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The estimation of the "free" and "bound" acetylcholine content of rat brain

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Rat brains, not frozen, were homogenized in saline solution containing physostigmine, and also cupric chloride to inhibit choline acetyltransferase. When brains were homogenized for periods of up to 3 min, the amount of acetylcholine extracted was proportional to the duration of homogenization. After 3 min, there was no further significant increase in acetylcholine. The acetylcholine extracted in this way was termed "free"; whereas that remaining in the brain tissue and extracted by acid-ethanol solution was termed "bound". The total amount obtained from each brain was not significantly different from the total amount extracted by a more conventional method from the brains of rats killed by rapid freezing in liquid air. This observation applied also to brains removed from animals during anaesthesia and convulsions.

It is desirable to be able to differentiate between a change in brain acetylcholine content that has arisen due to a variation in physiological activity and that which may have arisen from changes in the biochemical systems involved in acetylcholine metabolism. A method described by Crossland & Slater (1968) has shown that drugs may differ in the extent to which they affect two fractions—"free" and "bound"—of brain acetylcholine. The term "free" referred to acetylcholine extracted by simple homogenization in a saline medium under conditions where synthesis and destruction are prevented; "bound" referred to the second component that can be released and extracted only by protein precipitation. Other attempts to measure "free" acetylcholine have been made (Stone, 1955; Kurokawa, Machiyama & Kato, 1963) but the results show wide discrepancies. A possible reason for this is that although the breakdown of acetylcholine during the extraction procedure is usually prevented, the synthesis of more acetylcholine could continue because choline acetyltransferase activity was not usually inhibited.

The method of Crossland & Slater (1968) for estimating "free" and "bound" acetylcholine is now further evaluated.

METHODS

Female Wistar rats, 80–100 g were either killed by total immersion in liquid air or decapitated. Frozen brain tissue was chipped out, crushed and extracted with acid-alcohol according to Crossland (1951) to give total brain acetylcholine.

Non-frozen brains rapidly excised from decapitated animals were homogenized with the least possible delay in physiological saline solution containing physostigmine sulphate (15 μg/ml) and cupric chloride (17 μg/ml) using 5 ml of medium for each g of brain tissue. Homogenization was effected at 0° by an M.S.E. top-drive homogenizer with a stainless steel blade (diameter 1·5 cm) rotating at 1300 rev/min in a

15 ml capacity vortex flask. The blade speed was sufficient to ensure an even disintegration of the tissue within 2 to 3 min. The brain homogenate was immediately centrifuged at 5000 rev/min for 20 min to produce a clear supernatant solution largely free from particles of cell debris. The supernatant was decanted, adjusted to pH 4·0 with 0·5N hydrochloric acid and stood for 30 min, when any slight precipitate was removed. This clear solution then contained "free" acetylcholine. The tissue residue remaining after the extraction of the "free" acetylcholine was treated with acid-ethanol solution to release the "bound" acetylcholine (Crossland, 1951). The extracts were stored at 0° until required.

The assays were performed, using a bracketing dose technique, on the frog rectus abdominis preparation sensitized with neostigmine bromide (10^{-6}M). Half of each brain extract was adjusted to pH 12 and boiled for 2–3 min to destroy acetylcholine without affecting material that increases the response of the frog rectus to acetylcholine (Feldberg, 1945). Standard solutions of acetylcholine were added to the acetylcholine-free extract, adjusted to pH 7 and used in the assay against brain extracts.

Drugs administered to rats intraperitoneally were pentobarbitone sodium (35 mg/kg) and leptazol (75 mg/kg).

RESULTS

To determine the relation between the amount of "free" acetylcholine extracted and the duration of homogenization, brains removed from decapitated rats were homogenized under standard conditions for periods of between 1 and 8 min in the saline medium containing physostigmine and cupric ion. Fig. 1 shows that there was a rapid increase in "free" acetylcholine for the first 3 min, but very little subsequent increase. Approximately 2 min of homogenization was sufficient to reduce the brain tissue to a fine, even consistency, therefore for all subsequent determinations of "free" acetylcholine 3 min was chosen as being the optimum period for the homogenization.

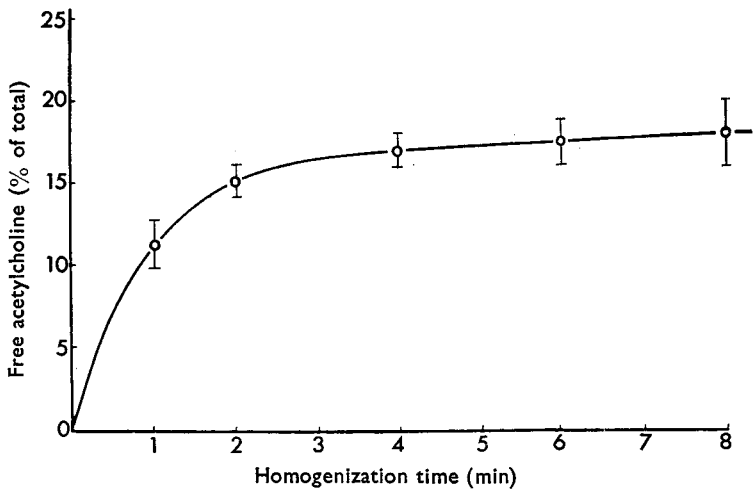

FIG. 1. The relation between the amount of "free" acetylcholine, expressed as percentage of total, obtained from rat brains subjected to saline homogenization for specified periods. The points represent the mean values from 4 determinations and each vertical line is one standard error. Further details of the homogenization procedure are given in the text.

The amount of "free" and "bound" acetylcholine extracted from rat brain under these standard conditions was determined. The mean concentration of "free" acetylcholine was 0·40 ± 0·03 μg/g (mean ± s.e. from 18 determinations) and the amount of "bound" was 2·82 ± 0·15 μg/g (18 determinations). The so-called "free" acetylcholine was therefore approximately 13% of the total amount obtained. These values are similar to those of Crossland & Slater (1968). The total acetylcholine content of rat brain by this method was therefore 3·22 ± 0·14 μg/g. The mean brain acetylcholine content of a group of 14 identical rats killed by immersion in liquid air was 3·49 ± 0·02 μg/g which is 8% greater than in the non-frozen brains. When analysed by Student's *t*-test the results of the two methods were not significantly different. It may be assumed therefore, that the use of non-frozen brain under conditions where both the synthesis and destruction of acetylcholine are prevented gives a fair estimate of the true acetylcholine content of brain as measured by using the rapid freezing technique. To investigate whether this conclusion remained valid for a drug-induced change in brain acetylcholine concentration, groups of rats were killed during pentobarbitone anaesthesia and leptazol convulsions. The amount of acetylcholine obtained using both methods are shown in Table 1. There was no

Table 1. *The total brain acetylcholine content of rats killed by either rapid freezing in liquid air or decapitation.*

Treatment	Time of killing	Brain acetylcholine content (μg/g fresh tissues ± s.e.) Rapid frozen	Decapitation
Normal rats	—	3·33 ± 0·14 (9)	3·22 ± 0·14 (18)
Anaesthetized rats .. .. .. (pentobarbitone sodium 35 mg/kg, i.p.)	30 min	4·72 ± 0·32*(5)	5·24 ± 0·34**(11)
Convulsing rats .. .. (leptazol 75 mg/kg, i.p.)	5 min	1·65 ± 0·12** (4)	1·88 ± 0·09** (4)

Significance of difference from normal $*P < 0{\cdot}02$, $**P < 0{\cdot}001$.
The number of animals used is shown in parentheses.

significant difference between the values obtained in the two instances from the normal untreated rats. During anaesthesia, both methods of extraction demonstrated increased amounts of brain acetylcholine with no difference between the two sets of results. Further, chemically-induced convulsions lowered the total amount of brain acetylcholine, similarly with no statistical difference between the results obtained using the two methods. The close correspondence between the results obtained with frozen and non-frozen brain therefore reflect the actual changes occurring in the amounts of brain acetylcholine in *vivo*. The method described for the estimation of "free" and "bound" acetylcholine appears to be effective in preventing both synthesis and destruction of acetylcholine during the homogenization and extraction.

DISCUSSION

Homogenization and extraction of non-frozen brain tissue is usually accompanied by a rapid synthesis of acetylcholine (Crossland, Pappius & Elliott, 1955). This is especially evident when the medium contains an anticholinesterase, with the result that the values for the total brain acetylcholine content may bear little resemblance to

those found *in vivo*. To overcome this, the technique of killing animals in liquid air and extracting the frozen brain was introduced (Crossland, 1951). It has been widely accepted that this method with more recent modifications introduced by Aprison & Takahashi (1965), gives values very close to the acetylcholine content of brain *in vivo*. But frozen brain cannot be used to measure "free" acetylcholine, since freezing and thawing brain tissue liberates a large proportion of the "bound" acetylcholine. However, from the findings presented here, extraction of non-frozen brain in a medium that adequately prevents synthesis of acetylcholine, gives results comparable to those obtained using frozen brain. Copper has been shown by Nachmansohn & Machado (1943) and Stone (1955) to prevent the synthesis of acetylcholine.

The concept of "free" acetylcholine has found little favour and has been described as an artifact that arises during the extraction of brain tissue. This is perhaps not unreasonable since, previous estimates of the "free" acetylcholine of brain have differed widely. Almost without exception, however, no precautions were taken to prevent synthesis of acetylcholine during the homogenization.

Mann, Tennenbaum & Quastel (1938) were the first to differentiate between free and bound acetylcholine. They found that during acetylcholine synthesis *in vitro* an equilibrium was set up between free acetylcholine in the medium and bound acetylcholine in brain slices. Tobias, Lipton & Lepinat (1946) reported that normal rat brain contained 20% of free acetylcholine when extracted in eserinized saline solution while Kurokawa, Machiyama & Kato (1963) obtained 18% of free acetylcholine from homogenates of mouse brain.

There is evidence that brain acetylcholine is associated with three fractions isolated by subcellular fractionation techniques. A "labile bound" fraction occurs in the cytoplasm of isolated synaptosomes and a "stable bound" fraction is associated with the synaptic vesicles (Whittaker, 1959; 1968). These two fractions represent between 70 and 90% of the total acetylcholine (Hebb & Whittaker, 1958), while the balance, corresponding to the free fraction, is recovered from the high-speed supernatant fraction, provided that a cholinesterase inhibitor is present.

Discussion about the "free" acetylcholine extracted using the method herein described is mainly speculative. It is certainly not entirely the result of the liberation of either form of bound acetylcholine, otherwise the proportion of "free" to "bound" would always remain constant. This does not happen because drugs such as physostigmine, tremorine and atropine affect the two fractions differently (Crossland & Slater, 1968). Other workers have come to the conclusion that free acetylcholine is not a simple artifact (Whittaker, 1959; Beani, Bianchi & others, 1969). Perhaps the most likely explanation is that the "free" acetylcholine is a mixture of acetylcholine from several sources. From the available knowledge it seems that acetylcholine is synthesized in the free form and is subsequently bound into the tissue storage sites. Part of the material extracted will include this newly synthesized acetylcholine. The remainder of the "free" fraction could consist of acetylcholine liberated as a result of nervous activity and that has not been destroyed by cholinesterase. There is also the possibility that a third fraction of the "free"acetylcholine may arise from the release of some of the bound material, and in particular the "labile bound" fraction, during the homogenization.

The results reported in this paper demonstrate that the proportion of "free" acetylcholine in rat brain is approximately 13% of the total. Since the amount

involved is small, only the most sensitive frog rectus preparation can be used for the assay. The slight fluctuations in the amounts of the "free" material appear to be due in part to difficulties in assay. It must however be emphasized that the term "free" and "bound" cannot yet be identified with any specific cell constituents.

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