EFFECTS OF INHIBITORS OF GLUTAMINE SYNTHESIS ON THE INHIBITION OF ACETYLCHOLINE SYNTHESIS IN BRAIN SLICES BY AMMONIUM IONS

by

BEATRIZ M. BRAGANCA, P. FAULKNER AND J. H. QUASTEL
Research Institute, Montreal General Hospital, Montreal (Canada)

INTRODUCTION

It was demonstrated by MANN, TENNENBAUM AND QUASTEL[1] that ammonium ions bring about an inhibition of the aerobic synthesis of acetylcholine by brain slices, the mechanism of this inhibitory action being at present unknown. KREBS[2] showed that actively respiring brain slices remove ammonium ions to form glutamine. It is now well known that both the synthesis of acetylcholine and of glutamine require adenosine-triphosphate[3,4]. Since these two processes proceed simultaneously in respiring brain slices the possibility that the inhibitory action of ammonium ions on acetylcholine synthesis by brain slices may be, in part, due to a competition between the acetylation process and that of glutamine formation for the available adenosine triphosphate has been investigated.

Results reported in this article show that substances which inhibit glutamine synthesis bring about a reduction of the inhibitory effect of ammonium ions on acetylcholine synthesis by rat brain slices.

METHODS AND MATERIALS

The technique employed was essentially that described by MANN *et al.*[1]. The rats were killed by decapitation, the slices were cut from the brain cortex by means of Stadie Riggs Slicer. The synthesis was allowed to take place in Warburg manometric vessels in 0.028 *M* bicarbonate-Locke Medium in an atmosphere of 93% O_2 and 7% CO_2 at 37° C. After incubation for 2 hours the slices were separated from the remaining fluid, which was assayed for free acetylcholine. The bound acetylcholine was determined directly in the slices after its conversion to the free form by placing in a boiling water bath for 10 minutes at pH 4. For this purpose the slices were placed in 3 ml of H_2O and acidified with 1 ml 0.3 *M* NaH_2PO_4. The tube containing the mixture was heated for 10 minutes in boiling water. The contents were then cooled, neutralised, and the assay carried out by utilising the contraction of eserinised leech muscle. In experiments in which acetylcholine synthesis was studied in presence of added adenosine triphosphate, this was removed prior to the assay, by the method of HARPUR AND QUASTEL[6]. None of the compounds themselves affected the leech. The values of acetylcholine synthesised are expressed in μg per gram of wet weight of tissue.

RESULTS

Effect of ammonium ions on aerobic synthesis of acetylcholine by rat brain slices

Results illustrating the effects of 0.027 *M* NH_4^+ on the aerobic synthesis of acetyl-

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choline by rat brain slices in presence of glucose are shown in Table I. It is seen from these results that the presence of ammonium ions brings about a considerable drop in the amount of combined acetylcholine formed. The effects, however, on the production of free acetylcholine are not so consistent. In some of the experiments, there is a slight increase in the rate of formation of free acetylcholine. This may be due largely to the breakdown of bound to free acetylcholine. The rate of total acetylcholine synthesis is almost always decreased in presence of ammonium ions.

TABLE I

EFFECTS OF ADDITION OF AMMONIUM IONS ON AEROBIC SYNTHESIS OF ACETYLCHOLINE BY RAT BRAIN SLICES IN 93% O_2 + 7% CO_2

(Locke-bicarbonate solution, 0.028 *M*; eserine, 0.005 *M*; choline chloride, 0.002 *M* and glucose, 0.01 *M*).

Expt. No.	NH_4^+	*μg Acetylcholine formed (μg/g tissue) 2 h 37 °C*			*% of control, with substrates in absence of added* NH_4^+		
		Free	*Bound*	*Total*	*Free*	*Bound*	*Total*
1	Nil	5.4	8.0	13.4			
	0.027 *M*	3.0	3.7	6.7	55.0	46.0	50.0
2	Nil	8.2	6.6	14.8			
	0.027 *M*	3.9	0.4	4.3	48.0	6.0	29.0
3	Nil	16.6	11.5	28.1			
	0.027 *M*	16.0	3.2	19.2	97.0	28.0	67.0
4	Nil	16.3	16.6	32.9			
	0.027 *M*	17.7	5.5	23.2	108.0	33.2	73.0
5	Nil	11.0	8.0	19.0			
	0.027 *M*	12.0	3.2	15.2	109.0	40.0	80.0
6	Nil	11.0	8.8	19.8			
	0.027 *M*	3.5	2.7	6.2	32.0	31.0	31.0

These observations, in general, confirm the earlier findings of MANN *et al.*[1]. The results reported here show a decrease in the synthesis of total acetylcholine in the majority of experiments and always a large decrease in the bound form.

Ammonium ions exerted no inhibition of acetylcholine synthesis in extracts of acetone powders of beef brain in the presence of adenosine triphosphate, confirming the observation of NACHMANSOHN AND MACHADO[3].

Effects of various inhibitors of glutamine synthesis on the ammonia inhibition of acetylcholine synthesis by rat brain slices

In respiring brain slices, where glutamate is being continually produced from α-ketoglutarate and ammonia, the presence of extra ammonium ions should enhance the synthesis of glutamine and should, therefore, reduce the available adenosine triphosphate required for the synthesis of acetylcholine. If this hypothesis is correct, it might be expected that substances, which inhibit the synthesis of glutamine, will tend to decrease the inhibitory action of ammonium ions on acetylcholine synthesis.

Typical results showing the effects of methionine sulphoxide, ethionine sulphoxide, methionine sulphoximine and glutamine on the inhibition produced by 0.027 *M* am-

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monium ions on the aerobic synthesis of acetylcholine by rat brain slices in presence of glucose are given in Table II.

TABLE II

EFFECTS OF ADDITION OF L-GLUTAMINE, DL-METHIONINE SULPHOXIDE, DL-ETHIONINE SULPHOXIDE, DL-METHIONINE SULPHOXIMINE AND AMMONIUM IONS ON AEROBIC SYNTHESIS OF ACETYLCHOLINE BY RAT BRAIN SLICES

(Incubation medium-Locke bicarbonate, 0.028 *M*; eserine, 0.005 *M*; choline chloride, 0.002 *M*; glucose, 0.01 *M*. Incubation time — 2 hours at 37° C. Gas phase 93% O_2/7% CO_2)

Expt. No.	NH_4+	*Test substance added*	*μg acetylcholine per g tissue*			*Ach synthesis (expressed as % control with substrates in absence of added* NH_4+		
			Free	*Bound*	*Total*	*Free*	*Bound*	*Total*
1	Nil	Nil	7.8	7.8	15.6			
	0.027 *M*	Nil	3.6	0.9	4.5	46	12	29
	Nil	0.01 *M* methionine sulphoxide	8.0	6.0	14.0			
	0.027 *M*	0.01 *M* methionine sulphoxide	4.9	4.8	9.7	61	80	69
2	Nil	Nil	6.6	9.1	15.7			
	0.027 *M*	Nil	10.8	2.3	13.1	163	25	84
	Nil	0.01 *M* ethionine sulphoxide	8.3	5.8	14.1			
	0.027 *M*	0.01 *M* ethionine sulphoxide	17.8	4.8	22.6	210	82	160
3	Nil	Nil	16.6	11.5	28.1			
	0.027 *M*	Nil	16.0	3.2	19.2	97	28	68
	Nil	0.01 *M* methionine sulphoximine	19.0	8.0	27.0			
	0.027 *M*	0.01 *M* methionine sulphoximine	15.0	6.2	21.2	79	78	78
4	Nil	Nil	16.0	13.3	29.3			
	0.027 *M*	Nil	16.0	4.0	20.0	100	30	68
	Nil	0.01 *M* L-glutamine	13.8	11.0	24.8			
	0.027 *M*	0.01 *M* L-glutamine	24.0	10.6	34.6	174	96	140

Methionine sulphoxide and ethionine sulphoxide are known to inhibit glutamine synthesis in bacteria[7]. The former compound also inhibits glutamine synthesis in preparations made from sheep brain[11]. It is seen from the typical results given in Table II (experiments 1 and 2) that both these compounds bring about a considerable reversal of the ammonia inhibition of acetylcholine synthesis. Ethionine sulphoxide has apparently no effect on the synthesizing system in absence of ammonium ions, but it produces an increased synthesis of acetylcholine when present together with ammonium ions.

Methionine-sulphoximine (0.01 *M*), the toxic factor from "agenized" flour which produces convulsive seizures in animals[8] inhibits glutamine synthesis, producing 43% inhibition of glutamine synthesis in beef brain extracts[12]. In Table II (experiment 3) are given the results of experiments showing the effects of methionine-sulphoximine on the relief of ammonia inhibition of acetylcholine synthesis. These indicate that methionine sulphoximine in absence of ammonium ions produces a slight inhibition of the synthesis of the bound acetylcholine. MCLENNAN AND ELLIOTT[5] have observed inhibitory effects of methionine sulphoximine at relatively large concentrations on acetylcholine synthesis. In presence of ammonium ions, however, methionine sulphoximine may bring about more than threefold stimulation of synthesis of bound acetylcholine.

In view of the possibility that the glutamine synthesising system is reversible, or

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of the possibility that glutamine may compete with glutamate for the enzyme involved, it might be expected that the presence of excess of glutamine would inhibit the rate of glutamine synthesis. The effect of adding glutamine to the acetylcholine synthesising system in presence of ammonium ions was, therefore, tested. The results obtained are illustrated in Table II (experiment 4). They show that glutamine brings about a partial reversal of the ammonium inhibition. In some of the experiments the reversal was almost complete.

Effects of addition of DL-*methionine,* L-*glutamate, and adenosinetriphosphate on the ammonia inhibition of acetylcholine synthesis by rat brain slices*

Typical results obtained with DL-methionine and L-glutamate are given in Table III (experiments 1 and 2). It is seen from these that both the amino acids produce an enhancement of acetylcholine synthesis. The increased synthesis observed in presence of L-glutamate is possibly due to the stimulating action of glutamate on respiration. It is, however, interesting that although L-glutamate tends to stimulate the synthesis of acetylcholine by rat brain slices, the percentage inhibition produced by ammonium ions is the same with and without the amino acid. DL-methionine has no effect on glutamine synthesis, and does not reverse ammonia inhibition of acetylcholine formation.

Experiments in which adenosine triphosphate was added to the slices, to determine whether it would reverse the inhibitory action of ammonium ions, were not conclusive. The characteristic results obtained are given in Table III (experiment 3). It is seen from these that adenosine triphosphate does not increase the synthesis of acetylcholine above that of the control, possibly due to its inability to penetrate the cell.

TABLE III

EFFECTS OF ADDITION OF DL-METHIONINE, L-GLUTAMATE, ADENOSINE TRIPHOSPHATE (ATP) IN PRESENCE OF AMMONIUM IONS ON THE AEROBIC SYNTHESIS OF ACETYLCHOLINE BY RAT BRAIN SLICES

(Incubation medium and time as for Table II)

Expt. No.	NH_4^+	*Test substance added*	*μg Acetylcholine formed μg/g tissue*			*Ach synthesis (expressed as % of the control) with substrates in absence of added* NH_4^+		
			Free	*Bound*	*Total*	*Free*	*Bound*	*Total*
1	Nil	Nil	6.9	11.4	18.3			
	0.027 *M*	Nil	13.0	3.8	16.8	189	33	93
	Nil	0.01 *M* methionine	11.0	14.0	25.0			
	0.027 *M*	0.01 *M* methionine	13.3	2.0	15.3	121	14.0	59
2	Nil	Nil	11.0	8.0	19.0			
	0.027 *M*	Nil	12.0	3.2	15.2	109	40	79
	Nil	0.01 *M* L-glutamate	13.7	12.3	26.0			
	0.027 *M*	0.01 *M* L-glutamate	16.3	5.3	21.6	119	43	81
3	Nil	Nil	8.4	13.0	21.4			
	0.027 *M*	Nil	15.1	4.0	19.1	180	31	90
	Nil	0.006 *M* ATP	8.0	13.2	21.2			
	0.027 *M*	0.006 *M* ATP	23.0	4.7	27.7	280	37	131

DISCUSSION

It is evident from these observations that the presence of ammonium ions produces

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a large decrease in the rate of acetylcholine synthesis by rat brain slices, this being most apparent with the bound form. It has no effect on the synthesis of acetylcholine by extracts of acetone powders of brain in presence of added adenosine triphosphate.

These results indicate that the toxic effect of ammonium ions is exerted only indirectly on the acetylcholine synthesizing system. Results obtained with various substances, namely methionine sulphoxide, ethionine sulphoxide, and methionine sulphoximine which are inhibitors of glutamine synthesis, show that their presence brings about a considerable relief of the inhibition produced by ammonium ions especially on the rate of formation of bound acetylcholine. The presence of the amino acids L-glutamate and DL-methionine, however, has no such effect. These findings may be explained on the hypothesis that in respiring brain slices the presence of extra ammonium ions increases the rate of formation of glutamine, thereby diminishing the available adenosine triphosphate and therefore the rate of synthesis of acetylcholine.

Conceivably, the mechanism by which ammonium ions inhibit acetylcholine synthesis in the nervous system may play a significant role in convulsive states, which are known to be accompanied by ammonia liberation in the brain[9]. The observations recorded by TOWER AND ELLIOTT[10], in which they show that, following partial anoxia, brain slices show a retarded rate of synthesis of bound acetylcholine which is accelerated by glutamine, are probably due to the effects of the presence of an increased concentration of ammonium ions.

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SUMMARY

1. The presence of ammonium ions produces a large decrease in the rate of synthesis of bound acetylcholine by rat brain slices but has no effect on the synthesis of acetylcholine by extracts of acetone powders of brain in presence of added adenosine triphosphate.

2. Various substances, namely methionine sulphoxide, ethionine sulphoxide, and methionine sulphoximine, which are inhibitors of glutamine synthesis, bring about a considerable relief of the inhibition produced by ammonium ions on the rate of formation of bound acetylcholine. L-glutamate and DL-methionine have no such effect.

3. It is suggested that in respiring brain slices the presence of extra ammonium ions increases the rate of formation of glutamine, thereby diminishing the available adenosine triphosphate and therefore the rate of synthesis of acetylcholine.

RÉSUMÉ

1. La présence d'ions ammonium provoque une décroissance considérable de la vitesse de synthèse d'acétylcholine liée dans des tranches de cerveau de rat mais n'affecte aucunement la synthèse d'acétylcholine par des extraits de poudres de cerveau (séchées à l'acétone) en présence d'adénosinetriphosphate ajouté.

2. Diverses substances, c.à.d. les sulphoxyde de méthionine et d'éthionine et la sulphoximine de méthionine qui sont des inhibiteurs de la synthèse de glutamine suppriment à un dégré considérable l'inhibition de la formation d'acétylcholine liée produite par les ions ammonium. L'acide L-glutamique et la DL-méthionine n'ont pas d'action de ce genre.

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3. Les auteurs suggèrent l'idée que dans les tranches de cerveau respirant le surplus d'ions ammonium accélère la formation de glutamine diminuant ainsi l'adénosinetriphosphate disponible et par conséquent la vitesse de synthèse d'acétylcholine.

ZUSAMMENFASSUNG

1. Ammoniumionen bewirken eine bedeutende Geschwindigkeitsabnahme der Synthese von gebundenem Acetylcholin in Scheiben von Rattenhirn, üben aber keine Wirkung auf die Acetylcholinsynthese durch Extrakte von Aceton-Hirnpulver in Gegenwart von zugefügtem Adenosintriphosphat aus.

2. Verschiedene Substanzen, nämlich Methionin- und Ethioninsulphoxyd sowie Methioninsulphoximin, welche Hemmstoffe der Glutaminsynthese sind, heben die, durch Ammoniumionen bewirkte Geschwindigkeitsabnahme der Acetylcholinbildung in bedeutendem Masse auf. L-Glutaminsäure und DL-Methionin haben keine derartige Wirkung.

3. In atmenden Hirnscheiben könnte die Gegenwart von Ammoniumionen die Glutaminbildung beschleunigen, wodurch das verfügbare Adenosintriphosphat vermindert und dadurch die Acetylcholinsynthese verlangsamt würde.

REFERENCES

[1] P. J. G. MANN, M. TENNENBAUM, AND J. H. QUASTEL, *Biochem. J.*, 33 (1939) 822.
[2] H. A. KREBS, *Biochem. J.*, 29 (1935) 1951.
[3] D. NACHMANSOHN AND A. L. MACHADO, *Neurophysiol.*, 6 (1943) 397.
[4] W. H. ELLIOT, *Nature*, 161 (1948) 128.
[5] H. MCLENNAN AND K. A. C. ELLIOTT, *J. Pharmacol. Exptl. Therap.*, 103 (1949) 35.
[6] R. P. HARPUR AND J. H. QUASTEL, *Nature*, 164 (1949) 779.
[7] H. WAELSCH, P. OWADES, K. H. MILLER AND E. BORECK, *J. Biol. Chem.*, 166 (1946) 273.
[8] E. MELLANBY, *Brit. Med. J.*, 2 (1947) 888.
[9] D. RICHTER, *J. Biol. Chem.*, 176 (1948) 1199.
[10] D. B. TOWER AND K. A. C. ELLIOTT, *Federation Proc.*, 10 (1951) 260.
[11] W. H. ELLIOT, *Biochem. J.*, 49 (1951) 106.
[12] B. M. BRAGANCA, J. H. QUASTEL AND R. SCHUCHER (in the press).

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