THE SYNTHESIS OF ACETYLCHOLINE BY ACETONE DRIED POWDERS FROM THE BRAINS OF NORMAL RATS AND OF THIAMINE-DEFICIENT RATS

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Received September 28, 1961

The rate of synthesis of acetylcholine by rat brains was reduced by thiamine deficiency. There was a reduction in available coenzyme A but not in choline acetylase activity.

SOME years ago, Mann and Quastel (1939) compared the rates at which acetylcholine was synthesised by the brains of normal and polyneuritic pigeons. They found lower rates than normal when the concentration of potassium ions in the medium was high. Added thiamine restored the rate of synthesis in the polyneuritic tissue, but failed to influence the normal.

In recent years the measurement of activity attributable to choline acetylase in tissues (Hebb, 1955; Hebb and Smallman, 1956) has not only been greatly improved but means have been provided by which the quantity of coenzyme A present may be determined. It therefore seemed desirable to reinvestigate the influence of thiamine deficiency on the rate of synthesis of acetylcholine in brain using modern techniques for measuring both the enzymic activity and the co-enzyme A available in the tissue. We have used rats for this purpose.

METHODS

Female rats of a single Wistar strain, weighing 150 to 200 g. were used. They were housed in a room maintained at 21 $\pm$ 0·5°, drank tap water and were fed the basic diet described by Fitzhugh, Knudsen and Nelson (1946). It consisted (per cent) of corn starch 60, casein 18, corn oil 6, dessicated whole liver powder 5, dried yeast 5 and U.S.P. salt mixture (XII, No. 2) 4, but the 2 per cent cod liver oil supplement was omitted. Instead, each rat received 0·5 ml. cod liver oil, orally by pipette each week. The thiamine content of this diet, assayed by the thiochrome method, was 138 μg. per 100 g.

A diet deficient in thiamine was prepared from the basic diet by additon of 0·6 per cent sodium metabisulphite. It was used within 7 weeks of preparation. This treatment reduced the thiamine in the diet to less than 1 μg./100 g. within 2 weeks of the sulphiting process. Rats fed the sulphited diet began to lose weight by the third or fourth week, and finally developed polyneuritis accompanied by bradycardia in the fifth or sixth week when they were ready for use, in parallel with control animals fed the basic unsulphited diet.

Assay of Choline Acetylase and Coenzyme A in Brain

The preparation of acetone dried powder from brains. The rats were killed by a single blow at the base of the neck, and decapitated. The whole brain was removed and ground in a cold mortar with 50 to 100 vol. of dry acetone at −4°. The sediment was collected by filtration using a No. 54 Whatman filter paper on a Buchner funnel. The resulting powder was kept over phosphorus pentoxide in a vacuum dessicator at −4° for 4–5 hr. before use. A separate powder was prepared from the brain of each rat.

Preparation of enzyme. The powder (10 mg./ml.) was suspended in normal saline containing 6 mg. l-cysteine hydrochloride per ml. The supernatant fluid was collected after centrifuging at 10,000 *g* at 1° for 3 hr.

Estimation of enzyme activity. The tubes prepared for incubation each contained enzyme derived from 25 mg. of acetone dried brain powder; l-cysteine hydrochloride, 15 mg.; sodium fluoride, 2 mg.; potassium chloride, 6 mg.; magnesium chloride, 4 mg.; eserine sulphate, 0·5 mg.; 0·3 ml. phosphate buffer, M/15, pH 7·0; choline chloride, 4 mg.; sodium citrate, 16·4 mg.; the disodium salt of adenosine triphosphate (ATP), 4 mg.; and coenzyme A, 100 μg. (equivalent to 30 Lipmann units). Each tube was plugged with cotton wool and was incubated for 1 hr. in a water bath at 37°. Enzyme activity was then arrested by the addition of 0·5 ml. 0·3N HCl followed by rapid boiling and cooling. The tubes were stored at −10° overnight, and were neutralised to litmus as external indicator with 0·3N NaOH and brought to a volume of 7 ml. immediately before biological assay for acetylcholine content.

Estimation of coenzyme A content of brains. Estimates of the coenzyme in the individual rat brains differed in method from estimates of choline acetylase only in the following points. First, the enzyme used throughout was provided by a single, well mixed sample of acetone dried powder obtained from the brains of a number of normal rats. Secondly, coenzyme A was omitted from the incubation mixture and was replaced by 2 ml. of boiled extract of acetone dried powder (12 mg./ml.) from individual rat brains.

Assay of acetylcholine. The eserinised frog rectus preparation of Chang and Gaddum (1933) was used taking the precautions advised by Feldberg (1945), Feldberg and Mann (1945, 1946), and Feldberg and Hebb (1947) to avoid errors due to substances in the extracts which may potentiate the effects of acetylcholine. Throughout, 2 × 2 assays of Latin square design have been used for comparison of the quantities of acetylcholine formed by the enzyme or coenzyme A in the brain of a thiamine deficient rat with that synthesised by the brain of a normal rat. In addition, the sensitivity of each rectus preparation toward acetylcholine was assessed in order that a rough estimate of concentration should accompany the more accurate knowledge of relative potency.

Investigation of the optimum conditions for the synthesis of acetylcholine in extracts of acetone dried powders made from normal rat brain. Acetone dried powders prepared from normal rat brains were used to establish conditions needed for the high rates of synthesis of acetylcholine recorded

by former workers. Previous investigators have employed either citrate (Feldberg and Mann, 1946; Barker, 1951) or acetate (Hebb, 1955) as substrates for the acetylation of coenzyme A. Citrate was used hence the reaction medium contained ATP and coenzyme A. The enzyme used initially was prepared from the acetone dried powders as described by Feldberg and Mann (1946). Without l-cysteine the yield was 922·5 ± 26·6 (4) μg./g. dried powder/hr.

This finding is in good accord with the early observation of Feldberg and Mann. Purification of the enzyme by high speed centrifugation, introduced by Lipton (1946), and the addition of cysteine as stabiliser gave a rate of synthesis of acetylcholine of 1925 ± 14·3 μg./g./hr. This compared satisfactorily with reported figures. Though reserpine was added to the reaction medium throughout this work to prevent breakdown of acetylcholine by cholinesterases, this precaution may have been needless. Nachmansohn and Berman (1946) have shown that acetone-dried brain yields powders almost devoid of cholinesterase activity.

RESULTS

Two series of experiments were made in which the acetylcholine synthesised by centrifuged extracts of the acetone dried powders from the brains of normal rats was compared with that made in corresponding extracts from the brains of animals deficient in thiamine. In the first series the reaction mixture contained added coenzyme A: in the second series it did not. The results of these experiments are shown in Table I.

TABLE I

A COMPARISON OF THE QUANTITIES OF ACETYLCHOLINE SYNTHESISED BY EXTRACTS OF ACETONE-DRIED POWDERS FROM THE BRAIN OF NORMAL AND THIAMINE-DEFICIENT RATS

	Acetylcholine synthesised μg./g. powder/hr.			
		Deficient in thiamine		
			Significance of difference	
Condition of test	Normal	Normal per cent	*t* calc.	P
No added coenzyme A.. ..	700–1100	75·8 ± 9·3(8)	2·48	<0·05
Coenzyme A added	1700–2100	110·0 ± 9·8(7)	1·95	<0·01

There was no reduction in the choline acetylase activity of brains from thiamine-deficient animals: this is clearly shown by the results of experiments made in the presence of excess coenzyme. Thus the reduced rate of synthesis of acetylcholine by extracts of thiamine-deficient brains to which no coenzyme A has been added is attributed to reduced coenzyme A content.

This conclusion was examined in a third series of eleven experiments. The available coenzyme A in the brains of normal and of thiamine-deficient rats was compared by measurement of acetylcholine synthesised by aliquots of a single enzyme preparation when standardised boiled extracts of these brains replaced coenzyme A in the reaction mixture. In these experiments

the quantity of acetylcholine synthesised when boiled extracts of the brains from thiamine-deficient rats provided the coenzyme was 73·7 ± 9·3 (11) per cent of that found when boiled extracts of normal brains were used. The difference was significant ($t = 2{\cdot}82$; $P = <0{\cdot}05$).

Discussion

There was no reduction in choline acetylase activity in the brains of rats made deficient in thiamine (Table I), but the rate of synthesis of acetylcholine in extracts of these brains is subnormal until coenzyme A is added. This fact indicates that a reduction in the coenzyme A present in the brain is responsible for the subnormal rate of synthesis. The reduced rate of synthesis of acetylcholine which we have observed in the brains of thiamine-deficient rats can explain the lowered concentrations of acetylcholine found by Lissák, Kovács and Nagy (1943) in the brain and cord of thiamine-deficient animals.

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