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AN ENZYME ASSAY FOR ACETYLCHOLINE*

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Abstract—A specific and sensitive enzymatic assay for acetylcholine has been developed. The acetylcholine was isolated from tissue extracts by electrophoresis, hydrolyzed to choline, and reacetylated by the transfer of the acetyl-^{14}C group of acetyl-^{14}C-coenzyme A to the choline molecule by the enzyme, choline acetyltransferase. The rate of formation of the product, acetyl-^{14}C-choline, was linearly related to the concentration of substrate choline up to 30·0 μg per ml. The assay can be used to measure acetylcholine in as little as 10 mg brain tissue (0·01 μg acetylcholine).

THERE is a need for a more specific assay for acetylcholine (ACh). The present bioassay methods are sufficiently sensitive, but become increasingly complicated when specificity is desired. The tedium and unaccountable variation in sensitivity from one animal to another also limit the usefulness of a bioassay. Bioassays are particularly unreliable when studying the effects of drugs on the ACh levels in the brain tissue of experimental animals. Many drugs penetrate the blood–brain barrier and, when extracted, have effects of their own on the muscle preparations used in bioassays.

There are some newer gas chromatographic methods which are inherently more specific and are as sensitive as the bioassay.[1, 2] At the present time, however, the reliability of these techniques is difficult to demonstrate with ACh extracted from biological tissue.

This is a report on a sensitive and specific enzymatic assay for acetylcholine extracted from biological tissue. The principle of the assay is relatively simple. The ACh from brain tissue extracts is isolated by paper electrophoresis. The ACh is eluted, hydrolyzed and the resulting choline (Ch) reacetylated with radioactive acetyl-^{14}C-coenzyme A in the presence of the enzyme, choline acetyltransferase. The rate of enzymatic reacetylation is linearly related to the choline concentration in the range of interest. The lower limit of sensitivity of the assay is presently about 10 nanograms. The theoretical lower limit is about 0·1 ng.

EXPERIMENTAL

Preparation of choline acetyltransferase. Choline acetyltransferase was prepared on the same day in which the assay was performed. All procedures were carried out at 0–5°. Five male CF_1 mice (Carworth Farms) weighing 18–25 g were killed by cervical fracture. The whole brains were rapidly removed and chilled on clean crushed ice.

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The cerebral cortices were reflected laterally on each side of the midline with a small sharp spatula so as to exclude tissue from subadjacent structures. The cortical tissue was homogenized in 5 ml of 1% Triton X-100 with a Virtis blade homogenizer (1 min at ½ full speed in a Packard liquid scintillation vial). The homogenate was spun at 15,000 rpm (27,000 *g*) for 1 hr in a Sorvall preparative centrifuge. The supernatant was dialyzed twice for 1·5 hr each against 1 l. of 0·1 M sodium phosphate buffer (pH 7·4) containing 0·1 mM Na_2 EDTA. During dialysis, 2 g of dry G-25 Sephadex (coarse, beaded) was weighed and placed in a 10-ml Beckman polyallomer tube. After dialysis, the supernatant volume was adjusted to 6 ml with 0·1 M sodium phosphate buffer, the pH was adjusted to 7·0–7·2 with solid K_2HPO_4 (approx. 100 mg), and then the supernatant was added to the dry Sephadex with rapid mixing. The beads were allowed to swell for 5 min in the cold. A small pinhole was made in the bottom of the polyallomer tube with a 26 gauge needle and the punctured tube was force-fitted into another polyallomer tube. The tubes were then aligned and centrifuged at 1000 rpm (236 *g*) for 10 min in an MSE-4L refrigerated centrifuge with a Universal swing-out head. The recovery of concentrated enzyme in the bottom tube was usually about 1 ml. An equal volume of cold 0·1 M sodium phosphate buffer was added, the enzyme was made 3×10^{-4}M in physostigmine and stored at 1–3° (not frozen) until use.

Preparation of brain samples. Individual mouse brains were removed as rapidly as possible and placed on dry ice covered with aluminum foil. The frozen brains were weighed on a torsion balance (range 0–500 mg) and then homogenized with approximately 1·5 vol. of chilled 0·1 N perchloric acid in all-glass microhomogenizers (Micro-Metric Instrument Co.). When discrete areas of the brain were analyzed, whole brains were placed under a dissecting microscope in a petri dish surrounded with crushed ice. The area of interest was excised as rapidly as possible, frozen on dry ice and treated as above. The smaller areas were weighed on a more sensitive balance (range 0–15 mg). After homogenization, the sample suspensions were allowed to sit for ½ hr in an ice bath and then centrifuged in the homogenizing vessels at 2500 rpm (1473 *g*) for 30 min in the MSE-4L centrifuge. The supernatant was taken for analysis. Volume corrections for the brain solids were estimated to be 78·9 per cent water by weight from freeze-drying data.

Preparation of standards. Stock solutions of AChCl containing 0, 1, 2, 3 and 4 μg/ml in 0·1 mM HCl were prepared periodically and stored in 100-μl aliquots at −70°. From this point on, the standards were treated in the same manner as tissue samples. At least one blank and four standard concentrations were run with each 25-tissue samples.

Isolation of acetylcholine by paper electrophoresis. ACh was isolated from free choline (Ch) and other choline-containing compounds by paper electrophoresis by using Schleicher and Schnell electrophoresis strips (2043A) obtained from Beckmann Instruments, Inc. The buffer was that of Potter and Murphy.[3]

A center line was drawn in pencil on the number of strips needed. Ten μl of a 3 mg/ml solution of tetraethylammonium bromide (TEA) was evenly applied with a 10-μl disposable micropipette and allowed to dry completely. The standards or tissue sample supernatants were then evenly applied on top of the dry TEA band as shown in Fig. 1. Up to 30 μl could be applied as long as only 10 μl was spotted at a time and the band was allowed to dry between applications. With more than 30 μl, the salts

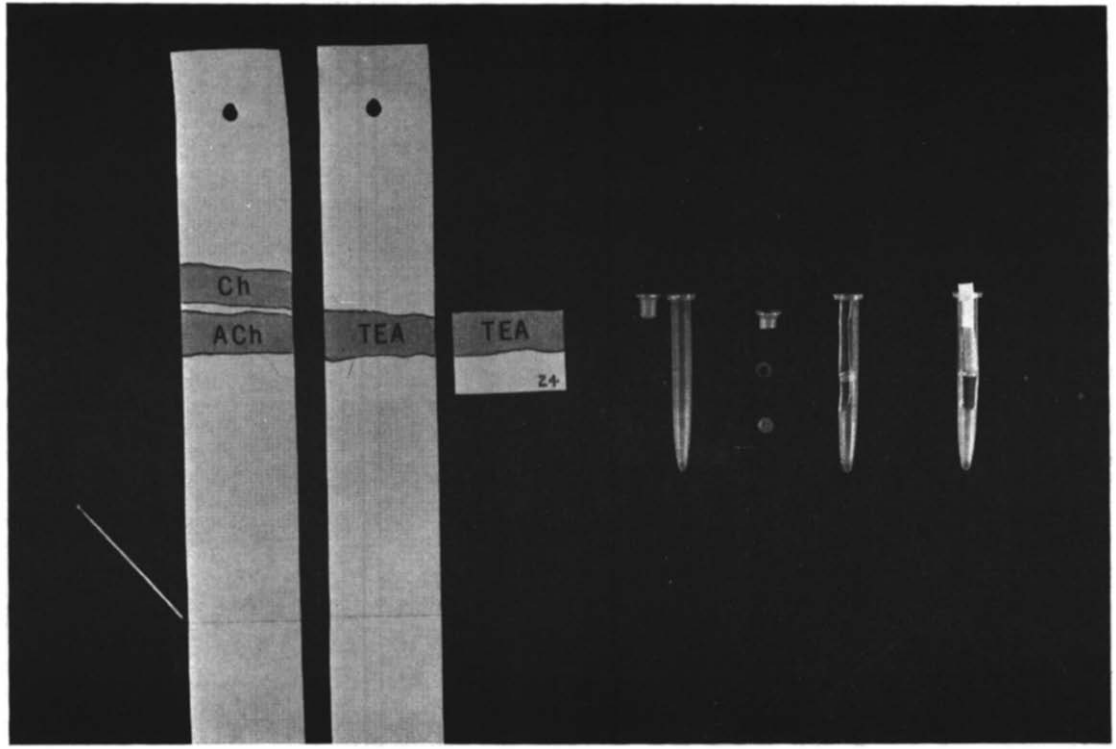

FIG. 1. Migration rates of choline, acetylcholine and tetraethylammonium using paper electrophoresis. Apparatus for isolation and elution using microtubes.

and other soluble materials in the tissue sample supernatants tended to interfere with the electrophoretic separation of the Ch and ACh. After the last sample had dried, the paper strips were placed in Beckmann Durrum cells and the paper was moistened with the buffer to within 1 cm of either side of the center line. When the cells were covered, the remaining dry paper near the center line was soon moistened by the ascending chromatographic flow of buffer from the wicks. Electrophoresis was carried out for 1 hr at 500 volts. The strips were air-dried in a hood and lightly stained in an I_2 vapor chamber while still slightly damp. As can be seen in Fig. 1, TEA and ACh have identical migration rates. The edges of the TEA bands were carefully outlined in pencil and the strips returned to the hood overnight. All the I_2 had evaporated by then, leaving a white band outlined in pencil. On each strip another pencil line was drawn 2·3 cm from the outer edge of the TEA band toward the center line such that an approximate square was formed with the TEA band inside and lining one edge of the square. The sample was identified in pencil in the square and the square was cut out with a pair of forceps and surgical scissors.

Elution and hydrolysis of the isolated acetylcholine. Beckmann microcentrifuge tubes have an attached cap. For each sample the cap was removed and the unflared end sliced off with a scalpel (see Fig. 1). Another slice was made approximately 1·5 mm above the freshly cut end to form a ring. The ring was force-fitted into the tube until it rested 2·0 cm from the top. The square piece of electrophoresis paper was folded three times into a cylindrical shape and slid into the tube until the outer edge of the TEA band rested against the ring. Approximately 3 mm of the paper cylinder protuded above the lip of the tube. The tubes with the samples in them were inverted and slid into small glass test tubes containing 50 μl of dry spectrograde acetone. The acetone was rapidly absorbed by the paper, collecting any remaining acetic and formic acid without disturbing the TEA and ACh. Samples were centrifuged in a Beckmann microcentrifuge for 5 min. The process was repeated with 100 μl acetone. Virtually all the acid was removed by this procedure. The samples were then transferred to fresh microcentrifuge tubes with retaining rings. The TEA and ACh were then eluted in similar fashion with a 7:1 mixture of methanol–concentrated NH_4OH, again starting with 50 μl. The fluid was rapidly absorbed by the paper edge and simultaneously chromatographed the TEA band even closer to the ring. Samples were centrifuged in a Beckmann microcentrifuge for 5 min. The process was repeated with 100 μl of the methanol–NH_4OH solution. The paper cylinders and the retaining rings were removed, leaving the sample in approximately 120 μl of the methanol-NH_4OH solution. The samples were placed in a 70° water bath. When the methanol had evaporated (approx. 5 min), the temperature of the water bath was raised to 95°. At this temperature the NH_4OH rapidly hydrolyzed the ACh and within 30 min the tubes were dry, leaving a small residue of TEA and Ch.

Reacetylation of the choline. The residues were redissolved in 15 μl of the 0·1 M sodium phosphate buffer (pH 7·4). The samples were chilled on crushed ice, then 15 μl of the enzyme solution and 15 μl of 187·5 μM acetyl-^{14}C-coenzyme A (sp. act., 30–50 mc/m-mole; New England Nuclear) were added. The individual tubes were vigorously mixed with a vortex mixer and then incubated in a water bath at 37° for 30 min. The reaction was stopped by placing the samples back on to the crushed ice. A 20-μl aliquot of the reaction mixture was applied to new strips of electrophoresis paper previously spotted with 10 μl of 3 mg per ml TEA. The electrophoretic separation

of the newly formed acetyl-^{14}C-choline from the unused acetyl-^{14}C-coenzyme A was carried out in identical fashion as the initial isolation of endogenous ACh described above. After staining, the TEA band was cut out and placed in 4 ml of 100% ethanol and 10 ml of toluene-based phosphor. Samples were counted for 10,000 counts in a Nuclear Chicago Unilux liquid scintillation spectrometer. The efficiency for each sample was determined by the channel ratio method. All data were expressed in disintegrations per minute (dpm).

RESULTS

Substrate specificity. The initial electrophoretic isolation of ACh is responsible for most of the specificity of the assay. As indicated by Potter and Murphy,[3] the ACh was easily separated from other choline-containing compounds tested (see Table 1).

TABLE 1. SEPARATION OF QUATERNARY AMINES BY PAPER ELECTROPHORESIS

Amine	Migration rate (cm/hr)
Cytidine diphosphocholine	0·3
Phosphocholine	0·7
Phosphatidylcholine	3·3
Choline	9·6
Acetylcholine	8·5
Acetyl coenzyme A	0·5

The migration rate of acetyl carnitine is 6·5 cm/hr in this electrophoresis system. Carnitine itself could not be acetylated by the enzyme system used here in concentrations up to 10 mg/ml. The separation of free choline and acetylcholine was the same as that quoted by Potter and Murphy,[3] about 99·6 per cent.

There was also specificity associated with the isolation of the acetyl-^{14}C-choline in the final electrophoresis step. Unknown acetyl-^{14}C acceptors could be present in the TEA band giving rise to false values. However, by specifically isolating ACh again in the last step, the possibility of picking up other ^{14}C-acetylated products was minimized.

Enzymatic O-acetylation of choline. When acetylcholine in concentrations of 0, 1 2, 3 and 4 μg/ml was isolated and hydrolyzed as described in the Methods section and the resulting choline was incubated with acetyl-^{14}C-coenzyme A and the enzyme choline acetyltransferase, a linear relationship between substrate choline and product acetyl-^{14}C-choline was obtained. A typical calibration curve is shown in Fig. 2.

Recoveries. The manipulations and per cent recovery in each step are shown in Table 2. These values were determined by using acetyl-^{14}C-choline in amounts equivalent to that found in the tissue samples. It has been pointed out that the overlap of choline into the ACh band was less than 0·5 per cent. However, the loss of ACh in the Ch band and the area separating the two bands was about 20 per cent. Since the ACh standards were subjected to the same manipulations as the tissue samples, the calibration curve was directly applicable to the tissue sample values. All the other manipulations were virtually 100 per cent efficient.

In order to be sure that ACh was not destroyed or preferentially bound to the precipitated proteins during the preparation of tissue samples, the recovery of a known amount of nonradioactive ACh added to a tissue sample was estimated. Hydrolyzed extracts of brain tissue were also prepared, that is, hydrolyzed before the

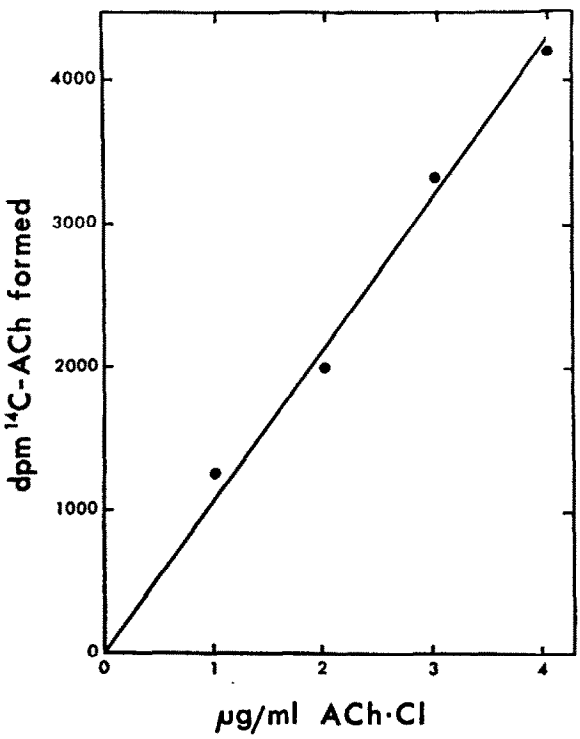

FIG. 2. Typical calibration curve for enzymatic assay system. Each point represents one standard sample to indicate the amount of variation in regressions with single estimates. The blank value for this set of estimates was 421 dpm.

TABLE 2. RECOVERY OF ACETYL-^{14}C-CHOLINE IN THE VARIOUS STEPS OF THE ASSAY INVOLVING POSSIBLE LOSS

Manipulation	% Recovery of individual step	% Running recovery
1. Initial electrophoretic isolation of acetylcholine	80·0	80·0
2. Acetylcholine remaining on paper after acetone wash	99·2	79·9
3. Elution of acetylcholine into microtubes	98·5	78·6
4. Hydrolysis of acetylcholine to choline	98·7	77·6
5. Final electrophoretic isolation of acetyl-^{14}C-choline	100·0	77·6

first electrophoresis. The results are shown in Table 3. The recovery of a known amount of ACh added to a tissue sample was approximately 100 per cent. Hydrolyzed extracts of brain tissue gave values indentical with the blank.

TABLE 3. RECOVERY OF KNOWN AMOUNTS OF ACETYLCHOLINE ADDED TO TISSUE SAMPLES

Sample	Volume (μl)	dpm
1. Acetylcholine, 1·0 μg/ml	10	936
2. Tissue extract	10	1037
3. Acetylcholine, 1·0 μg/ml, plus tissue extract	10 10	1984 (101% recovery)
4. Blank value	10	435
5. Hydrolyzed tissue extract	10	429

Measurement of acetylcholine levels in mouse brain. The method has been applied to discrete areas of the mouse brain. The major problem was not in the assay *per se*, but in the clean dissection and rapid excision of the samples. The results in Table 4 represent preliminary data, subject to verification with more refined dissection techniques. As in most other mammals, the hypothalamus contained the highest and the cerebellum the lowest microgram per gram level of ACh. The caudate-putamen,

TABLE 4. ACETYLCHOLINE CONTENT OF WHOLE AND DISCRETE AREAS OF MOUSE BRAIN*

Area	Average acetylcholine (free base) (μg/g ± S.E.)
Whole brain (37 brains)	2·35 ± 0·16†
Whole brain‡	2·31
Cortex	2·13
Hypothalamus (anterior and posterior)	4·12
Midbrain	1·19
Brain stem (including pons and tegmentum)	1·84
Olfactory bulb	1·49
Cerebellum	0·94
Reconstructed whole brain‡	1·86

* Except where noted, each value represents the average of samples taken from the left half of three individual mouse brains.
† Literature values using bioassay range from 1·86 to 2·35 μg/g.[4–9]
‡ Whole brain values obtained in experiments where parts were also measured.

usually found to contain the highest concentration of ACh, was part of the "cortex" and therefore was diluted with other cortical tissue.

Effect of physostigmine. Table 5 shows the effect of physostigmine on whole-brain ACh as measured by the enzyme system. Physostigmine (0·3 mg/kg), injected i.p. 1 hr prior to sacrifice, caused an 80 per cent increase in whole-brain ACh.

TABLE 5. EFFECT OF PHYSOSTIGMINE ON WHOLE BRAIN LEVELS OF ACETYLCHOLINE (a)

Drug	Average μg/g acetylcholine (free base) ± S.E.
Control	2·25 ± 0·28 (b)
Physostigmine (0·3 mg/kg	4·06 ± 0·71 (b)

(a) Assays were performed on individual mouse brains.
(b) Each figure represents the average of eight brains (two estimates per brain).

DISCUSSION

Acetylcholine extracted from tissue was isolated, hydrolyzed and reacetylated by using acetyl-^{14}C-coenzyme A and choline acetyltransferase. The product, acetyl-^{14}C-choline, was linearly related to substrate choline in the concentration range of interest as indicated in Fig. 2.

This enzymatic assay for acetylcholine is inherently more specific than currently used bioassay systems. Choline acetyltransferase will catalyze the transfer of acetyl-^{14}C groups only to choline in the concentration range of interest. Interfering choline esters and drugs are removed in the initial isolation of acetylcholine from the tissue samples. This is particularly useful in studies of the brain levels of acetylcholine where drugs have been used which interfere with bioassays.

This enzymatic assay is equally as sensitive as currently used bioassay systems. Because the assay system is done on a microscale, the ACh content in as little as 10 mg brain tissue can be measured (approximately 0·01 μg).* It must be understood, however, that this is because such a small sample volume (20 μl) is required. The concentration of ACh in that volume must be at least 0·4 μg per ml. Experiments are in progress to increase the sensitivity of the assay.

In steps where loss could be expected to occur, acetyl-^{14}C-choline in amounts equivalent to that found in tissue samples was used to determine recoveries. As shown in Table 2, only the initial isolation of ACh presents a significant loss and since the ACh standards are subjected to identical manipulations, the calibration curve is directly applicable to tissue sample values. The recovery of a known amount of ACh added to a tissue sample was 101 per cent.

Table 4 lists the ACh content of whole and discrete areas of mouse brain. Takahashi and Aprison[8] have demonstrated differences in the acetylcholine content of brain tissue depending on the method of sacrificing the animal. As an extension of this finding, it seems reasonable to attribute the 20 per cent lower ACh content of the reconstructed whole brain to time and temperature factors involved in dissection. The near-freezing method of Takahashi and Aprison[8] is now being considered.

Table 5 shows that whole brain ACh was increased 80 per cent by the i.p. injection of 0·3 mg/kg of physostigmine.

* The experiments reported in this paper were done with enzyme solutions prepared on the same day they were used. On some days the blank was higher than others, but there was always a corresponding shift in the slope of the calibration curve such that at least twice the blank was obtained for 0·01 μg AChCl.

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