choline and acetylcholine:

handbook of chemical assay methods

edited by
Israel Hanin

Choline and Acetylcholine: Handbook of Chemical Assay Methods

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Preface

Studies of the mammalian cholinergic system have lagged substantially behind those of the catecholaminergic system. One major contributing factor to explain this phenomenon has been the lack, until recently, of good sensitive and specific chemical methods for the assay of choline and acetylcholine. Bioassay has been the major approach used to measure acetylcholine activity in tissue.

Bioassay techniques used to measure tissue choline and acetylcholine are extremely sensitive. However, they are time consuming, require repetitive controls for accuracy and reproducibility of the data, and—last but not least—are not chemically specific.

Within the past two decades, and especially within the past few years, several chemical methods for assaying tissue choline and acetylcholine have been developed which seriously rival the best bioassay methods. Although some are not as sensitive as bioassay, they all exhibit properties of reproducibility and chemical specificity. Furthermore, most of these methods are capable of assaying simultaneously choline and acetylcholine in tissue extracts.

The idea for this book was spurred by the recent marked upsurge in the study of the metabolism and function of acetylcholine in the mammalian system. This is most evident from the steady and consistent increase in the number of volunteer papers on topics related to cholinergic mechanisms which were presented at the ASPET, FASEB, and IUPHAR meetings between the fall of 1971 and summer of 1972. It was therefore felt that it would be of great value if scientists studying cholinergic phenomena were to have access to a monograph such as this one, which incorporates under one cover all the available, sensitive working methods for the chemical assay of choline and acetylcholine.

The objective of this Handbook is to appeal to a wide spectrum of investigators interested in establishing a chemical method for the assay of acetylcholine and/or choline in their laboratories. This consideration consequently underlies the entire format of the book.

The book consists of 14 chapters, each describing a different chemical approach for the assay of choline and/or acetylcholine, which rivals bio-

assay in its limit of sensitivity. Enzymatic, fluorometric, gas chromatographic, photometric, and polarographic methods are described.

Emphasis has been placed on detail and a considerable amount of simplification. The authors have specifically attempted to incorporate in their chapters all details essential to enable an interested investigator to set up the entire procedure by following the instructions and directions contained in each chapter.

In order to achieve uniformity throughout the book, a set structural organization has generally been adhered to in each chapter. Each chapter has been subdivided according to the following guidelines:

1. *Introduction,* which confines itself to the methodological approach to be used with only a brief allusion to the historical aspect of methodology used for the assay of acetylcholine and choline. This avoids inevitable repetition throughout the various chapters, and more importantly, this immediately serves to give the reader an indication of the subject matter and approach to be taken in the chapter.
2. *Reactions Involved.*
3. *Preparation of Reagents.* Purification techniques and preparation of enzymes are included in this section whenever applicable. A "cookbook" style approach has been adopted in this section to achieve maximal detail and clarity.
4. *Methodology.* In this section are discussed steps involved in extracting choline and acetylcholine from tissue; preparation of choline and its esters for chemical assay; and the chemical assay of choline and acetylcholine.
5. *Advantages and Disadvantages of the Method.* Here we have asked the authors to present a candid and fairly stated analysis of the method in comparison with the other chemical assays for choline and acetylcholine. This includes information regarding the sensitivity of the method, its ease of operation, its limitations and/or advantages in various types of applications, the number of assays possible per unit time, etc.
6. *Applications—Current and Projected.*
7. *Summary.*

It is hoped that a complete novice would be able to use each and any of the chapters as a guide for setting up the method of his or her choice without referring to other outside sources. This Handbook will have served its purpose if this goal is achieved.

The major effort in putting this book together was achieved while I was a member of the staff of the Laboratory of Preclinical Pharmacology, NIMH, St. Elizabeths Hospital, in Washington, D.C. I would like to thank Dr. Erminio Costa for his encouragement of this effort. I would also like to extend my appreciation to the authors of these chapters for adhering so

closely to the guidelines with which they were confronted, and for completing their manuscripts in a relatively short period of time. Last, but certainly not least, the efficient secretarial assistance of Mrs. Judy Davis and Ms. Deborah Behun is warmly appreciated.

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Contents

Choline and Acetylcholine: Handbook of Chemical Assay Methods
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Determination of Acetylcholine and Choline by Enzymatic Radioassay

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I. INTRODUCTION

The first radioassay method for the determination of tissue acetylcholine was that of Feigenson and Saelens (1969). Acetylcholine was extracted from brain, isolated by paper electrophoresis, eluted, and hydrolyzed. The resulting choline was then enzymatically acetylated with radioacetylcoenzyme A and choline acetyltransferase, and the radioacetylcholine formed was isolated by paper electrophoresis and quantitated.

A subsequent paper by Saelens, Allen, and Simke (1970) included the measurement of choline, as well as acetylcholine, in the same tissue sample. Moreover, a preparation of choline acetyltransferase was described wherein the enzyme could be obtained in large amounts and stored in lyophilized form for long periods. This increased the utility of the method by shortening the working time and insuring uniformly high enzymatic activity over a series of at least several hundred assays.

This chapter reports a description of the method of Saelens et al. (1970) incorporating some modifications that we have made during the past 2 years. A double-isotope technique for quantitating the small overlap of choline during electrophoresis which may occur under certain specific conditions was developed in our laboratory (Ladinsky, Consolo, and Bareggi, 1972*a*) and will be described.

II. REACTION INVOLVED

The reaction catalyzed by choline acetyltransferase is:

^{14}C-acetylcoenzyme A + choline → ^{14}C-acetylcholine + coenzyme A.

III. PREPARATION OF REAGENTS

A. Preparation of Choline Acetyltransferase

All steps are performed at 0 to 5°C. The whole brains from 40 male albino Swiss mice are removed as quickly as possible after decapitation

and placed in 80 ml of ice-cold 0.1 mM Na_2EDTA containing 160 μl of 1 N NH_4OH and 800 μl of n-butanol. The mixture is homogenized in an Omni-Mixer (Sorvall) for 1 min, chilled on crushed ice for 15 min, and again homogenized for 30 sec. The butanol and NH_4OH help to solubilize the enzyme by raising the pH and disrupting enzyme-containing particles (Potter, Glover, and Saelens, 1968).

The homogenate is then spun at 47,000 rpm for 1 hr in a Spinco L-2 ultracentrifuge, and the supernatants are pooled.

Choline acetyltransferase is partially purified and concentrated by isoelectric precipitation. We titrate three 1-ml aliquots of the pooled supernatant with 10, 11, and 12 μl of 1 N acetic acid. Good yield of the enzyme is obtained when a pink pellet and clear pink supernatant are produced after low-speed centrifugation. The addition of too much or too little acid results in white or whitish pellets and murky supernatants. Usually 11.5 to 12-μl samples of 1 N acetic acid per ml of supernatant produce the best yields of enzyme.

The pooled supernatant is then divided into 8-ml aliquots in 15-ml conical test tubes, and the proportional amount of 1 N acetic acid added. The suspension is vortexed every 5 min for 15 min and spun in a table-model centrifuge for 15 min at 3,500 rpm. The supernatant is discarded, and the pink precipitate is washed three times with 10 ml of ice-cold distilled water. Between each washing the suspensions are vortexed and spun for 15 min. This washing procedure should not remove any of the color.

The precipitate in each tube is dissolved in 1.25 ml of 50 mM sodium phosphate buffer, pH 7.4. The solutions are pooled and dialyzed overnight against 2 liters of 50 mM sodium phosphate buffer, pH 7.4. Physostigmine sulfate, 1.4 mg/ml, is added at a volume of 70 μl/ml of dialysate to give a final concentration of 3×10^{-4} M. The solution is spun at low speed to remove any insoluble material, and the dialysate is placed in vials in 500-μl aliquots and lyophilized. The protein concentration before lyophilization is usually 15 to 20 mg/ml. The lyophilized vials are sealed and stored at −20°C. In this condition we have found the enzyme to be stable for up to 3 months. Within this time span there is a slow loss of activity in some preparations so that longer storage is not recommended. We have found that the enzyme is equally active and stable when dialyzed against 0.1 M Tris, pH 7.4.

Approximately 25 vials can be obtained from 40 mouse brains, and each vial contains enough material for 30 to 35 assays.

These preparations usually have a specific activity (V_{max}) of 0.072 μmole of product per hr per mg of protein.

B. Preparation of Standards

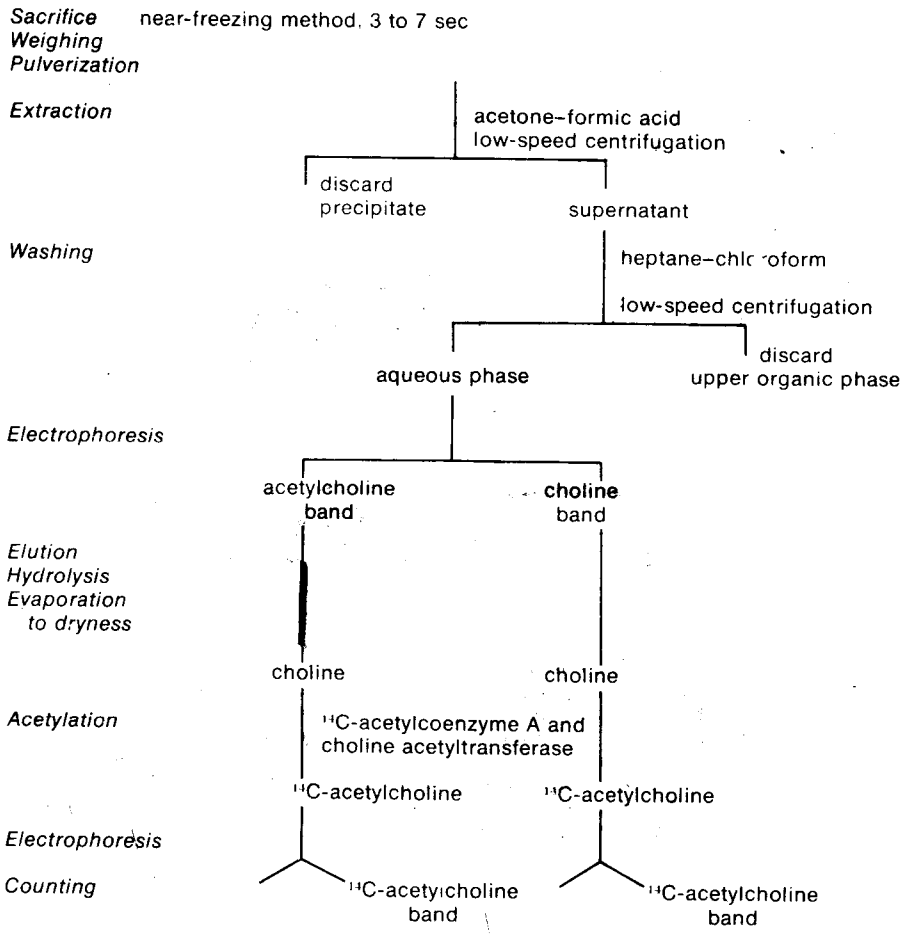
Acetylcholine and choline standards are prepared freshly each week in 0.1 mM HCl and stored in 5-ml aliquots at −20°C until use. Each aliquot is

used once and the rest discarded. Acetylcholine iodide (Fluka) is prepared in concentrations of 0.5 to 3 μg free base/ml (3.4 to 20.5 nmoles/ml) and choline chloride (Fluka) 1.5 to 5 μg free base/ml (14.4 to 48.1 nmoles/ml). Ten-μl aliquots of three concentrations of each standard, in duplicate, are electrophoresed and run for each experiment.

IV. METHODOLOGY

A flow sheet of the method used is shown in Table 1.

TABLE 1. *Flow sheet for the enzymatic radioassay of acetylcholine and choline*



A. Sacrifice

Small animals are sacrificed by the near-freezing method of Takahashi and Aprison (1964) by dipping them into liquid nitrogen for 3 to 7 sec, depending upon their size.

The tissue is rapidly removed, placed on a tared gauze square, frozen in liquid nitrogen, and weighed in the frozen state. At this stage the tissues may be stored in liquid nitrogen for at least 1 week.

B. Pulverization

The frozen tissue is pulverized in a mortar and pestle containing a removable base plate which permits rapid and quantitative transfer of the frozen powder to test tubes for subsequent extraction (Figs. 1 and 2; Ladinsky, Consolo, and Sanvito, 1972*b*). The pulverizer is selected relative to the tissue volume. The diameter of the test tube used for subsequent extraction of the tissue should be slightly larger than the base plate so that the powdered tissue can be shaken off easily into the tube. Upon removal from the liquid nitrogen, a tissue sample can be pulverized and quantitatively transferred within 80 sec.

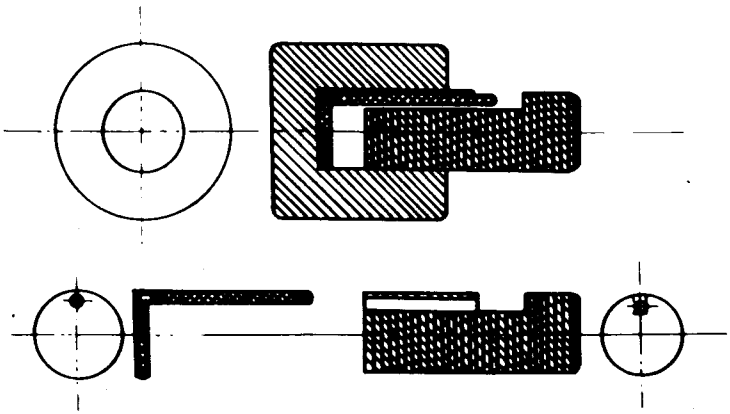


FIG. 1. Detail of pulverizer which consists of a mortar, pestle, and mortar base plate. The base plate fits snugly into the mortar and is controlled by a handle attached perpendicularly to its edge. A hole is drilled along the longitudinal axis of the pestle so that the pestle can slide down the base plate handle and fit snugly into the mortar above the base plate. Frozen tissue is placed on the base plate in the mortar and than the pestle is placed over the tissue. The tissue is finely pulverized by several hammer taps. After removal of the pestle, the base plate and frozen powder are removed, and the powder is rapidly and quantitatively transferred to vessels containing extractant solvents. (From Ladinsky et al., 1972*b*, with permission of the publisher.)

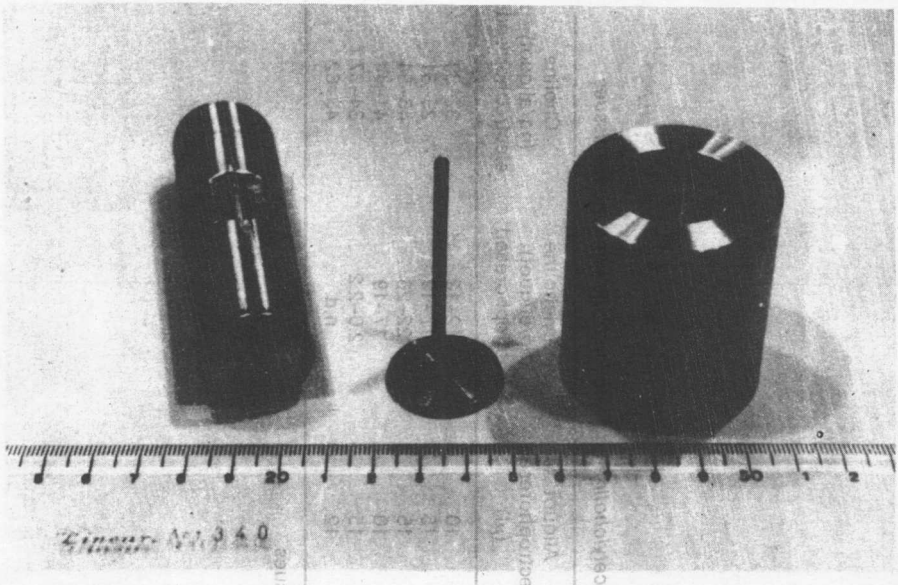


FIG. 2. Pulverizer components. Pestle, mortar base plate, and mortar are shown from left to right. (From Ladinsky et al., 1972*b*, with permission of the publisher.)

C. Extraction

Acetylcholine and choline are extracted into 85% acetone–15% aqueous 1 N formic acid (Toru and Aprison, 1966). We have varied the proportions of extractant volume to tissue weight depending upon the concentration of these amines in the tissue and their ratio (Table 2). After it is shaken for 30 min in ice, the mixture is centrifuged for 10 min at 2,000 rpm. The precipitate is discarded, and the clear supernatant is transferred to other plastic conical 15-ml tubes and partitioned twice with 5 ml of heptane–chloroform (4:1). The organic phase is aspirated and discarded. The lipid interphase is kept after the first wash but discarded after the second. The heptane-chloroform removes some lipid, liposoluble choline-containing material, and the acetone from the extraction mixture, thus reducing the volume by 85%. The tissue amines are now present in the 15% aqueous phase of the original extractant plus the water content of the tissue.

D. Electrophoresis

Acetylcholine is separated from choline by horizontal paper electrophoresis at 500 V (Elvi 32 power supply) for 60 min using S&S 2043A

TABLE 2. *Volume of acetone-formic acid used for extraction of acetylcholine and choline from some mouse tissues*

Tissue	Weight (mg)	Extractant volume (ml)	Ratio (extractant vol/tissue wt)	Aliquot electrophoresed (μl)	Acetylcholine (ng/aliquot) electrophoresed	Choline (ng/aliquot) electrophoresed
Whole brain	460–520	3	6	10	12–13	37–39
Hemispheres	260–290	3	10	15	17–19	29–31
Diencephalon	60– 80	0.5	6–8	15	22–26	46–54
Mesencephalon	80– 90	0.5	6	10	17–19	41–44
Cerebellum	60– 70	0.6	10	15	2.0–2.2	34–37
Spleen	110–180	2	10–20	12	n.d.	43–62

See Table 6 for acetylcholine and choline concentrations in these tissues.
n.d. = not detectable

paper strips (30 × 300 mm). The buffer is that of Potter and Murphy (1967) prepared by mixing 25 ml of concentrated formic acid and 75 ml of glacial acetic acid with 900 ml of glass-distilled water.

To prepare the sample for electrophoresis, a thin, centered pencil line is drawn on the paper strip and tetraethylammonium (TEA), 3 mg/ml, is applied evenly with a 10-μl disposable micropipette. TEA acts as a marker for acetylcholine since the migration rates of both quaternary amines are almost identical (Potter and Murphy, 1967; Feigenson and Saelens, 1969). Aliquots of the aqueous extracts are streaked on the paper on top of the dry TEA band in 5- to 20-μl volumes, depending on the concentrations of acetylcholine and choline present in the extract (see Table 2).

At this point acetylcholine and choline standards (see section III) are spotted on paper strips for electrophoresis. Blanks consist of strips containing only TEA.

After air drying, the strips are wetted to within 1 cm on each side of the center line and placed on the horizontal bridges in the electrophoretic cells. The solutions move toward the origin by capillary action and concentrate the samples at the origin.

After electrophoresis, the papers are dried in an air-circulated oven at 100°C for 5 min; the TEA band is then lightly stained with iodine vapor. The outer edge of the stained TEA band is carefully delineated by a thin pencil line using a soft lead. For acetylcholine, 2.3 cm is measured from the outer edge toward the origin, whereas for choline 2.3. cm is measured from the outer edge away from the origin. The paper is allowed to air-dry overnight. This has the added advantage of permitting evaporation of the iodine.

We have found that acetylcholine and choline can be quantitatively separated under these conditions with an overlap of acetylcholine onto the choline band of less than 2%. Figure 3 shows the relationship between the percentage overlap of choline onto the acetylcholine band and the amount of choline placed on the paper. Less than 0.5% trailing was observed up to about 100 ng (1 nmole) of choline, but it then increased logarithmically to reach 3.5% overlap at about 270 ng (2.6 nmoles) choline. Since electrophoresed tissue aliquots contain less than 60 ng (0.6 nmole) of choline (Table 2), errors due to overlap are negligible (see section V).

E. Elution and Hydrolysis

The bands are carefully cut out along the pencil lines and folded three times into a cylindrical shape. The paper cylinders are placed into 450-μl Beckman polyethylene microtubes. Each tube has a force-fitted ring inserted 2.0 cm from the top. The rings are made from the stoppers attached to the