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The Distribution of Bound Acetylcholine and Choline Acetylase in Rat and Pigeon Brain

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Mann, Tennenbaum & Quastel (1938) presented evidence that about three-quarters of the acetylcholine in guinea-pig brain was bound to some protein constituent of the cell, and later the same authors (1939) suggested that this was a complex of acetylcholine and its synthesizing enzyme. It has since been pointed out (Braganca & Quastel, 1952) that bound acetylcholine could be acetylcholine held within the mitochondria, and from indirect evidence del Castillo & Katz (1956) conclude that intracellular granules containing acetylcholine may function in synaptic transmission.

Hebb & Whittaker (1958) have located bound acetylcholine and choline acetylase in particulate fractions from guinea-pig and rabbit brain. Bound acetylcholine and choline acetylase have also been found in mitochondrial preparations from locust-head tissue (Bellamy, 1958).

This paper records the distribution of bound acetylcholine and choline acetylase in rat- and pigeon-brain homogenates fractionated by differential centrifuging and provides evidence that both the enzyme and its stored product occur in the same particulate fraction.

METHODS

Preparation and fractionation of the homogenates. Brains from rat or pigeon were forced through a chilled stainless-steel Fisher mincer (Jouan, Paris) and homogenized with 4 vol. of 0.25M-sucrose soln. at 0° in a stainless-steel homogenizer of the Potter-Elvehjem type (working volume 40 ml.). The clearance of the pestle in the cylinder was 0.005 in. and it was moved up and down eight times, revolving at a speed of 2000 rev./min.

The homogenate was diluted with a further 2 vol. of 0.25M-sucrose soln. and fractionated at the gravitational forces and times described later. Each fraction was

examined by phase-contrast microscopy and it was found that the nuclear and microsomal fractions were not entirely free from mitochondria. After sedimentation of each fraction it was washed three times with 0.25M-sucrose soln. (one-third of the original volume of the tissue mince) and the washings were added to the supernatant fluid from that fraction. The last centrifuging of the fractions was carried out at 25 000 g for 15 min. and the tightly packed residue resuspended in 9 vol. of 0.25M-sucrose soln. The microsomal fraction was not washed but resuspended immediately in 9 vol. of 0.25M-sucrose soln. All the operations were carried out as close to 0° as possible.

Preparation of electron micrographs. Small samples of the mitochondrial and microsomal pellets were fixed in 1% osmic acid adjusted to 0.25 osmolar with sucrose, then dehydrated and embedded in butylmethacrylate as described by Bellamy (1958). Sections were cut and examined under the electron microscope by Dr G. Meek of the Department of Anatomy, Oxford.

Extraction and estimation of acetylcholine. Acetylcholine in the tissue preparations was extracted with 1% trichloroacetic acid soln. after brief heat treatment as described previously (Bellamy, 1958), except that the ion-exchange resin was not used. The extracted acetylcholine was measured with the frog rectus abdominis-muscle preparation described by Chang & Gaddum (1933), with the controls suggested by Feldberg (1942-43).

Determination of dry weights. The dry weights of tissue samples were determined as acid-insoluble dry matter according to the method of Werkheiser & Bartley (1957).

RESULTS

Distribution of bound acetylcholine in brain tissue. No inhibitor of acetylcholine esterase was added during the preparation of the homogenate or during the fractionation procedure. This ensured that only acetylcholine bound to tissue components was present (Mann *et al.* 1939). A sample of pigeon-brain homogenate contained 13.6 µg. of bound

acetylcholine/g. dry wt. and of rat brain 5.8 $\mu\text{g./g.}$ dry wt.

In rat brain 70 % of the bound acetylcholine was recovered in the mitochondrial fraction against 54 % in pigeon brain (Table 1). The concentration of bound acetylcholine in these fractions was $3\frac{1}{2}$ and $2\frac{1}{2}$ times that of the whole homogenate respectively (18 and 33 $\mu\text{g./g.}$ dry wt.).

About 14 % of the total acetylcholine of the homogenate was recovered in the microsomal fraction of rat brain and the concentration was a quarter of that in the homogenate. The pigeon-brain microsomal fraction contained 7 % of the acetylcholine of the homogenate at a concentration as high as in the mitochondrial fraction.

Nearly 40 % of the acetylcholine of pigeon brain was found in the combined cell debris and nuclear fraction. The concentration was lower than that in the homogenate and microscopic observation showed that this fraction contained more incompletely broken cells than the corresponding fraction from rat brain.

Distribution of enzymes forming acetylcholine. Acetylcholine synthesis is expressed as the total acetylcholine formed/g. dry wt. on incubating the tissue suspension 1 hr. at 38°. The rate of synthesis was not linear over this period; the amount of acetylcholine synthesized in 30 min. was generally two-thirds of that formed after 1 hr.

Eserine sulphate (mm) was added to all incubation mixtures. On incubating the brain homogenates in the presence of adenosine triphosphate (ATP) and choline (Table 2) there was a slight rise in the total acetylcholine. This was augmented on adding acetate, reaching a value of 8.1 $\mu\text{g.}$ and 11.1 $\mu\text{g./g./hr.}$ for rat and pigeon respectively. In the subcellular particulate fractions acetylcholine synthesis occurred only when acetate was included in the incubation medium. The supernatant fraction did not synthesize acetylcholine under the conditions used, a finding which contrasts with that of Hebb & Smallman (1956) on rabbit brain, where 25 % of the enzyme activity appeared in the supernatant fraction.

Table 1. *Distribution of bound acetylcholine in fractionated homogenates of rat and pigeon brain*

Rat brains (8 g. wet wt.) were homogenized in 32 ml. of 0.25M-sucrose soln. and diluted and fractionated as described in the text. The cell debris was sedimented at 1000 g for 10 min., the nuclear fraction at 2000 g for 10 min., the mitochondrial fraction at 15 000 g for 10 min. and the microsomal fraction at 25 000 g for 40 min. Pigeon brains (20 g. wet wt.) were homogenized and fractionated as described above except that the mitochondrial fraction was sedimented between 4000 and 14 000 g in 15 min.

	Acetylcholine content of tissues			
	Rat ($\mu\text{g.}$ in fraction)	Pigeon	Rat ($\mu\text{g./g.}$ dry wt.)	Pigeon
Whole homogenate	14.1	46.0	5.4	13.6
Fractions:				
Cell debris and nuclei	2.9	18.3	5.8	9.9
Mitochondria	9.8	25.0	18.7	33.2
Microsomes	2.2	3.3	1.2	32.8
Supernatant	0	0	—	—
Recovery	14.9	46.6	—	—

Table 2. *Synthesis of acetylcholine by fractionated brain homogenates*

Homogenates were fractionated as described in Table 1. Incubation medium: 4 ml., containing 0.017M-potassium phosphate buffer, pH 7.4, 0.013M-MgCl₂, 2 mM-ATP, 0.033M-choline chloride and mM-eserine sulphate. Acetate was added to a final concn. of 0.033M where indicated. After addition of the particle suspension (2 ml. containing 100 mg. dry wt.) the medium was adjusted to 0.25 osmolar with sucrose, and the incubation was carried out in air at 38° for 1 hr.

	Total acetylcholine synthesized ($\mu\text{g./g.}$ dry wt./hr.)			
	Rat		Pigeon	
	Endogenous	With acetate	Endogenous	With acetate
Homogenate	3.3	8.1	1.5	11.7
Fraction:				
Cell debris and nuclei	0	1.5	0	1.3
Mitochondria	0	27.1	0	76.5
Microsomes	0	12.4	0	60.7
Supernatant	0	0	0	0

The rate of acetylcholine formation in the mitochondrial fraction was greater than in the homogenates. It was three times greater in the rat and seven times greater in the pigeon (27 and 76 $\mu\text{g./g.}$ dry wt./hr. respectively). The increase in the total amount of acetylcholine in both rat- and pigeon-brain mitochondrial fractions after incubating them for 1 hr. at 38° (in the presence of acetate, ATP and choline) was threefold (Table 3). As already mentioned no synthesis occurred if acetate was omitted and a considerable amount of the bound acetylcholine was released into the surrounding medium (64% for pigeon and 46% for rat). The bound acetylcholine of the particles fell on incubation with choline and ATP. In rat-brain particles the amount of bound acetylcholine fell during incubation whether acetate was present or not. In pigeon brain the addition of acetate maintained the initial acetylcholine content of the particles.

The microsomal fraction also formed acetylcholine faster than the homogenate. This was most marked in the pigeon, where the activity of the enzymes in the microsomal fraction was nearly as high as in the mitochondrial fraction. In pigeon brain all of the activity of the homogenate was recovered in the derived fractions (Table 4). About 53% of the total activity was found in the mitochondrial fraction. On fractionation of rat-brain homogenate only 60% of the total enzyme activity was recovered in the derived fractions.

Relationship between bound acetylcholine and enzymes forming acetylcholine. The distribution of bound acetylcholine between the fractions derived

from pigeon brain was the same as that of the enzymes forming acetylcholine (Table 4). The results indicate that the mitochondrial and microsomal fractions are the main binding sites.

Electron micrographs of sections through these fractions show particles with a typical mitochondrial structure (Witter, Watson & Cottone, 1955; Lever & Chappell, 1958). Small unidentified vesicles distinct from mitochondria were seen in both mitochondrial and microsomal fractions, and from this evidence it is impossible to say that one type of subcellular particle only is associated with bound acetylcholine and choline acetylase.

Since the completion of this work, Whittaker (1958) has reported the separation of particles rich in acetylcholine and choline acetylase from particles containing the oxidative enzymes thought to be characteristic of mitochondria. The separated particles are structurally distinct from mitochondria and appear to be similar to the unidentified vesicles seen in the particulate fractions from rat and pigeon brain.

DISCUSSION

The localization of choline acetylase within the nerve cells bears on the question of the physiological role of acetylcholine in the transmission of impulses. However, as it is not yet possible to correlate the isolated particles containing acetylcholine and choline acetylase with known structures of the nerve cells no inferences can be made from the present findings about the function of acetylcholine.

Table 3. *Synthesis of acetylcholine in the mitochondrial fractions prepared from rat and pigeon brain*

The mitochondrial fractions were incubated as in Table 2. After 1 hr. the mitochondria were isolated from the incubation mixture by centrifuging at 14 000 g for 15 min. The sedimented particles were washed once with 10 vol. of 0.25M-sucrose soln. and the acetylcholine in the particles and the supernatant medium was determined.

Additions	Total acetylcholine in the incubation mixture ($\mu\text{g./g.}$ dry wt.)		Percentage distribution of acetylcholine in the incubation mixture			
			Mitochondria		Suspending medium	
	Rat	Pigeon	Rat	Pigeon	Rat	Pigeon
Nil	15.1	37.7	54	36	46	64
0.033M-Acetate	42.1	113	19	39	81	61

Table 4. *Recovery of bound acetylcholine and acetylcholine-forming enzymes of the whole homogenate*

Figures are given as the percentage of the total amount of bound acetylcholine and enzymes forming acetylcholine (choline acetylase) of the homogenate recovered in each fraction.

	Rat		Pigeon	
	Acetylcholine	Choline acetylase	Acetylcholine	Choline acetylase
Cell debris and nuclei	21	20	40	44
Mitochondria	70	34	54	53
Microsomes	16	6	7	7
Supernatant	0	0	0	0
Total recovery	107	60	101	104

One of the problems is the mechanism which controls the quantities of acetylcholine synthesized under physiological conditions. The synthesis is probably not continuous but, during the resting state of the tissue, comes to a standstill when certain amounts have accumulated. The key substances required for synthesis in the intact tissue include acetyl-coenzyme A and choline. Acetyl-coenzyme A cannot be a controlling factor since it is produced in relatively large quantities by the normal oxidative metabolism. Nor is the amount of choline likely to be a limiting factor because the level of free choline in rat brain has been estimated at $1.4 \mu\text{moles/g.}$ fresh weight (Leucke & Pearson, 1944), which is about 200 times the average level of total acetylcholine in the brain. The present experiments show some parallelism between the rate of acetylcholine synthesis, the capacity of brain particles for binding acetylcholine and the rate of release of the bound ester, in that all are greater in the pigeon brain than in the rat brain. This might be taken to suggest that the enzyme system also provides the binding site and that the enzyme system becomes inactivated when the binding sites are saturated with acetylcholine.

SUMMARY

1. Homogenates of rat and pigeon brain in 0.25M -sucrose solution have been fractionated by differential centrifuging and the distribution of bound acetylcholine and choline acetylase in the fractions has been determined.

2. The mitochondrial fractions from rat and pigeon brain contained respectively 70 % and 54 % of the total bound acetylcholine of the whole homogenate (on a dry-weight basis these figures correspond to 18 and $33 \mu\text{g./g.}$).

3. Choline acetylase activity was detected in all fractions of the homogenate except the supernatant fractions. Activities of the enzymes (per g. dry wt.) in the mitochondrial and microsomal fractions were higher than the activity of the respective homogenates. For rat- and pigeon-brain mitochondrial fractions the respective rates were $27 \mu\text{g.}$ and

$76 \mu\text{g./g.}$ dry wt./hr. at 38° , equivalent to three and seven times those in the whole homogenates.

4. On incubating the brain-mitochondrial suspension with choline no synthesis of acetylcholine occurred but between 64 % (pigeon brain) and 46 % (rat brain) of the bound acetylcholine was released into the surrounding medium. On incubation of pigeon-brain particles with choline plus acetate a synthesis of acetylcholine took place and the initial level of bound acetylcholine was maintained.

5. The rate of acetylcholine synthesis, the capacity of the particles for binding acetylcholine and the rate of release of the bound ester were all greater in pigeon brain than in rat brain.

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