is known to be that of a phosphate transferring catalyst. It was thought that ATP might act similarly in the synthesis of acetylcholine, by transferring its labile phosphate groups to an intermediary product of glycolysis, so as to produce a phosphorylated compound essential for the synthesis. To test this possibility, a number of sugars and sugar-phosphoric acid derivatives have been tested. None of these compounds, however, was active. On the contrary, both glucose and fructose (but not sucrose) exhibited, in the presence of ATP, a definite inhibitory effect on the synthesis, both under anaerobic and aerobic conditions. The same strong inhibition was observed with the Embden-ester (6-phospho-hexose), and

TABLE 5. Effect of glucose, fructose and phosphohexoses on the synthesis of acetylcholine and on the metabolism of ATP

Anaerobic incubation, 1 hr., in presence of eserine, ATP, NaF, KCl, choline and Na-acetate. Each sample contains extract from 50 mg. acetone powder and was made up to 3·3 ml.

Compounds added	μg. acetylcholine/g. acetone powder	Phosphorus in samples	
		μg. P_0	μg. P_7
—	330	136	192
4 mg. glucose	20	117	157
4 mg. fructose	30	128	150
8·9 mg. Embden-ester (Na-salt)	18	50	165
8·3 mg. Cori-ester (K-salt)	92	28	124
10 mg. Harden-Young-ester (Na-salt)	136	—	—

a smaller effect was noticed with the Cori-ester (1-phospho-glucose). The Harden-Young-ester (1,6-diphospho-fructose) caused least inhibition (Table 5).

The determinations of phosphorus brought out two phenomena which might provide some explanation for the behaviour of these compounds: a marked decrease in the liberation of inorganic phosphate from ATP and a decrease in the ATP-P_7 value, through the formation of phosphoric acid esters which are difficult to hydrolyse. Such esterifications diminish the amount of ATP available for the synthesis of acetylcholine.

TABLE 6. Effect of varying concentrations of glucose on the synthesis of acetylcholine

Anaerobic incubation, 1 hr., in presence of eserine, ATP, NaF, KCl, choline and Na-acetate. Each sample contains extract from 50 mg. acetone powder and was made up to 3·5 ml.

Concentration of glucose mg./ml.	μg. acetylcholine/g. acetone powder	
	With 0·2 mg. ATP-P_7	With 0·4 mg. ATP-P_7
0	250	300
0·1	44	215
0·4	22	35
1·0	23	38

Table 6 shows the effect of various concentrations of glucose on the synthesis of acetylcholine. Even with such a low concentration as 0·1 mg. glucose per ml. there was a considerable decrease in the yield of acetylcholine. However, the concentration of glucose was not the sole determining factor. The degree of inhibition by glucose varied with the concentration of ATP. With 0·2 mg. ATP-P_7 the inhibition was 76 %, with 0·4 mg. ATP-P_7 only 28 %. In the presence of reduced glutathione, glucose retained its inhibitory action on the formation of acetylcholine.

Adenosine-monophosphoric acid (muscle adenylic acid) was unable to replace ATP in either the presence, or the absence of phosphoglyceric acid. Adenosine-diphosphoric acid (ADP) was only slightly effective. 20μg. acetylcholine/g. were formed anaerobically in the presence of ADP as compared with 183μg./g., formed in the presence of the corresponding amount of ATP and with 3μg./g., formed in the absence of either of these two adenyl derivatives. Inosinetriphosphoric acid (ITP), which differs from ATP by the absence of the

TABLE 7. Synthesis of acetylcholine in presence of adenosinetriphosphate (ATP) and inosinetriphosphate (ITP)

Each sample contains extract from 50 mg. acetone powder. Anaerobic incubation, 1 hr., in presence of eserine, NaF, KCl, choline and Na-acetate

Exp. no.	μg. acetylcholine/g. acetone powder			Content of ATP or ITP in μg. P_7 per each sample
	With ATP	With ITP	Without ATP or ITP	
1	205	28	<4	0·2
	267	28	<4	0·4
2	133	54	10	0·3
	180	83	10	0·6

amino group, was more effective than ADP but less than ATP (Table 7). This shows that the NH_2 group is also of some importance with regard to the synthesis of acetylcholine. We have investigated, therefore, whether ammonia is liberated from ATP on incubation with extracts from brain acetone powder. It was found that the deaminase activity of such extracts was small. During 1 hr., at 37° C., only 0·016 mg. NH_3-N was split off from 0·099 mg. ATP-NH_3-N under the action of 3 ml. extract made from 50 mg. acetone powder.

Aneurin. Neither aneurin nor aneurin diphosphate (cocarboxylase) could replace ATP in the synthesis of acetylcholine. In the presence of ATP aneurin inhibited the formation of acetylcholine.

TABLE 8. Effect of potassium ions on the synthesis of acetylcholine

One hr. incubation in presence of eserine, ATP, choline and Na-acetate

	μg. acetylcholine/g. acetone powder		
Exp. no.	Without KCl	With 0·02 *M* KCl	Incubation
1	127*	253	Anaerobic in presence of NaF
2	133	307	
3	120	240	
4	145	310	
5	180	360	
6	77	150	Anaerobic in absence of NaF
7	133	165	
8	120	200	
9	50	60	Anaerobic in absence of NaF, but in presence of 0·0018 *M* $CaCl_2$
10	33	42	Anaerobic in absence of NaF, but in presence of 0·0055 *M* $CaCl_2$
11	43	80	Aerobic in presence of NaF
12	70	177	

* This sample contained 0·005 *M* KCl.

The action of K and Ca ions. Potassium chloride had a stimulating effect on the synthesis of acetylcholine, both under aerobic and anaerobic conditions (Table 8). With calcium chloride, on the other hand, there was a marked inhibition (Table 9). In presence of cysteine, K^+ and Ca^{++} had similar effects. A study of the action which Ca^{++} and K^+ have on the metabolism of ATP revealed that the Ca-ions inhibit the breakdown of ATP to inorganic phosphate, and that this inhibition is not affected by the simultaneous presence of K-ions. K-ions alone did not affect the dephosphorylation of ATP to inorganic phosphate. This shows that the antagonistic action which the two ions, Ca and K, exert on the synthesis of acetylcholine is not solely dependent on the manner in which they affect the dephosphorylation of ATP. There may be yet another process connected with the synthesis of acetylcholine in which the two ions are involved. That this may be the case is suggested by the results of experiments in which the action of each of the two ions was examined separately, in the presence and in the absence of the other ion.

TABLE 9. Effect of calcium ions on the synthesis of acetylcholine

Anaerobic incubation, 1 hr., in presence of eserine, ATP, choline and Na-acetate

Exp. no.	μg. acetylcholine/g. acetone powder: (*a*) Without calcium	(*b*) With calcium	Molar concentration of $CaCl_2$ in (*b*)	Incubation
1	113	30	0·007	In absence of KCl
2	120	30	0·007	
3	140	46	0·007	
4	64	14	0·007	
5	77	33	0·0055	
6	77	50	0·0018	
7	200	40	0·007	In presence of 0·02 *M* KCl
8	130	50	0·007	
9	150	42	0·0055	
10	150	60	0·0018	

Extracts containing both Ca^{++} and K^+ produced only a little more acetylcholine than those which had Ca^{++} alone (Exps. 9 and 10, Table 8). This shows that the inhibition caused by the Ca-ions is not reversed by K-ions. On the other hand, extracts containing both Ca^{++} and K^+ produced much less acetylcholine than those which had K^+ alone (Table 9). This is particularly noticeable when the yields of acetylcholine of the following samples are compared:

5 (*a*) Without Ca^{++} and without K^+	77μg.
5 (*b*) With Ca^{++} but without K^+	33μg.
9 (*a*) Without Ca^{++} but with K^+	150μg.
9 (*b*) With Ca^{++} and with K^+	40μg.

Similar differences stand out if samples 6 (*a*), 6 (*b*), 10 (*a*) and 10 (*b*) are compared. It should be pointed out that all eight samples were incubated simultaneously and contained extracts prepared from the same acetone powder.

DISCUSSION

Since 1931, when the isolation of adenosinetriphosphate was successfully accomplished by Lohmann, there has been a continuous flow of contributions on the subject of the physiological role and metabolism of this important nucleotide. ATP has been found to react in tissues with creatine and thus to promote the formation of phosphocreatine (Lohmann, 1934). The labile groups of ATP were found to be of paramount importance in the formation and breakdown of several intermediary products of glycolysis, both in muscle (Parnas, Ostern & Mann, 1934) and in yeast (Lutwak-Mann & Mann, 1935). ATP has been recognized as the substance the breakdown of which supplies the energy of muscle contraction, and the dephosphorylation of ATP was shown to be closely associated with myosin (Engelhardt & Ljubimowa, 1939). A new and interesting feature of the action of ATP was described by Green (1943) and

by Bielschowsky & Green (1943) who identified as ATP the muscle component which causes severe shock when injected into animals.

The most recent contribution to the subject of the function of ATP comes from Nachmansohn & Machado (1943) who found that homogenized brain tissue can build up acetylcholine from choline, provided that ATP is added and the mixture is incubated anaerobically. This we were able to confirm. At the same time, however, we noticed that very little acetylcholine was formed in the presence of ATP, if the mixtures were incubated aerobically, and yet it is well known that the formation of acetylcholine in brain slices and pulp occurs in the presence of oxygen. This discrepancy remained to be explained.

In our experiments we used mainly saline extracts prepared from acetone-dried and powdered brains. Their use made it possible to demonstrate the essential role of ATP in the aerobic synthesis of acetylcholine and, moreover, that a definite function in this process must be assigned to sulphydryl compounds. The function of ATP appears to be specific. Even closely related derivatives such as adenosinediphosphate or inosinetriphosphate are but poor substitutes for ATP in the synthesis of acetylcholine. It is too early to state precisely which part of the ATP molecule is involved in the formation of acetylcholine. The evidence available so far, suggests that the most likely change is the conversion of ATP to ADP.

It has been observed that glucose, fructose and also some phospho-hexoses significantly decrease the synthesis of acetylcholine. This we attribute to esterification taking place between ATP and the various sugar compounds, with the result that less ATP remains available for the synthesis of acetylcholine.

It is interesting to note that low as well as high concentrations of glucose are capable of inhibiting the formation of acetylcholine. In this respect the results differ from those obtained with brain slices and pulp, where glucose acts as an inhibitor, only if present in high concentrations (Feldberg, 1945), whereas low concentrations stimulate and even appear to be essential for the synthesis (Mann, Tennenbaum & Quastel, 1939). Such differences, however, are not surprising if we bear in mind the fact that the conditions under which acetylcholine is synthesized in the brain tissue differ substantially from those which prevail in the cell-free extracts. For instance, whereas in the brain tissue the formation of acetylcholine is closely linked with its release, no release is possible in the extracts. In addition, the formation of acetylcholine in the brain tissue, unlike that in the extracts, occurs under conditions where respiration is a dominant factor. The inhibitory effect of glucose on the formation of acetylcholine in the extracts has been shown to be due primarily to an enzymic esterification of the labile phosphate groups of ATP. There is, as yet, no evidence that a similar mechanism is involved in the inhibitory action of glucose on the formation of acetylcholine in the brain tissue.

It has been shown previously that K-ions enhance, and Ca-ions diminish the formation of acetylcholine (Mann *et al.* 1939; Feldberg, 1945). Nachmansohn & Machado failed to observe the stimulating effect of K-ions on the formation of acetylcholine in homogenized brain and stated that the previously observed effect of K^+ was not connected directly with the formation of acetylcholine, but was due to certain changes in cell permeability. Our experiments, however, show that K-ions have a strong stimulating effect on the formation of acetylcholine both in homogenized brain and in extracts from acetone powder. The solutions used by Nachmansohn & Machado apparently already contained K-ions in a concentration sufficient to stimulate the formation of acetylcholine.

The fact that K-ions are involved in the formation of acetylcholine is of special interest in connexion with the role which K^+ plays in the mechanism of chemical transmission of nerve impulses by acetylcholine. The release of acetylcholine from cholinergic nerve fibres is generally regarded as the result of 'a mobilization of K-ions' which occurs during the passage of a nerve impulse. The release is followed by a resynthesis of acetylcholine which is said to be independent of the passage of impulses. Our results indicate that the 'mobilization of K-ions' is not only connected with the release, but that it also directly affects the resynthesis.

Summary

1. The observation of Nachmansohn & Machado (1943), that homogenized brain tissue synthesizes acetylcholine anaerobically in the presence of adenosinetriphosphate (ATP) and choline, has been confirmed. Scarcely any acetylcholine was formed under aerobic conditions.

2. The enzyme system which catalyses the formation of acetylcholine has been separated from the particulate matter of brain and obtained in the form of cell-free solutions, prepared by saline extraction of acetone-dried and powdered brain (rat).

3. The formation of acetylcholine in such extracts was better in anaerobic than in aerobic conditions. In 1 hr., at 37° C., 140–400μg. acetylcholine was formed anaerobically by the extract obtained from 1 g. acetone powder, but only half this amount was produced aerobically.

4. Reduced glutathione and cysteine strongly activated the aerobic formation of acetylcholine. On the other hand, oxidized glutathione and cysteine inhibited both the aerobic and the anaerobic formation of acetylcholine. Thus adenosinetriphosphate and SH— compounds are both essential for the aerobic formation of acetylcholine.

5. The breakdown of ATP in the extract from the brain acetone-powder has been followed in the course of the synthesis of acetylcholine. ATP could not be replaced by either adenosinediphosphate, inosinetriphosphate or adenylate.

6. The formation of acetylcholine was inhibited in the presence of glucose, fructose and certain phosphohexoses. The effect of sugars was found to be due to an enzymic esterification of the labile phosphate groups of ATP.

7. The formation of acetylcholine was enhanced by K-ions and diminished by Ca-ions.

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REFERENCES

Bailey, K. (1942). *Biochem. J.* **36**, 121.
Bielschowsky, M. & Green, H. N. (1943). *Lancet*, **2**, 153.
Engelhardt, V. A. & Ljubimowa, M. M. (1939). *Nature, Lond.*, **144**, 668.
Feldberg, W. (1945). *J. Physiol.* **103**, 367.
Green, H. N. (1943). *Lancet*, **2**, 147.
Lohmann, K. (1931). *Biochem. Z.* **237**, 445.
Lohmann, K. (1934). *Biochem. Z.* **271**, 264.
Lohmann, K. & Jendrassik, L. (1926). *Biochem. Z.* **178**, 419.
Lutwak-Mann, C. & Mann, T. (1935). *Biochem. Z.* **281**, 140.
Mann, P. J. G., Tennenbaum, M. & Quastel, J. H. (1939). *Biochem. J.* **33**, 1506.
Nachmansohn, D. & Machado, A. L. (1943). *J. Neurophysiol.* **6**, 397.
Parnas, J. K., Ostern, P. & Mann, T. (1934). *Biochem. Z.* **272**, 64.
Quastel, J. H., Tennenbaum, M. & Wheatley, H. M. (1936). *Biochem. J.* **30**, 1668.
Stedman, E. & Stedman, E. (1937). *Biochem. J.* **31**, 817.