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Institute of Biochemistry,
School of Medicine,
University of Buenos Aires, Argentina.

B. N. BADANO
A. O. M. STOPPANI

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The fluorometric determination of acetylcholine

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PROBLEMS under investigation in this laboratory on certain aspects of acetylcholine (ACh) metabolism indicated the need for a sensitive chemical assay for the neurohumor. The principle of the method that has been devised is as follows: acetylcholine is reduced under mild conditions by the use of potassium borohydride and lithium choloride to yield ethanol and choline. The ethanol is then oxidized to acetaldehyde by alcohol dehydrogenase with the concomitant reduction of NAD (DPN) to NADH (DPNH); finally, the NADH is determined fluorometrically.

The procedure, which may be modified to accommodate any volume that is convenient, is outlined below.

Reagents. (1) Acetylcholine: a stock solution of acetylcholine chloride or perchlorate of 1 mg/ml is prepared and suitably diluted to serve as a standard. (2) Reducing solution: per milliliter, this solution contains 30 mg of potassium borohydride and 10 mg of lithium chloride and is prepared just prior to use; sodium borohydride may be used in place of the potassium salt. The borohydrides may be obtained from Metal Hydrides, Inc. (3) Glycine buffer: this solution contains 22·5 g of glycine,* 3·75 g of semicarbazide HCl and alkali to pH 9·6. The solution is freeze-dried to remove traces of alcohol in the reagents and finally diluted to 100 ml; to prevent deterioration, it should be stored in

* Most commercial samples of glycine contain a fluorescent impurity; this contaminant may be removed by treatment with charcoal.

the frozen state. Prior to use, the volume of buffer that will be needed in the determination is saturated with mannitol because hydrochloric acid converts borohydride to borate and the latter complexes with NAD; the mannitol is necessary to remove the borate. (4) NAD: the preparation of Sigma Chemical Co. is recommended because of its low fluorescent blank. A solution is prepared, freeze-dried to remove contaminating traces of alcohol, and then diluted to yield a concentration of 10 mg/ml (5) Alcohol dehydrogenase (ADH): yeast ADH prepared by Boehringer and Soehne (obtainable from California Corp. for Biochemical Research) is recommended. Where a unit of enzyme activity is defined according to Racker,[1] but in a final volume of 1 ml, it has been found that approximately 1,600 to 2,300 units of enzyme are optimal in the determination of acetylcholine. With the usual commercial enzyme preparation, this means that the preparation of ADH may be diluted 1:50 and an aliquot of 0·03 ml (approximately 18 μg) is necessary for each sample; the solution of the enzyme should be diluted just before using. The use of more ADH than is recommended above will result in a larger blank (see below). Since the suspension of ADH tends to deteriorate with time, it should be assayed every two weeks.

PROCEDURE

To 0·5 ml of a neutralized solution of acetylcholine in a small test tube is added 0·05 ml of the reducing solution. The contents of the tube are mixed and incubated for 20 min at 37°, at which time the sample is removed to room temperature, and 0·1 ml of 0·5 N HCl is added to destroy the borohydride. After thorough mixing, the tube is allowed to stand for 10 min, but with intermittent shaking, to ensure complete destruction of the borohydride. The sample is transferred by Pasteur pipet to a test tube containing 0·2 ml of the glycine–mannitol solution; 0·02 ml of NAD and 0·03 ml of crystalline yeast alcohol dehydrogenase, diluted 1:50, are added, and the contents of the tube are gently mixed. The sample is allowed to stand for 60 min at room temperature, to permit complete oxidation of the ethanol generated from acetylcholine. At this point water may be added to make whatever final volume is desired. The solution is transferred to a calibrated fluorometer tube, and the native fluorescence of the NADH is measured. The activation filter is Turner 110-816 (Corning 7-60); the fluorescence filters are Turner 110-817 (Kodak Wratten 2A) and Turner 110-831 (Kodak Wratten 48). When a spectrophotofluorometer is employed, the activation wavelength is 340 mμ and the fluorescence wavelength is 460 mμ. If more than a few micrograms of ACh are to be determined, the concentration of NADH may be conveniently assayed in a spectrophotometer at a wavelength of 340 mμ. A standard solution of acetylcholine is carried through the procedure, together with the unknown solutions, and is used as the basis for calculating the concentration of acetylcholine in the experimental samples.

RESULTS AND DISCUSSION

The linear relationship between fluorescence and concentration of acetylcholine is shown in Table 1. These results were obtained with a Turner fluorometer with microcuvet adaptor, but equivalent values resulted when the Aminco–Bowman spectrophotofluorometer was employed.

A comparison of the specificity of the chemical assay with that of the bioassay for the neurohumor is recorded in Table 2. For a compound to interfere in the chemical assay, two criteria must be met.

TABLE 1. PROPORTIONALITY BETWEEN CONCENTRATION OF ACETYLCHOLINE AND FLUORESCENCE.*

ACh (μg)	Fluorescent units
0·1	9·0 ± 2·0
0·2	18·0 ± 1·3
0·4	35·0 ± 2·0
0·8	69·0 ± 3·0

* Conditions as described in text.

First, the compound must be reduced to yield an alcohol under the above conditions, and second, the alcohol must be a substrate for yeast ADH. From Table 2 it is apparent that the chemical assay has about the same degree of specificity as that in the bioassay. It is interesting to note that the reducing conditions are such that neither carboxylic acids nor amides are reduced.

TABLE 2. A COMPARISON OF THE SPECIFICITY OF THE ASSAY WITH THAT OF BIOASSAY

	Assay*	
	Biological	Chemical
Acetylcholine	+	+
Propionylcholine	+	+
Butyrylcholine	+	+
Choline	+	—
γ-Butyrobetaine	+	—
Acetate	—	—
N-Acetylaspartic acid	—	—
Acetone	—	+
Acetyl CoA	—	+

* Positive response, +; no interference, —.

Because physostigmine (eserine) produces a pink color at an alkaline pH, it will interfere in the assay; however, neither prostigmine (neostigmine) nor diisopropylfluorophosphate (DFP) causes interference.

There are two drawbacks to the determination of acetylcholine in the manner described in this paper. The first of these is the lack of sensitivity, as compared to bioassay procedures. From the stoichiometry of the reactions utilized in this method and from a knowledge of the fluorescent properties of NADH, it should be possible, in theory, to determine approximately 20 mμg of acetycholine in a volume of 1 ml. As may be noted in Table 1, however, the lowest level of the neurohumor that may be determined with accuracy is about 10 times this value. There are two reasons for this discrepancy. First, in the reduction step that occurs at a slightly alkaline pH, there is some hydrolysis of acetylcholine, so that the overall yield of NADH per mole of acetylcholine is approximately 60 to 70%. The major reason for the limited sensitivity, however, is attributable to a large reagent blank; this is caused by an apparent reaction between NAD and ADH to yield NADH. Thus, in the absence of substrate and under conditions that have excluded all traces of ethanol from the reagents, one can observe the appearance of NADH in either a fluorometer or a spectrophotometer. That the major source of this blank is NADH can be demonstrated by mixing an alkaline buffer with NAD and ADH, allowing the reaction to go to completion, and then adding acetaldehyde. This addition results in the disappearance of the bulk of the blank.

The second drawback to this determination is the unbelievable avidity with which the reagents including ADH, pick up traces of alcohol in the air. For this reason it is almost mandatory to work in an isolated room that is completely free of solvents. It is also advisable to use glass-distilled water and to check any new reagent for possible contamination with alcohol.

On the other hand, the simplicity of this procedure, as compared to those of bioassays, coupled with its specificity, suggests that it may be of considerable value in studies of choline esterase and choline acetylase. Unfortunately, some unknown fluorescent material in brain extracts precludes the use of this method in determining ACh in brain. Attempts to remove this material have met with little success.

Department of Pharmacology,
Yale University School of Medicine,
New Haven, Conn., U.S.A.

JACK R. COOPER

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