A METHOD FOR DIFFERENTIAL MEASUREMENT OF FREE AND BOUND ACETYLCHOLINE IN THE SMALL INTESTINE

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It is well known that almost all acetylcholine (ACh) in tissues is present in a bound form and is released during excitation. In order to study the physiological role of ACh, the free form should be distinguishable from that of the bound. In previous papers (1-4) the amount of ACh diffused into the bathing solution of the intestine was considered to exhibit the entire amount of ACh released from tissue, however, there remains the possibility of retainment. Extraction of free ACh would be more satisfactory if the tissue were sliced before extraction. In this report, an attempt has been made to determine a method which would demonstrate the total picture of the tissue bound and free ACh in one operation.

Five to two segments of intestine about 4 cm long were taken from a female chicken aged 6 to 8 weeks. These were weighed after blotting both sides of the intestinal wall with dried filter paper. The preparations thus obtained were suspended in a medium containing 50 μ M physostigmine, incubated at 38°C for 30 min under aeration and then cooled in ice water. After 15 min, the segment of the intestine was sliced by means of McIlwain's auto-chopper (5) into an appropriate width (0.4 or 0.1 mm), resuspended in the incubation medium and allowed to stand in cold environment for 15 to 60 min. Free ACh was determined by assaying an aliquot of the suspension on the frog rectus abdominis muscle sensitized with 50 μ M physostigmine (6). To determine total ACh, the bound ACh was first released by treating the sample at pH 4 and 100°C for 10 min (7). The sample treated above was neutralized to pH 6.8 before assaying. The difference between the total and free ACh content of a sample was taken to represent the bound ACh therein.

The active substance was identified as ACh based on the following evidence. 1) The tissue extract with eserine free solution failed to contract frog rectus muscle. 2) The active substance lost activity after being boiled for a few minutes in an alkaline medium. 3) ACh and the active substance were equally antagonized by small doses of atropine.

Width of slices: Unsliced segment and sliced segment of 0.4 mm or 0.1 mm width were compared. In the unsliced, only a trace or no ACh was detectable, while in sliced preparations significant amounts of free ACh were observed. These results suggest that the wall of the intestine was too thick to extract released ACh completely and that the ACh assay in the bathing solution doesn't necessarily give the true picture of free ACh. Either 0.4 mm or 0.1 mm slices were adequate to extract the free ACh released. (Table 1, column

TABLE 1. Effects of slice width and extraction period on free ACh measurement.

Age in weeks	Un sliced		Sliced		
	Extraction Period		0.1 mm	0.4 mm	0.4 mm
	30 min (1)	60 min (2)	15 min (3)	15 min (4)	60 min (5)
	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$	$\mu\text{g/g}$
6	none	trace	0.4	0.4	0.3
6	none	trace	0.5	0.4	0.4
6	none	trace	0.5	0.5	0.6
8	none	trace	0.9	0.8	0.8
8	none	0.2	1.1	1.0	1.1
8	none	0.2	1.0	0.9	0.9
Mean $\pm$ S. D.			0.7 $\pm$ 0.3	0.7 $\pm$ 0.3	0.7 $\pm$ 0.3

Preparations were incubated in isotonic sucrose solution at 38°C for 30 min.

Values of ACh are expressed as μg of the chloride per gm of original tissue. Six chickens were used.

3,4) Preparations from aged chicks appeared to release more ACh, but further experiments are required to validate a conclusion.

Effect of extraction period: The sliced tissue preparations of 0.4 mm width were divided into two parts. One part was suspended in the medium for 15 min and the other 60 min. In both cases the amount of extracted ACh was $0.7 \pm 0.3 \mu\text{g/g}$ tissue. (Table 1, column 4,5) The results show that a 15 min suspension suffices to extract free ACh completely.

Comparison with other methods: Among several different methods (8-10) which have been described for the extraction and determination of the total amount of tissue ACh, the trichloroacetic acid (TCA) method (8) has been generally utilized. Total amount of tissue ACh measured using our method was compared with that of TCA. As shown in Table 2, the method herein was comparable in sensitivity to the TCA method and is relatively

TABLE 2. Comparison of TCA- and slice-method in the measurement of tissue ACh.

	TCA method	Slice method
	$\mu\text{g/g}$	$\mu\text{g/g}$
	1.8	1.5
	1.7	1.5
	2.4	2.4
	3.0	2.5
	4.0	3.7
	3.2	3.2
	3.1	3.2
Mean $\pm$ S. D.	2.7 $\pm$ 0.82	2.6 $\pm$ 0.85

Values of ACh are expressed as μg of the chloride per gm of original tissue. Seven chickens were used.

simple to perform.

It could be argued that the greater amount of free ACh obtained with the sliced preparations was due only to the mechanical shocks when slicing the tissue; however, as Table I shows, approximately the same amount of free ACh was estimated both in 0.4 mm and 0.1 mm slices, hence does not appear to be due to the slicing artifact.

With the development of a simple method for determining free ACh from that of bound it is now possible to investigate metabolic aspects of the tissue which have been mechanically or chemically stimulated. Such studies have been carried out and will be reported in the near future.

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EFFECT OF CONSECUTIVE DOSING OF THIAMPHENICOL ON ITS OWN SERUM CONCENTRATION AND LIVER DRUG METABOLIZING ENZYME ACTIVITIES

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It has been reported that many drugs stimulate their own metabolism as the result of enhancement of liver drug metabolizing enzyme activities (1,2) and that chloramphenicol (CP) inhibits liver drug metabolizing enzyme activities in rats (3, 4) and humans (5).

Recently, Della Bella et al. (6) observed in rats that pretreatment with phenobarbital enhanced decreases of the drug concentration in serum and chemotherapeutic activity of CP but did not affect those of thiamphenicol (TP).

On the other hand, although a study of the serum concentration of single dose of thiamphenicol glycinate hydrochloride (TPG) in rabbits has been reported (7), studies are not available as to the effects of multiple dosing of TPG on its own fluctuation of blood level and on liver drug metabolizing enzymes in rabbits.