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"Free" and "bound" acetylcholine concentrations in rat brain: variability in determination of "free" acetylcholine fraction

Crossland & Slater (1968) have reported a method for simple fractionation of the brain acetylcholine into "free" (extracted with eserinized saline) and "bound" (extracted with acid-ethanol) components and have described the effect of some drugs on these fractions. Although the identity and the physiological significance of these fractions was not clear, they were, nevertheless, differentially affected by various groups of drugs.

We now describe our experience with determination of the "free" acetylcholine fraction using a modified approach.

Male Sprague-Dawley rats, 180–220 g were killed by dipping into liquid nitrogen for 10 s ("near-freezing" method of Takahashi & Aprison, 1964). Freezing and subsequent thawing of brain tissue under this condition does not occur. After decapitation the brains were carefully removed from the skull and weighed rapidly. The "free" and "bound" acetylcholine from the whole brain (without cerebellum, pons and medulla) were then extracted according to the procedure of Crossland & Slater (1968), or in other experiments, the "total" fraction from whole brain was extracted with acid-ethanol using the method of Crossland (1961). Assays were performed using the frog (*Rana temporaria*) rectus abdominis muscle sensitized with eserine sulphate ($1{\cdot}6 \times 10^{-5}$ M). Samples of tissue extracts were tested in a double-bracketed assay against standard solution of acetylcholine iodide prepared in alkali-inactivated parts of the same extracts (Feldberg, 1945). The recovery of acetylcholine added to the tissue homogenate was 90%.

During the bioassays we consistently noticed that the response of the frog rectus to the "free" acetylcholine samples declined after the first exposures of the muscle to this extract. This was not so when the acid-ethanol extracted samples of "bound" or "total" acetylcholine were assayed. This observation suggested to us that the direct estimation of the "free" fraction from the supernatant, obtained after extraction of the brain tissue with eserinized saline may yield false low values. We therefore decided to estimate directly the amount of "total" and "bound" fractions, both extracted with acid-ethanol from pooled opposing halves of the brains of a pair of animals ($R_1 + L_2$ for "total", $R_2 + L_1$ for "bound"). The values for the "free" fractions were then calculated by subtracting the values of "bound" from the values of "total" acetylcholine. The results are in Table 1.

Table 1. *Comparison of the values of "free", "bound" and "total" acetylcholine in whole rat brain.* Method 1: Direct estimation of "free" and "bound" acetylcholine and calculation of the value for "total" acetylcholine. Method 2: Direct estimation of "total" and "bound" acetylcholine and calculation of the value for "free" acetylcholine.

	"Free" ACh	"Bound" ACh	"Total" ACh	Ratio (%)		
	nmol/g ± s.e.			F/T	F/B	B/T
Method 1	3·38 ± 0·33 (7)	11·74 ± 0·87 (7)	15·12 ± 0·88[a] (7)	22·3	28·8	77·6
Method 2	6·98 ± 0·45[b] (12)*	10·16 ± 0·48 (12)	17·75 ± 0·48 (16)	39·3	68·7	57·2

[a] Calculated value obtained by adding the individual values of "free" and "bound" acetylcholine.
[b] Calculated values obtained by subtracting the individual values of "bound" from the values of "total" acetylcholine.
* Significantly different from the value obtained by method 1; $P < 0{\cdot}001$.
In parentheses: number of animals.
Ratio F/T—"free"/"total": F/B—"free"/"bound": B/T—"bound"/"total" acetylcholine.

The discrepancy between the values of "free" acetylcholine estimated directly and by using the second approach are obvious. The same applies for the ratio "free"/"total" fractions. Lower values of directly estimated "free" fraction may account for lower values of calculated "total" as compared with directly measured "total" amounts. The latter values are in good agreement with those reported by Crossland, Pappius & Elliot (1955), Stone (1955) and Takahashi & Aprison (1964) using the same method of extraction and assay. The reported "free"/"total" ratio of rat brain varies. Crossland & Slater (1968) found it to be in the range 9·2–15%, Milosevic (1970) found 30% and Richter & Goldstein (1970) 25%. We found 22·3% Using a different approach (calculating the values for "free" from values of "total" and "bound" acetylcholine) we obtained a ratio of 39·3%.

Direct determination of the "free" fraction is subject to high degree of variability due presumably to varying efficiency of extraction procedure (Richter & Goldstein, 1970). Our experiments further emphasize problems involved in attributing physiological significance to the fractionation of brain acetylcholine to "free" and "bound" portions. Such fractionation has, however, shown some utility in studying the effect of drugs on brain concentrations of acetylcholine.

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