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RADIO-ENZYMATIC ASSAY OF ACETYLCHOLINE IN TISSUES

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Abstract—A method for the determination of ACh in tissues and subcellular fractions is described. The ACh is extracted by an ion pair exchange method, purified by paper chromatography and determined by enzymatic phosphorylation with choline kinase after enzymatic hydrolysis with AChE.

Until recently, biological assay was the only method with sufficient sensitivity to measure ACh in tissues and tissue perfusates. The results of such assays are sometimes unreliable because other substances—both endogenous and exogenous—may interfere with the assay. Therefore, several attempts have been made to develop chemical assay methods of sufficient sensitivity and selectivity. The methods can be classified as radio-isotopic [1], fluorimetric [2], gas chromatographic or gas chromatographic-mass spectrometric [3–5].

The present paper describes a radio-enzymatic method for the determination of ACh in pmole amounts. The ACh content has been determined in cat ventral roots, cat sciatic nerves, rat diaphragms, rat hearts and rat brain cortex.

EXPERIMENTAL

Tissue preparation and ACh extraction. Samples (1 g) of all tissues, except rat brain cortex, were homogenized in 10 ml 0·25 M sucrose, pH 7·4 (10 mM Tris base buffer) with an Ultra Turrax homogenizer. Microsomal pellets were prepared by precentrifugation at 12,000 *g* for 15 min (P_1), followed by a second centrifugation of the supernatant at 125,000 *g* for 30 min. The high-speed pellets (P_2) were then resuspended in 2 ml 0·25 M sucrose,. After adjusting to pH $\simeq$ 4 with 50 μl concentrated acetic acid, the ACh was released by boiling for 10 min. Before further extraction, the samples were adjusted to pH 8 with Tris base buffer. All operations were carried out at 0°.

Rat brain was prepared as follows. The brain was quickly removed after cervical fracture. 0·5 *g* of the cortex was scraped off, weighed and homogenized in 1 ml acetonitrile [6]. Precipitated proteins were centrifuged down at 4000 *g* for 10 min and the pellets re-extracted with 1 ml acetonitrile. The acetonitrile fractions were pooled and evaporated. Before extracting ACh into the allylcyanide phase as described below, 100 μl of a 50 mM Tris base buffer (pH 8·0) was added.

The following extraction procedure was used for all tissues studied, except in the case of the rat brain, where the solvent volumes were one fifth of those given below. The ACh was extracted from the sucrose solutions, or in the case of the rat brain from the Tris base buffer (pH 8·0) with 1 ml allylcyanide containing 12·5 mg/kg sodium tetraphenyloboron (Na-TPB) [7]. The phases were mixed thoroughly for 1 min and then separated by centrifuging at 18,000 *g* for 20 min. 0·75 ml of the organic phase was transferred to new tubes containing 2·5 ml of 0·4 N HCl. The phases were mixed and separated and the organic phase was then discarded. The HCl phase was washed with 2 × 5 ml ether and then freeze-dried. To remove choline and contaminating salts emanating from the organic phase, the lyophilized pellets were dissolved in ethanol and subjected to paper chromatography: Whatman No. 1 paper; solvent mixture: *n*-butanol–ethanol–acetic acid–water (8:2:3:1)[8]. ACh-chloride (15 μg) and choline-chloride (10 μg) were run as reference substances at the sides 5 cm apart from the samples to be measured. The spots corresponding to the ACh reference were cut out, eluted with methanol (3 × 300 μl) and the eluate then evaporated in plastic microtubes. If a small portion of the Ch-spot is included in cutting out the ACh-spot, this is of no great importance since contaminating traces of choline are phosphorylated in the first step of the incubation procedure.

ACh determination. Remaining traces of choline were removed by phosphorylation with choline kinase and non-radioactive ATP. The ACh was then hydrolysed with AChE and the resulting choline was phosphorylated with [γ-^{32}P]ATP; the incubation steps were carried out according to the procedure of Goldberg *et al.* [9]. Both reactions go to completion.

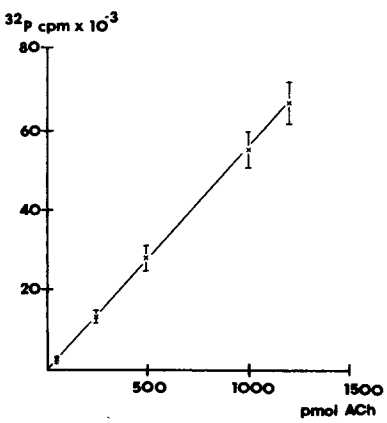

Fig. 1. ACh standard curve constructed by extracting known amounts of ACh from 0·25 M sucrose solutions. Blank values (180 ± 27) have been subtracted. Standard deviations are shown in the figure (n = 6, $P < 0{\cdot}05$). There was a similar linear correlation down to 10–15 pmoles (not shown in figure).

Table 1. Recovery at the different steps in the method

Step in the procedure	ACh content (pmoles)	Radioactivity (cpm)
Tissue homogenate	(1640)*	11,125 ± 143 (100 ± 1%)
Na-TPB extraction	not measureable	10,767 ± 131 (97 ± 1%)
Total recovery	1399 ± 98 (85 ± 6%)	9573 ± 1244 (86 ± 11%)

1000 pmoles (10 nCi) of [^{3}H]ACh was added to each of six aliquots of a sciatic nerve homogenate which had been boiled for 10 min before the addition of ACh.

* Calculated from the endogenous amount (640 ± 68 pmoles, mean ± S.D., $n = 4$) + the known amount of radioactive ACh (1000 pmoles) added.

The ACh content in the samples was calculated from the ^{32}P-counts and a standard curve which was constructed by incubating known amounts of ACh. This standard curve was linear up to 900–1000 pmoles of ACh (see Fig. 1). Losses of ACh throughout the procedure were determined from the recovery of [methyl-^{3}H]ACh (5 nCi) added to the original samples. A correction for overhearing from the ^{32}P-channel into the ^{3}H-channel was made.

For comparison, ACh was assayed by the leech-assay method described by Whittaker *et al.* [10] and by gas chromatography–mass spectrometry according to Karlén *et al.* [5]. A Student's *t*-test was used for calculations of significance.

MATERIALS

[γ-^{32}P]Adenosine-5'-triphosphate (sp. act. > 10 Ci/m-mole) was purchased from New England Nuclear, West Germany. [*N*-methyl-^{3}H]choline chloride (sp. act. > 10 Ci/m-mole) was purchased from the Radiochemical Centre, Amersham, England. (*N*-methyl-^{3}H)ACh (sp. act. > 10 Ci/m-mole) was prepared from acetyl chloride and [*N*-methyl-^{3}H]choline [11]. The product was purified by recrystallization from ethanol/ether and subsequent paper chromatography. AChE was obtained from Sigma Chemical Co. Choline kinase was prepared from brewer's yeast [12]. Only enzyme preparations with a specific activity higher than 3 m-moles/g protein were used. All other substances were obtained from usual commercial sources.

RESULTS

Efficiency of ACh extraction. Recoveries of ACh in the different steps are shown in Table 1.

Sensitivity. The maximum sensitivity of the method is 10–15 pmoles, depending on the purity and the specific activity of the radioactive ATP; impure ATP preparations gave high blank values.

Specificity. The specificity was checked by adding known amounts of ACh to tissue homogenates (Table 1) and using a leech assay procedure and gas chromatography combined with mass spectrometry (Table 2). The ACh level found in rat brain cortex was between 17 and 23 nmoles/g fresh tissue. This is well within the range of values reported in the literature.

DISCUSSION

The radio-enzymatic ACh assay described here has been successfully used for the determination of ACh in various tissues and subcellular fractions. The results are in good agreement with those obtained using other methods, such as the leech assay (after paper chromatography) and a gas chromatographic method (Table 2).

The specificity of this method is mainly based in (1) ion pair extraction [7], (2) paper chromatography and (3) incubation with choline phosphokinase. Choline analogues, such as 2-aminoethanol and 2-methylethanol are substrates for choline phosphokinase. However, since they are not quarternary ammonium compounds they are extracted with Na-TPB to only a minor degree. In addition, they will be eliminated in the chromatographic step. Should other choline esters be present they will also be eliminated in the chromatographic step. The threshold sensitivity is about 10 pmoles, which is lower than the sensitivity reported by Feigenson and Saelens [1] for their radioisotopic method. The threshold sensitivity of the gas chromatographic methods is about 50 pmoles. How-

Table 2. ACh content in various tissue fractions measured by three different methods

Tissue fraction	ACh content (nmoles) (mean ± S.D.)		
	Choline kinase assay	Leech assay	Gas chrom.–mass spectrometry
Heart, microsomal pellet (P_2) (from 1·5 g tissue)	2·1 ± 0·2 ($n = 6$)	2·4 ± 0·5 ($n = 6$)	2·2 2·1
Diaphragm, microsomal pellet (from 2·0 g tissue)	0·05 ± 0·01 ($n = 6$)	not measurable	0·6 0·05
Ventral roots, total homogenate (from 0·1 g tissue)	15 ± 1·6 ($n = 6$)	19 ± 5 ($n = 6$)	—
Ventral roots, microsomal pellets (P_2) (from 0·1 g tissue)	5 ± 0·6 ($n = 6$)	6 ± 2 ($n = 6$)	—
Sciatic nerve, total homogenate (from 1·0 g tissue)	39 ± 2·7 ($n = 6$)	43 ± 8 ($n = 6$)	—
Sciatic nerve, microsomal pellet (P_2) (from 1·0 g tissue)	14 ± 1·6 ($n = 6$)	18 ± 3 ($n = 6$)	—

ever, these are optimal values, since recoveries from tissues are much lower.

Thus the method presented here is specific, reliable and relatively easy to perform. It is probably more specific than the leech assay. The method seems to be as specific and reliable as the combined gas chromatographic–mass spectrometric methods but is less time-consuming and less expensive.

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