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THE EFFECTS OF MAGNESIUM IONS AND OF CREATINE PHOSPHATE ON THE SYNTHESIS OF ACETYLCHOLINE

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This paper deals with the influence of divalent cations on the synthesis of acetylcholine and with the problem of determining whether creatine phosphate (CrP) can replace adenosine triphosphate (ATP) in this synthesis.

In previous experiments in which studies were made of the synthesis of acetylcholine by homogenized brain tissue (Nachmansohn & Machado, 1943) or by acetone-dried brain (Feldberg & Mann, 1945) in the presence of ATP, it was found that magnesium ions had little or no action. In later experiments (Feldberg & Hebb, 1945), in which *dialysed* enzyme preparations of acetone-dried brain tissue were used, Mg ions were found to have a strong accelerating action on the synthesis of acetylcholine; it appeared as though dialysis had removed from the system the Mg ions, in the absence of which synthesis was greatly depressed. This explanation is only partially true. Dialysis is not the sole determining factor. A distinction has to be made between the synthesis which is accelerated by citrate and the synthesis which occurs in its absence. It has now been found that only the synthesis accelerated by citrate is potentiated by Mg ions, although, in order to obtain the maximum effect, dialysed enzyme preparations have to be used. We have further shown that Mg ions can be replaced by manganese ions, but not by any of the other divalent cations examined.

The suggestion has been made (v. Muralt, 1943; Nachmansohn, Cox, Coates & Machado, 1943) that not only ATP but also CrP may act in the synthesis of acetylcholine in a manner analogous to their action in muscle metabolism. We have, in fact, found that, under certain conditions, CrP can replace ATP in the synthesis of acetylcholine. A short account of these results has been published (Feldberg & Hebb, 1946). Since then Torda & Wolff (1946), using minced frog's brain as the source of their enzyme preparations, have found

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independently that CrP increases the formation of acetylcholine by about 50% and that creatine produces about half this effect. With our method the accelerating effect of CrP is incomparably greater, but creatine is inactive.

METHODS

The experimental methods were essentially the same as those previously described by Feldberg & Mann (1945, 1946). Non-dialysed or dialysed saline extracts were prepared from the acetone-dried brain of the rat or the guinea-pig, and incubated aerobically. In a few experiments incubation was carried out anaerobically. The incubated samples always had a volume of 4·5 c.c. and included, unless otherwise stated, the following: saline extract of 50 mg. acetone-dried brain powder, 6 mg. KCl, 3 mg. choline, 2 mg. NaF, 4·5 mg. cysteine, 0·5 mg. eserine sulphate and either 1·5 mg. $MgSO_4$ or 2–4 mg. $MgCl_2$. In testing the effects of other divalent cations, the salts used were $CaCl_2$, $MnCl_2$, $ZnSO_4$ and $Co(NO_3)_2$. Activator when required was prepared by boiling saline extracts of acetone-dried brain tissue; the supernatant fluid of the boiled extract, after centrifugation, was added to the sample in an amount equivalent to 100 mg. acetone powder. Citrate when used was added in an amount of 15 mg. sodium citrate; similarly, the amount of ATP per sample, when it was used, was 0·4 mg. ATP-pyro P. About 5 mg. of the barium salt (converted to the sodium salt before use) correspond to 1 mg. ATP-pyro P. CrP was kindly prepared for us as the Ba salt by Dr P. Eggleton and Dr R. N. Smith, to whom we should like to express our thanks. The barium salt was also converted to the sodium salt before use, and the amounts given in each sample are expressed in mg. creatine phosphate phosphorus (CrP-P); about 12 mg. of the barium salt correspond to 1 mg. Cr P-P. We should like also to express our thanks to Dr Malcolm Dixon for supplying us with a sample of muscle adenylic acid and to Dr S. Bach for supplying us with a sample of *l*(+)-glutamic acid.

RESULTS

Divalent cations

Magnesium ions. When synthesis of acetylcholine is produced in saline extracts of acetone-dried brain in the presence of ATP, KCl, NaF, choline, cysteine and eserine, Mg ions have no effect. If, however, citrate is added to the synthesizing medium as well, Mg ions exert a relatively strong accelerating action on the formation of acetylcholine. This difference is shown in the experiments of Table 1. In none of the nine experiments in which the synthesis was studied in the absence of citrate did Mg^{++} accelerate the synthesis, but in all but one of the fifteen experiments in which it was studied in the presence of citrate Mg ions had a strong action of this kind. This action was not affected by fluoride; it could be demonstrated in the absence of cysteine under anaerobic conditions (Table 1).

In Fig. 1 is seen the effect of varying concentrations of Mg ions on the synthesis of acetylcholine in the presence of citrate. A concentration of 0·0001 M increases the synthesis by about 40%; the optimal effect, an increase of about 300%, was obtained with a concentration of 0·004 M. With higher concentrations of Mg ions the rate of synthesis declined.

The action of Mg ions on the synthesis of acetylcholine in the presence of citrate was even more pronounced when the saline extracts used for incubation

TABLE 1. Effect of Mg ions on the formation of acetylcholine in non-dialysed saline extracts of acetone-dried brain in the absence and in the presence of citrate

	μg. acetylcholine formed in 1 hr./g. acetone powder				
	Without citrate		With citrate		
Exp.	(a) No Mg	(b) 0·002 M-Mg	(c) No Mg	(d) 0·002 M-Mg	Notes
1	80	85	280	650	Anaerobically without cysteine
2	140	155	—	—	—
3	180	165	360	1050	—
4	180	190	430	1050	—
5	260	250	400	1100	—
6	450	330	720	1240	Activator added to samples
7	470	400	800	1100	Activator added to samples
8	180	190	330	700	Ca (0·002 M) added to samples
9	450	450	600	880	Activator and Ca (0·002 M) added to samples
10	—	—	140	420	NaF omitted from samples
11	—	—	310	880	—
12	—	—	460	510	NaF omitted from samples
13	—	—	520	880	NaF omitted from samples
14	—	—	560	900	—
15	—	—	610	1260	NaF omitted from samples
16	—	—	430	1250*	—

* Sample contained 0·004 M-Mg.

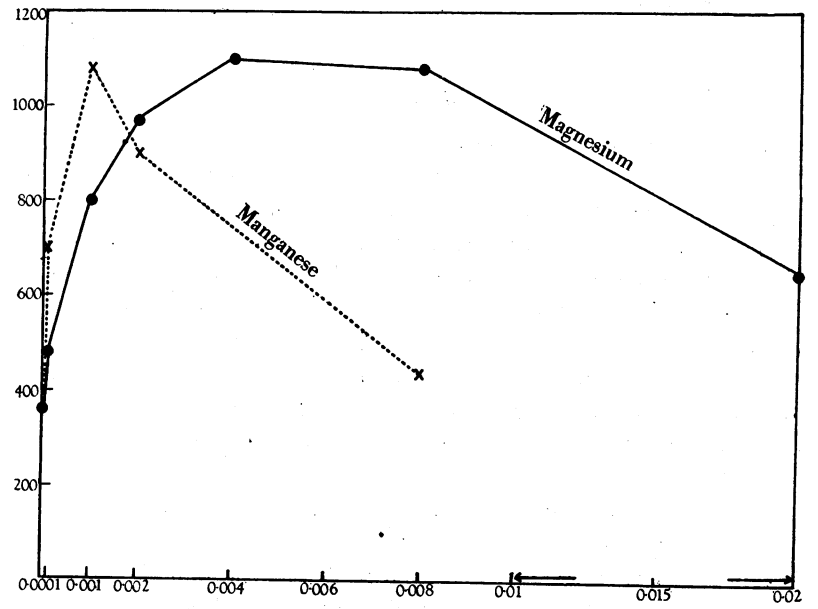

Fig. 1. Effect of varying concentrations of Mg ions (continuous line) and of Mn ions (dotted line) on the formation of acetylcholine by *non-dialysed* saline extracts of acetone-dried brain in the presence of ATP and citrate. Abscissae: molar concentration of Mg and Mn ions. Ordinates: μg./g./hr. acetylcholine formed.

were first dialysed. Again, fluoride did not affect the result. In such dialysed extracts Mg ions may increase the synthesis of acetylcholine by as much as 20 times (Table 2). In fact, the strong reactivating effect that citrate has on the synthesis in dialysed brain extracts in the presence of ATP depends on the Mg ions. For instance, in most experiments of Table 2, citrate increased the synthesis of acetylcholine by not more than 2 to 3 times in the absence, but 10–20 times in the presence of Mg ions. From Exps. 1 and 2 of Table 2 it will be seen that in dialysed extracts Mg ions have some accelerating effect on the synthesis of acetylcholine independent of citrate; the effect, however, is small compared to that obtained in the presence of citrate.

TABLE 2. Effect of Mg and Mn ions on the formation of acetylcholine in *dialysed* saline extracts of acetone dried brain in the absence and in the presence of citrate

	μg. acetylcholine formed in 1 hr./g. acetone powder				
	Without citrate		With citrate		
Exp.	No Mg	0·002–0·004 M-Mg	No Mg	0·002–0·004 M-Mg	0·001–0·002 M-Mn
1	12	28	24	440	—
2	18	35	55	580	—
3	20	—	30	600	—
4	12	—	38	380	500
5	12	—	55	500	620
6	20	—	25	330	420
7	24	—	64	430	520
8	40	—	150	650	720

In Fig. 2 is seen the effect of varying concentrations of Mg ions on the synthesis of acetylcholine in dialysed extracts and in the presence of citrate. Without Mg ions 24 μg. acetylcholine per g. tissue were formed in 1 hr.; a concentration of 0·00005 M-Mg ions, however, increased this value to 42, and a concentration of 0·002–0·004 M was usually necessary to obtain the maximal value which, in this experiment, was about 400 μg. With a concentration of Mg ions stronger than 0·004 M the synthesis of acetylcholine showed no further improvement, but was in fact slightly depressed.

The action of Mg ions when tested in dialysed saline extracts to which activator had been added is indistinguishable from its action on the synthesis in non-dialysed extracts, i.e. in the presence of citrate 0·002 M-Mg ions increased the synthesis about 2–3 times; in the absence of citrate it had no effect, or might even depress it. This was the case when large amounts of activator were added to the dialysed as well as to the non-dialysed enzyme extracts (see Exps. 6 and 7, Table 1).

Calcium ions. Ca ions are known to inhibit the synthesis of acetylcholine. According to Greville & Lehmann (1944) and to Bailey & Webb (1944), Ca ions and Mg ions have an antagonistic action on certain enzymes which is due to their being competitive with one another. This explanation does not account

for the opposing actions of Ca and Mg ions on the synthesis of acetylcholine. The accelerating action of Mg ions is dependent on citrate; the inhibiting action of Ca ions is not; in addition, Mg ions exert the accelerating effect in the presence of calcium ions (see Exps. 8 and 9, Table 1).

Manganese ions. Mn ions can replace Mg ions in the synthesis of acetylcholine. Like Mg ions the action of Mn ions is dependent on the presence of citrate. Of the two ions, Mn is the more powerful. In the experiment shown in Fig. 1, for instance, 0·001 M-Mn ions had the same optimal accelerating action on the synthesis of acetylcholine as Mg ions in a concentration of 0·004 M. Similarly, the depression of the synthesis which occurs with higher

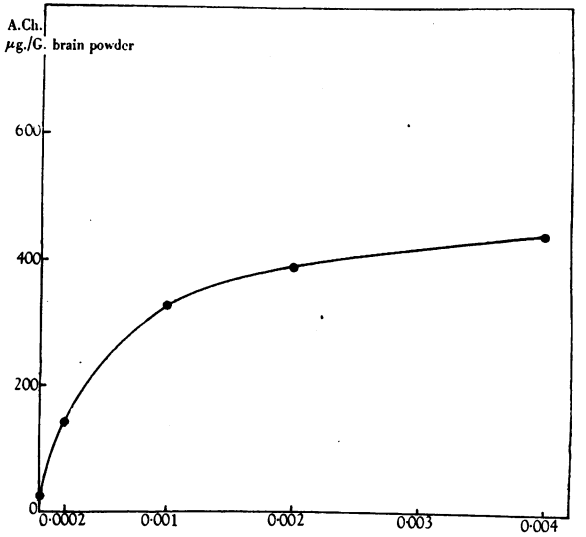

Fig. 2. Effect of varying concentrations of Mg ions on the formation of acetylcholine by *dialysed* saline extracts of acetone-dried brain in the presence of ATP and citrate. Abscissae: molar concentration of Mg ions. Ordinates: μg./g./hr. acetylcholine formed.

concentrations of Mg ions begins at 0·002 M-Mn ions. For the formation of acetylcholine in dialysed enzyme extracts also, the optimal concentration of Mn^{++} is lower than that of Mg^{++}. In the five experiments of Table 2, in which the effect of Mn and Mg ions were compared with each other, the amounts of acetylcholine formed in the presence of Mn were each time more than those formed in the presence of Mg^{++}, although the concentration of Mg^{++} was twice that of Mn^{++}.

By combining the two ions, Mg and Mn, in half-optimal concentrations, the synthesis of acetylcholine may be increased to values corresponding to those obtained with the optimal concentration of one ion alone. It is not possible, however, to increase the synthesis any further by combining the two ions each in optimal concentration. In fact, under such conditions, the synthesis of

acetylcholine decreases again, as it would do if one of the two ions alone were used in double the optimal concentration. From these experiments, which were carried out with dialysed enzyme extracts, it appears that the two ions Mg and Mn are interchangeable in their mode of action on the synthesis of acetylcholine.

Cobalt and zinc ions. These ions in a concentration of 0·0005–0·0001 M have no action on the synthesis of acetylcholine in dialysed or non-dialysed enzyme extracts, either in the presence or absence of citrate. Actually 0·004 M-Zn^{++} inhibited the synthesis.

Creatine phosphate (CrP)

Non-dialysed extracts. CrP may be used to replace ATP in the synthesis of acetylcholine by non-dialysed extracts prepared from acetone-dried brain powder. Of the two, CrP usually has the more powerful action. The maximal effect of CrP is obtained when it is used in a concentration of about 0·1 mg./c.c. CrP-P; some action may be observed even with a fiftieth of this concentration. The effects of this range of concentrations of CrP are seen in Table 3. For each

TABLE 3. Effect of different concentrations of CrP on the formation of acetylcholine in saline extracts of acetone-dried brain

Concentration of CrP-P in mg./c.c.	μg. acetylcholine formed in 1 hr./g. acetone-powder	
	Without citrate	With citrate
0	23	45
0·002	45	75
0·01	170	230
0·05	250	900
0·1	300	1100

of the given concentrations of CrP, values are given for the amounts of acetylcholine synthesized both in the presence and absence of citrate, Mg ions being added in both cases. The synthesis of acetylcholine was much greater in the presence of citrate, but the effect of citrate depended largely on the addition of Mg ions as well. In this and other ways the action of CrP is analogous to that of ATP. For example, when CrP is used to replace ATP, the synthesis occurs also in the absence of fluoride and under anaerobic conditions; it is slightly increased by *l*(+)-glutamic and inhibited by pyruvic acid.

When one of the two, CrP or ATP, is already present in about optimal concentration, the synthesis is not very much increased by addition of the other. In the experiment of Table 4 are shown the effects of CrP and of ATP added either separately or together. A concentration of 0·08 mg./c.c. CrP-P brought the synthesis of acetylcholine to the value of 290 μg./g. in the absence of citrate and to 1200 μg./g. in the presence of citrate. With 0·09 mg./c.c. ATP-P the corresponding values were 145 and 900, and with 0·009 mg./c.c.

ATP-P 50 and 270 μg./g./hr. respectively. When the smaller concentration of ATP was given together with the CrP, the synthesis was increased by only 3%: when the stronger concentration was given together with CrP, by only 17% above the level of CrP alone.

TABLE 4. Effect of ATP and CrP on the formation of acetylcholine in saline extracts of acetone-dried brain

Additions of CrP-P or ATP-P in mg./c.c.	μg. acetylcholine formed in 1 hr./g. acetone powder	
	Without citrate	With citrate
None	26	80
0·009 ATP-P	50	270
0·09 ATP-P	145	900
0·08 CrP-P	290	1200
0·08 Cr-P, 0·009 ATP-P	300	1240
0·08 Cr-P, 0·09 ATP-P	340	1400

Dialysed extracts. On dialysis, the saline extracts of acetone-dried brain lose their ability to synthesize acetylcholine or retain this ability to only a slight extent, even in the presence of ATP. They are reactivated, however, by adding either ATP together with activator, or ATP together with citrate and Mg ions (Feldberg & Mann, 1946). In corresponding experiments with CrP, it was found that CrP together with activator reactivated the dialysed extract. CrP together with citrate and Mg ions on the other hand had no such effect. If, however, muscle adenylic acid were added as well, large amounts of acetylcholine were then formed. We must therefore conclude that CrP in order to be effective as a phosphorylating agent in the synthesis of acetylcholine acts in combination with muscle adenylic acid, probably by the formation of ATP.

The reactivation of dialysed enzyme extracts by CrP with activator is shown in the following two experiments: in the one, the dialysed extract when incubated for 1 hr. with CrP alone synthesized 15 μg./g., but when incubated with activator as well it produced 360 μg./g./hr.; in the other, the corresponding rates were 30 and 360 μg./g./hr.

By comparison with the effect of activator, citrate combined with CrP and Mg ions had a relatively weak action and, as shown in Table 5, did not re-

TABLE 5. Formation of acetylcholine in *dialysed* saline extracts of acetone-dried brain. Effect of CrP with and without muscle and yeast adenylic acid or adenosine

Additions to samples	μg. acetylcholine formed in 1 hr./g. acetone powder		
	Exp. 1	Exp. 2	Exp. 3
Citrate	38	40	—
CrP, citrate	65	50	22
CrP, citrate, muscle adenylic acid	720	650	320
CrP, citrate, yeast adenylic acid	—	50	30
CrP, citrate, adenosine	—	50	25
CrP, citrate, activator	1200	—	—
CrP, citrate, activator muscle adenylic acid	1500	—	—

activate the dialysed extract to any extent. Thus only 22–65 μg./g./hr. were formed in samples incubated with CrP, citrate and Mg ions together. These values increased to between 320 and 850 when muscle adenylic acid was added as well.

The optimal effect of muscle adenylic acid is obtained with relatively low concentrations, since its action is again depressed with concentrations greater than 0·01 mg./c.c. adenylic acid-P. This result may be seen in Fig. 3, in which the different concentrations of muscle adenylic acid are plotted against the percentage increase of acetylcholine above that found in the absence of adenylic acid. Some of the points are the mean values of two or three observations.

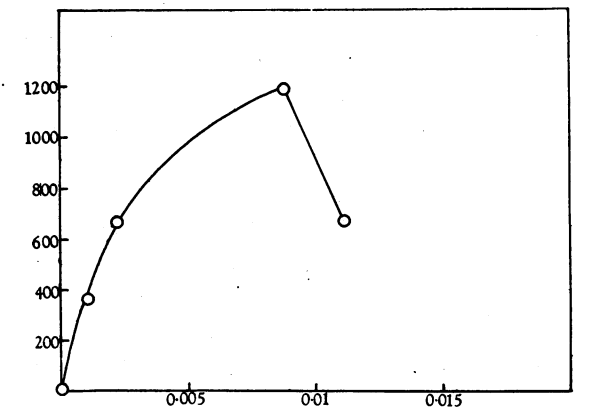

Fig. 3. Effect of varying concentrations of muscle adenylic acid on the formation of acetylcholine by *dialysed* saline extracts of acetone dried brain in the presence of CrP, citrate and Mg ions. Abscissae: concentration of muscle adenylic acid-P in mg./c.c. Ordinates: percentage increase of acetylcholine above level found in the absence of adenylic acid.

The effectiveness of muscle adenylic acid in increasing the synthesis of acetylcholine depends upon its action in combination with CrP. If muscle adenylic acid is given alone, or given with creatine plus inorganic phosphate, or given with ATP, it has no effect.

The effect of muscle adenylic acid is specific; it cannot be replaced by yeast adenylic acid or by adenosine (see Table 5).

DISCUSSION

Our experiments show that Mg ions have a great accelerating effect on the synthesis of acetylcholine, but only when the system includes citrate. In such a system, the presence of either Mg ions, or an ion which like Mn^{++} can replace Mg^{++}, is necessary to obtain the full effect which citrate can have. The effect of Mg ions has hitherto been overlooked; in earlier experiments it was examined on the synthesis of acetylcholine in the absence of citrate, and the effects of citrate were examined always in the presence of Mg^{++} only.

When synthesis of acetylcholine is studied in a system which includes the activator instead of citrate, the presence of Mg ions is of little importance. This suggests that the mechanisms of synthesis, the one involving activator, the other involving citrate are independent of one another. Any suspicion, therefore, that the activator can be identical with citrate is removed by these considerations, if the evidence given previously (Feldberg & Mann, 1946) were not already sufficient to show this.

Creatine phosphate (CrP) like ATP appears to be a normal constituent of nerves and brain (Gerard & Wallen, 1929; Holmes, 1933; Kerr, 1935). According to Kerr it is present in mammalian brain tissue in a concentration of between 0·08 and 0·14 mg./g. CrP-P. The optimal concentration for the synthesis of acetylcholine in our experiments was of the same order, i.e. 0·1 mg./c.c. CrP-P. Lohmann (1935) and Torres (1935) have demonstrated that the CrP metabolism in nervous tissue involves adenylic acid as a phosphate carrier, the ATP formed being the phosphate donator. It is probable that a similar interpretation can be given to our finding that, in dialysed saline extracts of acetone-dried brain tissue, CrP can act with citrate and Mg ions to replace ATP, only if muscle adenylic acid is added as well. It is also likely that our preparations of activator, which are obtained by boiling saline extracts of brain tissue, contain muscle adenylic acid since it is both heat-stable and dialysable, and that the formation of ATP when either activator or muscle adenylic acid as such is added, is the means by which CrP acts in the synthesis of acetylcholine.

SUMMARY

1. Mg ions accelerate the synthesis of acetylcholine by saline extracts of acetone-dried brain in the presence of citrate. In non-dialysed saline extracts Mg ions increase the synthesis 2–3 times, in dialysed extracts sometimes as much as 20 times. Without citrate, Mg ions have little or no effect. The strong accelerating action which citrate itself has on the synthesis of acetylcholine is, in fact, dependent on the presence of Mg ions. Mg ions can be replaced by Mn, but not by Ca, Zn or Co ions.

2. Creatine phosphate (CrP) can replace ATP in the synthesis of acetylcholine by *non-dialysed* extracts prepared from acetone-dried brain.

3. CrP does not replace ATP in the synthesis of acetylcholine by *dialysed* extracts unless either activator or muscle adenylic acid is added as well. The effect of muscle adenylic acid is specific, and it cannot be replaced by yeast adenylic acid or adenosine. The theory is discussed that CrP, in order to act in the synthesis of acetylcholine, involves muscle adenylic acid as a phosphate carrier, according to the Lohmann reaction, the ATP formed being the phosphate donator.

Note added in proof. ATP sensitizes the frog rectus muscle to acetylcholine. This was observed by Torda & Wolff (1945) and later independently by Babsky & Minajew (1946). These

authors suggest that ATP may not stimulate the synthesis of acetylcholine but that the synthesis observed in brain extracts by Nachmansohn & Machado and by Feldberg & Mann may be nothing but this sensitizing effect of ATP. It is true that even the minute concentrations of ATP present in the diluted extracts used for the assay on the frog rectus muscle are sufficient to sensitize it to acetylcholine. When brain extracts which have been incubated with ATP are assayed against pure acetylcholine solutions the values obtained for acetylcholine are in fact somewhat too high (Feldberg & Mann, 1945). However, in the experiments of Feldberg & Mann as well as in our experiments, the brain extracts were assayed against acetylcholine solutions to which were added equivalent amounts of the same brain extract after it had been boiled for a moment in alkaline solution and then neutralized (see Feldberg & Mann, 1945). This treatment hydrolyses the acetylcholine but does not abolish the sensitizing action of ATP. We have found that known solutions of acetylcholine added to brain extract containing ATP can be determined quantitatively in this way. Therefore with the method of assay used by Feldberg & Mann and by ourselves the sensitizing effect of ATP is taken into account and does not interfere with the result. According to Torda & Wolff the sensitizing effect of CrP compared to that of ATP is only slight; it would therefore scarcely affect the result even if it were not taken into account in the assay of acetylcholine.

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