Biochemical Pharmacology, 1961, Vol. 7, pp 88–95. Pergamon Press Ltd., Printed in Great Britain.

A NEW AND RAPID COLORIMETRIC DETERMINATION OF ACETYLCHOLINESTERASE ACTIVITY

GEORGE L. ELLMAN, K. DIANE COURTNEY, VALENTINO ANDRES, JR. and ROBERT M. FEATHERSTONE

Department of Pharmacology, University of California Medical Center, San Francisco, California

(*Received* 14 *November* 1960)

Abstract—A photometric method for determining acetylcholinesterase activity of tissue extracts, homogenates, cell suspensions, etc., has been described. The enzyme activity is measured by following the increase of yellow color produced from thiocholine when it reacts with dithiobisnitrobenzoate ion. It is based on coupling of these reactions:

$$\text{acetylthiocholine} \xrightarrow{\text{(enzyme)}} \text{thiocholine} + \text{acetate}$$

$$\text{thiocholine} + \text{dithiobisnitrobenzoate} \longrightarrow \text{yellow color}$$

The latter reaction is rapid and the assay is sensitive (i.e. a 10 μl sample of blood is adequate). The use of a recorder has been most helpful, but is not essential. The method has been used to study the enzyme in human erythrocytes and homogenates of rat brain, kidney, lungs, liver and muscle tissue. Kinetic constants determined by this system for erythrocyte cholinesterase are presented. The data obtained with acetylthiocholine as substrate are similar to those with acetylcholine.

INTRODUCTION

A FEW years ago Bonting and Featherstone[1] introduced a modification of the Hestrin hydroxamic acid method[2] suitable for the determination of cholinesterase levels in small quantities of cells cultured *in vitro*. This modification was used successfully in several studies involving the control of enzyme levels in cells by manipulating the levels of substrates or closely related compounds present in the medium.[3] Several interesting areas of research were indicated by these studies. However, this modified method of enzyme assay, although scaled down to the micro-level, had several disadvantages. Among these was the fact that only a terminal figure could be obtained from the material in one tube of cultured cells. Thus, the time course of the reaction could not be followed without resorting to separate experimental tubes for each time interval desired. The method also had the disadvantage that the color measured was developed from the remainder of an added substrate—a procedure in which the possibility of error is relatively great when the level of enzyme activity is small.

Consideration of the relative merits of various methods which might be useful in studying the time-course of acetylcholinesterase activity in very small tissue samples led us to combine a method reported by Koelle[4] with a sulfhydryl reagent studied by Ellman.[5] This new method, which is presented here, is extremely sensitive and is applicable to either small amounts of tissue or to low concentrations of enzyme. It makes detailed kinetic studies of acetylcholinesterase activity possible. The progress of the hydrolysis is followed by the measurement of a product of the reaction.

Acetylthiocholine is used as the substrate. This analog of the natural substrate has been used most extensively by Koelle[4] for histochemical localization. Other workers[6, 7] have used the sulfur analog in the enzyme assay. Their work, in addition to data we shall present, suggests that this compound is a satisfactory substitute for the natural substrate, and differs much less than some of the synthetic substrates frequently used as in assays of phosphatases, trypsin, chymotrypsin, pepsin, etc.

The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolyzed. This is accomplished by the continuous reaction of the thiol with 5 : 5-dithiobis-2-nitrobenzoate ion (I)[5]

$$H_2O + (CH_3)_3\overset{+}{N}CH_2CH_2SCOCH_3 \xrightarrow{\text{(enzyme)}} (CH_3)_3\overset{+}{N}CH_2CH_2S^- + CH_3COO^- + 2H^+$$

$$(CH_3)_3\overset{+}{N}CH_2CH_2S^- + \underset{\text{(I)}}{RSSR} \longrightarrow (CH_3)_3\overset{+}{N}CH_2CH_2SSR + \underset{\text{(II)}}{RS^-}$$

$$R = O_2N-C_6H_3(COO^-)-$$

to produce the yellow anion of 5-thio-2-nitro-benzoic acid (II). The rate of color production is measured at 412 mμ in a photometer. The reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme, and in the concentrations used does not inhibit the enzymic hydrolysis. By recording the output of the photometer continuously, records of the complete assay can be obtained (Fig. 1).

We considered it desirable to establish that this method yields results comparable with other procedures. For this reason, the effects of inhibitors were examined; the kinetic constants were calculated and compared with those obtained by other methods. In addition, we were able to compare assays on blood samples by the ferric-hydroxamate method[8] and the present one.

METHODS

The reaction rates were recorded with a Beckman DU spectrophotometer equipped with a Beckman adapter and a Minneapolis–Honeywell recorder.

The general method used was to place buffer in the photocell and add concentrated solutions of reagents by means of micropipettes. The mixture was stirred by continued blowing through the pipettes while moving them around the bottom of the photometer cells. In this way, reagents were added, mixed, and the cover of the cell compartment replaced within 10–15 sec. Our photometer required 30–40 sec to become stabilized to new light conditions. Thus, there was about 1 min when the readings were due to a combination of factors (e.g. bubbles rising through the solutions, sulfhydryl material in the "enzyme", etc.) which were unrelated to the desired measurements. Subsequent readings were strictly dependent on the absorption of the solution under consideration and even rapid changes were followed by the recorder faithfully, as evidenced by the reproducibility of time-transmission curves (Fig. 1).

Solutions

Buffer. Phosphate, 0·1 M, pH 8·0.

Substrate. Acetylthiocholine iodide,* 0·075 M (21·67 mg/ml). This solution was used successfully for 10–15 days if kept refrigerated.

Reagent. Dithiobisnitrobenzoic acid (DTNB) 0·01 M of the 5 : 5-dithiobis-2-nitrobenzoic acid† prepared as described previously,[5] 39·6 mg were dissolved in 10 ml pH 7·0 phosphate buffer (0·1 M) and 15 mg of sodium bicarbonate were added. The reagent was made up in buffer of pH 7 in which it was more stable than in that of pH 8.

Enzyme. Bovine erythrocyte cholinesterase (Nutritional Biochem. Corp., 20,000 units) was dissolved in 20 ml of 1% gelatin. This solution was diluted 1 : 200 with water for use, yielding a solution of 5 units/ml.

General method

A typical run used: 3·0 ml pH 8·0 buffer, 20·0 μl substrate, 100·0 μl DTNB (reagent), 50·0 μl enzyme. The results of several runs are shown in Fig. 1. The blank for such a run consists of buffer, substrate, and DTNB solutions. The absorbances‡ were read from the strip charts and plotted on a rectangular graph paper, the best line drawn through the points and the slope measured. In a run such as that described above, the linear portion of the curve describing the hydrolysis was observed during the first 15–20 min of the reaction; the slope is the rate in absorbance units/min. At this pH level, there is an appreciable non-enzymic hydrolysis of the substrate, and for long runs it was necessary to correct for this. The rate of non-enzymic hydrolysis of acetylthiocholine at 25° was 0·0016 absorbance units per min.

The procedures have been extended to micro-size. A run comparable to those in Fig. 1 was made in a micro-cell (total solution volume was 0·317 ml). The rate was 0·102/min, the same as that determined in the larger cuvettes.

Since the extinction coefficient of the yellow anion (II) is known,[5] the rates can be converted to absolute units, viz.:

$$\text{rate (moles/l. per min)} = \frac{\Delta \text{ absorbance/min}}{1{\cdot}36 \times 10^4}$$

In dealing with cell extracts or suspensions, a blank consisting of extract or suspension, DTNB, and buffer may be required to correct for release of thiol material from the cells and the absorbance of the other materials in the suspension.

Method for blood

A fairly stable suspension was formed from whole blood or washed human erythrocytes. Since the acetylcholinesterase is on the cell membrane, hemolysis was not necessary. The assay of blood was carried out as follows:

(1) A suspension of the blood cells§ in phosphate buffer (pH 8·0, 0·1 M) was prepared. The most practical dilution was 1 : 600 (e.g. 10μl blood into 6 ml buffer).

(2) Exactly 3·0 ml of the suspension were pipetted into a cuvette.

* California Corporation for Biochemical Research, Los Angeles, California.

† This is now available from the Aldrich Chemical Co., 2369 No. 29th, Milwaukee 10, Wisconsin.

‡ Strip charts printed in absorbance units are available from Minneapolis–Honeywell Corporation, Chart 5871.

§ Red cell counts were performed by the clinical laboratory.

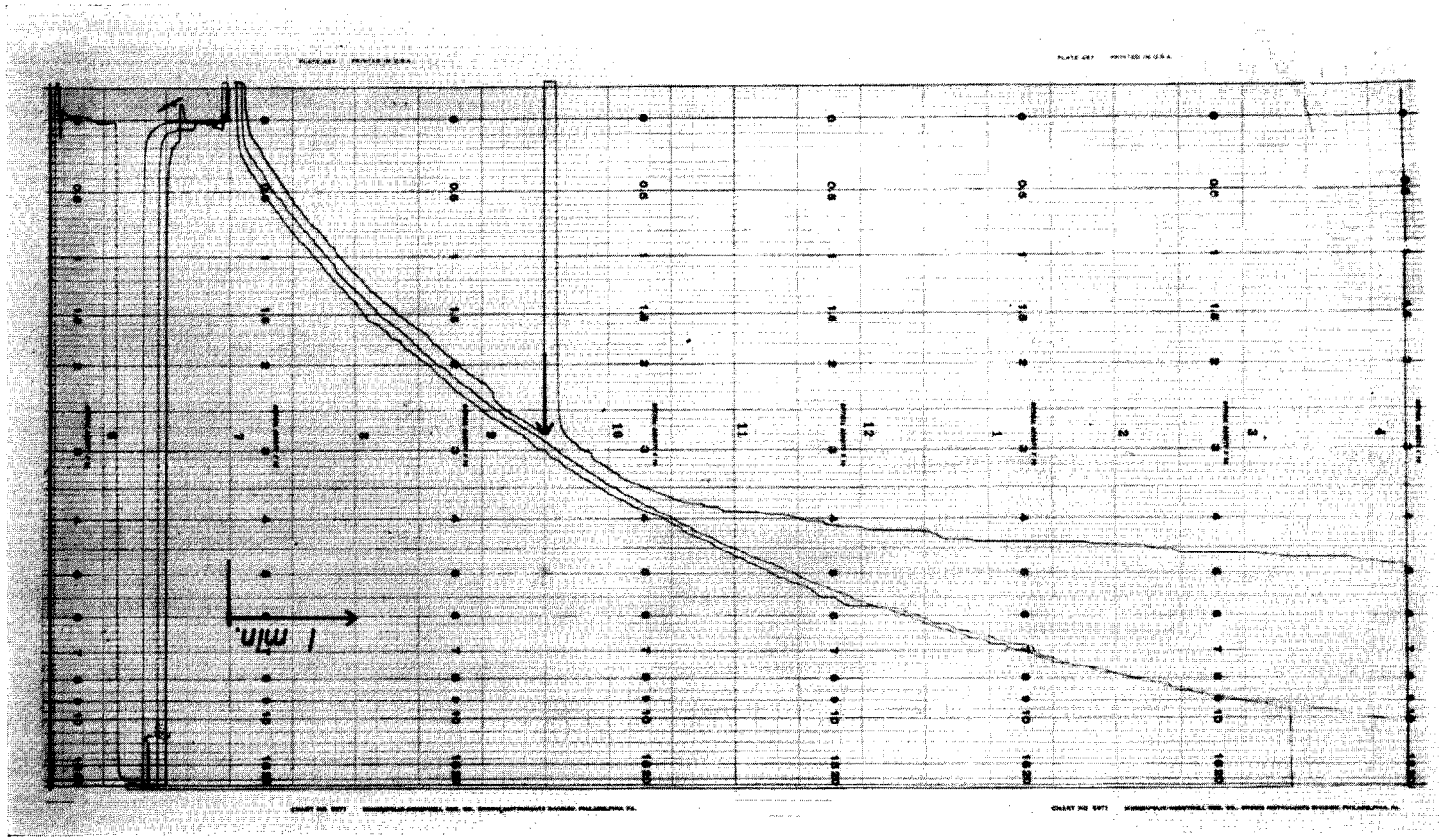

FIG. 1. Photograph of strip chart record of two identical assays. At the arrow, physostigmine salicylate (final concentration, 3×10^{-7} M) was added to a third replicate.

facing page 90

(3) If only red cell cholinesterase was desired, 10 μl of 0·1 per cent quinidine sulfate was added to inhibit plasma esterase.[8] If total esterase was desired, this step was omitted. See comment on quinidine in the discussion section.

(4) Of the DTNB reagent, 25 μl were added. The cuvette was placed in the photometer.

(5) The slit of the photometer was adjusted so that the absorbance (at 412 mμ) of the suspension in the cuvette was zero.

(6) Of the substrate, 20 μl were added to this cuvette. Changes in absorbance at 412 mμ were recorded for at least 6 min.

(7) Calculations:

$$\text{moles substrate hydrolyzed/min per R.B.C.} = (4{\cdot}41)\,(10^{-14})\,\frac{\Delta A}{\text{R.B.C.}}$$

where $4{\cdot}41\,(10^{-14})$ = factor for dilution, extinction coefficient, and changes in units;

$$= \frac{600}{13{,}600} \times \frac{1}{10^6\ \text{mm}^3/\text{l.}} \times \frac{1}{10^6\ \text{R.B.C.}};$$

ΔA = change in absorbance/min;
R.B.C. = red cell count (in millions per mm^3).

Other tissues

We have also assayed rat lung, liver, stomach, heart, muscle and brain by this procedure. The steps we used were these:

(1) The tissue was homogenized (approximately 20 mg of tissue per ml of phosphate buffer (pH 8·0, 0·1 M)) in a Potter–Elvehjem homogenizer. For muscular tissue, considerable mincing was necessary before homogenizing.

(2) A 0·4-ml aliquot of this homogenate was added to a cuvette containing 2·6 ml of phosphate buffer (pH 8·0, 0·1 M).

(3) Of the DTNB reagent, 100 μl were added to the photocell. The absorbance was measured at 412 mμ; when this had stopped increasing, the photometer slit was opened so that the absorbance was set to zero.

(4) Of the substrate, 20 μl were added. Changes in absorbance were recorded and the change in absorbance per min. was calculated.

(5) The rates were calculated as follows:

$$R = \frac{\Delta A}{1{\cdot}36\,(10^4)} \times \frac{1}{(400/3120)C_0} = 5{\cdot}74\,(10^{-4})\,\frac{\Delta A}{C_0}$$

where R = rate, in moles substrate hydrolyzed per min per g of tissue;
ΔA = change in absorbance per min;
C_0 = original concentration of tissue (mg/ml).

RESULTS AND DISCUSSION

Reproducibility

The results of repeated assays under various conditions are shown in Table 1. The standard deviations are about 4 per cent of the mean rates, a variability that could be accounted for as pipetting variation.

The rate of hydrolysis of acetylthiocholine by bovine erythrocyte esterase as a function of enzyme concentration is shown in Fig. 2. The unit of enzyme activity is that described by Ammon,[9] and is the amount which hydrolysed 0·01 mg of acetylcholine bromide in 1 min in 3 ml of 0·0092 M acetylcholine bromide. The observed rate was a linear function of enzyme concentration. The data were plotted in terms of the final concentration of enzyme.

TABLE 1. REPRODUCIBILITY OF RATE MEASUREMENTS

Substrate (moles/l.)	Enzyme (units)*	Mean rate†	Standard deviation	Replicates
$5{\cdot}0 \times 10^{-4}$	0·033	0·0402	0·0012	2
$5{\cdot}0 \times 10^{-4}$	0·083	0·1003	0·0038	9
$2{\cdot}5 \times 10^{-4}$	0·083	0·0842	0·0041	4

* Bovine erythrocyte esterase.
† Rate is given in change of absorbance/min. Other details are given under Procedures.

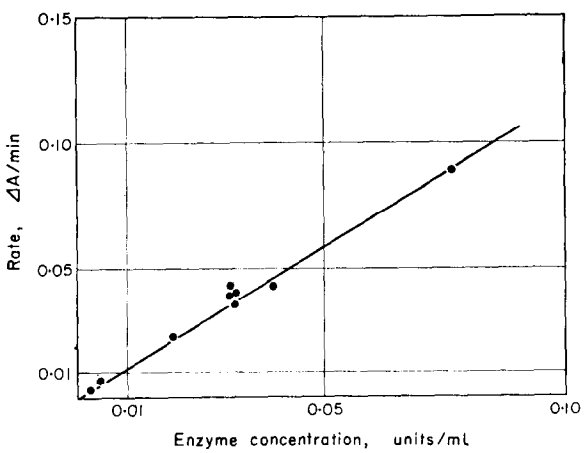

FIG. 2. Rate of thiocholine production as a function of enzyme concentration.

The rate of hydrolysis of acetylthiocholine by bovine erythrocyte cholinesterase as a function of substrate concentration is shown in Fig. 3. The substrate was hydrolysed at an appreciable rate without enzyme above 5×10^{-3} M. The rate plotted for the enzyme is thus the net rate after non-enzymic hydrolysis rate is subtracted from the observed rate. As expected for the "true" enzyme, substrate inhibition was observed, and the data in this experiment were analysed by a reaction sequence that includes this factor. The method of Wright and Sabine[10] was used to analyze these data. The results of the analysis, as well as inhibition constants (also evaluated by the method of Wright and Sabine), are shown in Table 2. The agreement with literature values is close enough that estimates of enzyme rates determined with acetylthiocholine may be considered good estimates of the rate for acetylcholine hydrolysis.

Twelve samples of human blood were assayed by our technique and the ferric hydroxamate method.[8] The comparison is shown in Fig. 4. It is apparent that the observed rates were directly proportional. Thus, the two assays were clearly measuring the same activity. We used quinidine sulfate (2×10^{-5} M, final concentration) to inhibit the plasma esterase.[8] Using the above technique with samples obtained from eighty-seven people, we obtained as the mean and standard deviation ($1{\cdot}08 \pm 0{\cdot}16$) $\times$ 10^{-15} moles substrate hydrolysed per min per red blood cell. Each assay took about 5 min. It is possible to determine at least fifty samples per day.

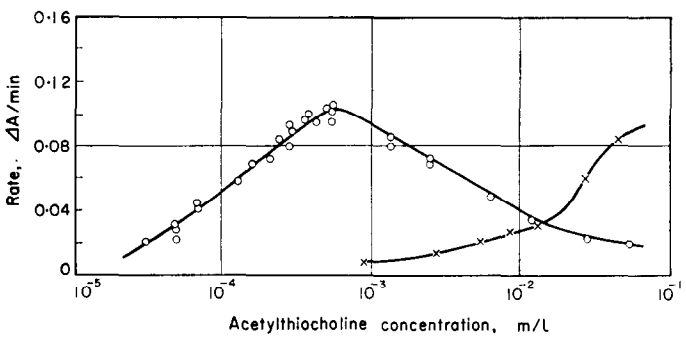

FIG. 3. Rate of thiocholine production as a function of substrate concentration; ○, enzymic ×, non-enzymic.

It should be noted that the concentration of quinidine used will not necessarily inhibit the non-specific esterase of all species or of all tissues (see e.g.[5]). It may, in fact, not be possible to inhibit all the non-specific esterase without inhibiting some of the acetylcholinesterase. It may be necessary, in the study of a given tissue to determine the appropriate concentration of a selective inhibitor (e.g. DFP), in order to use the method for determination of specific acetylcholinesterase.

TABLE 2. KINETIC CONSTANTS OF ERYTHROCYTE CHOLINESTERASE

Constant	Acetylthiocholine	Acetylcholine	Ref.
Michaelis (K_M)	$1{\cdot}4$ (10^{-4})	$2{\cdot}0$ (10^{-4})	10
Inhibition (K_I)			
Substrate	$2{\cdot}9$ (10^{-2})	$1{\cdot}5$ (10^{-2})	10
Decamethonium	$6{\cdot}1$ (10^{-5})	$2{\cdot}5$ (10^{-5})	12
Physostigmine	$2{\cdot}3$ (10^{-8})	$6{\cdot}1$ (10^{-8})	13
Quinidine	$8{\cdot}7$ (10^{-4})	$9{\cdot}6$ (10^{-4})	10

The activity of various rat tissues on acetyl- and butyryl-thiocholine was measured and results are presented in Table 3. These results were obtained in order to establish the applicability of the method, and are not intended to represent a study of these enzymes in these tissues. Since most of these tissues contain non-specific esterases (possibly thiolesterases), the non-cholinesterase hydrolysis might best be determined by incorporating the proper amount of physostigmine in an extra blank to be subtracted.[11]

The method described here is, in principle, similar to that of Gal and Roth.[7] However, we feel that our method has several advantages: (a) it is dependent on changes in the visible region of the spectrum, so that unusual changes in the absorbance can be checked immediately, e.g. appearance of turbidity, spills on the photocell windows, etc.; (b) since the extinction coefficient of the nitrothiobenzoate ion is 13,600, as against 5140 for acetylthiocholine chloride, there is at least a 2·5-fold potential increase in sensitivity available; (c) measurements of the appearance of products are usually more sensitive than disappearance of substrate; (d) homogenates of tissue do not require any special handling, i.e. precipitation of protein before readings, etc.; (e) the reagents required are commercially available. For these reasons, we believe that our method is more convenient than that of Gal and Roth.

TABLE 3. AVERAGE RATES OF HYDROLYSIS OF ACETYL- AND BUTYRYL-THIOCHOLINE BY RAT TISSUES

Tissue	Rates (moles/l. per min $\times 10^{-6}$ per g of tissue)	
	Acetyl	Butyryl
Lung	1·63	0·54
Liver	1·07	0·65
Muscle (thigh)	1·82	0·12
Kidney	0·28	0·21
Brain (whole)	10·31	0·31

The rates given are average values obtained from tissues of four Sprague–Dawley rats.

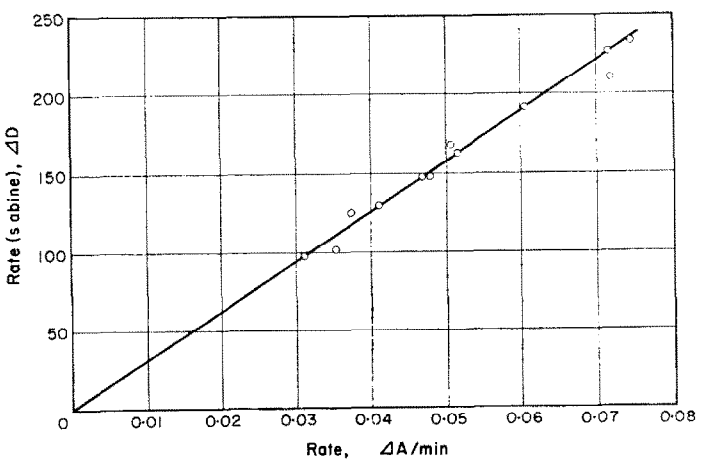

FIG. 4. Comparison of rates of R.B.C. cholinesterase obtained from twelve human R.B.C. samples when the present method and the Sabine[8] method were used.

The hydroxamate method[2] determines the residual substrate after a period of incubation. Hence, only one time period can be measured per analysis, and the execution of kinetic studies with this method becomes difficult. In addition, like the Gal–Roth technique, the disappearance of substrate is being measured. The acidimetric

and gasometric methods, while capable of being used to study kinetics, are inherently less sensitive, often requiring several hundred milligrams of tissue to be useful.

Acknowledgements—We gratefully acknowledge support from the U.S. Public Health Service, National Institutes of Health (Grant CY3861) and State of California Mental Hygiene Commission (Grant 60-1-1302). We wish to thank Dr. J. C. Sabine for her valuable advice and assistance.

REFERENCES

1. S. L. Bonting and R. M. Featherstone, *Arch. Biochem. Biophys.* **61**, 89 (1956).
2. S. Hestrin, *J. Biol. Chem.* **180**, 249 (1949).
3. M. Jones, R. M. Featherstone and S. L. Bonting, *J. Pharmacol.* **116**, 114 (1955).
4. G. B. Koelle, *J. Pharmacol.* **100**, 158 (1951).
5. G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
6. I. I. A. Tabachnik, *Biochim. Biophys. Acta* **21**, 580 (1956).
7. E. M. Gal and E. Roth, *Clin. Chim. Acta* **2**, 316 (1957).
8. J. C. Sabine, *Blood* **10**, 1132 (1955).
9. R. G. Ammon, *Pflüg. Arch. ges. Physiol.* **233**, 486 (1933).
10. C. I. Wright and J. C. Sabine, *J. Pharmacol.* **93**, 230 (1948).
11. G. B. Koelle, *J. Pharmacol.* **114**, 167 (1955).
12. F. Bergmann, I. B. Wilson and D. Nachmansohn, *Biochim. Biophys. Acta* **6**, 217 (1950).
13. D. Nachmansohn and I. M. Wilson, *Advanc. Enzymol.* **12**, 259 (1951).