AN ELECTROLYTIC TITRATOR METHOD FOR THE DETERMINATION OF ACETYLCHOLINE HYDROLASE ACTIVITY

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ABSTRACT

A rapid and sensitive method for measuring AChE activity is described. The instrumental design is a modification of the self-equilibrating electrolytic method for the determination of acid production rates by Einsel et al. (1956).

The acid produced by the enzymatic hydrolysis of an ester is titrated by means of internal electrogeneration of hydroxyl ion. The removal of hydrogen ions is accomplished automatically by a feed back mechanism. Experimental data is provided to show the validity of the system. After the elimination of any remaining technical problems the method will provide a most sensitive experimental approach to the study of reaction kinetics.

The sensitivity of the instrument is such that the production of three nanoequivalents of acid per minute may be easily detected. Sample sizes in the order of 0.6 to 10 µl of erythrocytes have been used.

The study of enzymatic activity as a function of temperature, pH and ionic strength can also be easily accomplished.

This technique is not limited to enzyme reactions but can be used to study the rate of hydrolysis of any ester. It can also be adapted to determine hydroxyl ion production rates.

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I. INTRODUCTION

The amount of attention which has been devoted to enzymes, since they were first discovered around the middle of the nineteenth century, could hardly be justified if the role of enzymes as simple catalysts were an end in itself. The fact that the action of one enzyme catalyzes a series of vital reactions for living organisms is the main reason why the interest of any scientist is aroused. Undoubtedly, the best experimental subject for man is himself. In studying any particular enzyme system man is eventually studying his own internal mechanism.

For a chemist, an enzyme system is challenging from a kinetic point of view, because of the speed at which enzyme catalysed reactions occur. The very large size of the enzyme molecules, the complexity of the entire system makes it difficult to resist. With the development of modern instruments and techniques, enzymes have become a little more accessible. However, the mode of action of any one enzyme, is still to be completely understood.

The present investigation will attempt to gain some information about the molecular nature of an extensively studied enzyme whose role, while understood in the nervous system, is still a matter of investigation in other organs.

A. General Principles on Acetylcholine Hydrolase

Acetylcholine hydrolase (3.1.1.7., I U P A C) or more commonly known as acetylcholinesterase (AChE), is an enzyme whose biological importance cannot be overestimated, because of its role in the transmission

of impulses in nerves and muscles. AChE catalyzes the following reaction:

Acetylcholine + Water $\rightleftharpoons$ Choline + Acetic acid

$$(CH_3)_3N^+CH_2CH_2OCOCH_3 + H_2O \rightleftharpoons (CH_3)_3N^+CH_2CH_2OH + CH_3COOH$$

A simplified picture of the nervous system will place this reaction in its proper perspective.

The nervous system has two major subdivisions:

A) the central nervous system, brain and spinal chord

B) the peripheral nervous system.

The peripheral nervous system is then divided into subsystems; (a) A system which delivers outgoing impulses from the central nervous system to voluntary muscles inducing appropriate movement in them. (b) A system which receives incoming impulses from external receptors and internal receptors so that the brain is kept informed of the stimuli from the outside world and of the condition of the body. (c) Two other systems which supply nerves to involuntary muscle systems and the glands. One of these two systems is known as the cholinergic system or, mediated by acetylcholine; the other, the adrenergic system or, mediated by adrenalin. All of these systems originate in the central nervous system. Messages to and from the brain travel and are delivered as nerve impulses by means of nerve cells. Nerve cells, or neurons, are fundamentally similar to other cells in the body. In addition, they possess structures which enable them to carry on the conduction of nerve impulses.

Consider a single unit from any of the acetylcholine mediated systems. When an impulse, originating in the brain, arrives by means of nerve cells at the junction between the neuron and the muscle (the neuromuscular junction) it releases a tiny "packet" of ACh which diffuses across the gap existing between the nerve cell and the muscle. The diffusion of

ACh stimulates either an organ effect or initiates events leading to a fresh impulse. If the ACh were allowed to persist, it would continue its stimulation and destroy the required proportionality between input stimulus and event. In practice, the ACh is destroyed very promptly by AChE, an enzyme which is present in large excess in all conductive organs. If the AChE should be prevented from acting, ACh will accumulate, causing at first an excessive stimulation, and finally complete disruption of the system. The result of inhibiting AChE will thus be: (a) interference with the neuromuscular junction, giving rise to rapid twitching of voluntary muscles; (b) interference with the system innervating involuntary muscle systems at the sites mediated by ACh, thus giving rise to lack of co-ordination of muscular responses. This is of particular importance in the respiratory system.

This brief description of the structure and function of the nervous system, demonstrates that the release and removal of ACh, are necessary processes in the chain of reactions which generate and conduct the nervous impulse. AChE is found in all conductive tissues (nerve and muscles) and is located in the neuronal surface where bioelectric phenomena occur. The speed at which the hydrolysis of ACh by AChE may occur is perhaps the most outstanding feature of this enzyme system when in connection with electrical manifestations. One molecule of enzyme may split one molecule of substrate, namely ACh, in three to four microseconds.

The toxicity of organophosphates is due to their action upon AChE. For instance, di-isopropyl-fluorophosphate (DFP), a potent nerve gas, is an irreversible inhibitor of AChE. The toxicity of a great number of organic phosphates is a function of their effectiveness as AChE inhibitors (O'Brian, 1960).

B. Preparation and Purification

The task of extracting and purifying AChE is a major one. As with any enzyme, the low concentration of AChE per gram of tissue and the difficulty in getting the enzyme in solution are two major factors responsible for the results obtained for AChE isolation. Acetylcholinesterase can be found in all conductive tissues and erythrocytes; it has well-defined properties and it is unmistakably different from other cholinesterases.

A relatively high concentration of the enzyme is found in the brain of mammals, squid head ganglia, the electric organs of the electric eel and red blood cells. Among the suitable species are the "Electrophorus Electricus" and the various "Torpedo Species". The association between AChE and electrical phenomena has been explained at least in part, though the role of this enzyme in the blood is still unknown (Nachmansohn & Wilson, 1955).

The purification of AChE is favoured, to a certain extent, by its great stability. This enzyme may be kept frozen for years without loss of activity (Nachmansohn & Wilson 1955). A number of methods are available describing the preparation and purification of AChE. The best results have been obtained using the electric organ of the electric eel as a source. One reason for this might be the relatively high concentration of the enzyme per gram of tissue as compared with the concentration in the erythrocytes. Also, there is the possibility, that the enzyme in the electric eel is not so firmly bound to the membrane of the cell as it is to the erythrocyte membrane.

A procedure for the purification of AChE was first described by Rothenberg and Nachmansohn (1947). Using the electric organ of the electric eel, they obtained, by a series of fractionations with ammonium sulfate, a preparation with a specific activity of 2.2×10^3 units per mg

TABLE I

Differences between Acetylcholinesterase and Pseudocholinesterase

	Acetylcholinesterase	Pseudocholinesterase
Substrate most rapidly hydrolyzed	Acetylcholine	Propionyl and butyrylcholine
Will not hydrolyze	Most noncholine esters, butyrylcholine, benzoylcholine	Acetyl-methyl choline
Excess substrate effect	Inhibited	Non inhibited
Main source	Erythrocytes nervous tissue	Serum
Alternative names	Specific cholinesterase True " e- " Acetyl " Aceto " Cholinesterase II	Non specific cholines Pseudo " s- " Cholinesterase Cholinesterase II Serum Cholinesterase

(O'Brian, 1966)

of protein, where one unit is defined as that amount which will catalyze the transformation of one micromole of acetylcholine per minute under specified pH, temperature, ionic strength and substrate concentration (Dixon & Webb, 1964). When the preparation of Rothenberg and Nachmansohn was ultracentrifuged, a deposit with a specific activity of about 6.7×10^3 units per mg of protein was obtained. The authors considered this preparation pure.

In 1953, Cohen and Warringa published a method for preparing a soluble AChE preparation from ox red cells; the specific activity of this preparation was reported as 5.9×10^3 units per mg of protein. In 1955, the same authors published a method which yields preparations with a specific activity of about 5.9×10^4 units per mg of protein or ten times greater than that of their previous method.

Zittle and Della Monica (1954) developed a procedure in which the important step was the solubilization of the esterase in the red cell stroma by the use of the neutral surface active agent polyoxyethylene sorbitan monolaurate (Tween 20). The final product had an activity of 1.1×10^3 units per mg of protein, 20% recovery of the initial activity, and 250—fold purification with respect to the starting material.

Lawler (1959), whose enzyme source was the electric organ, used a modified and more reproducible procedure for purifying AChE. He obtained a preparation of only 50-170 units per mg of protein.

More recently, Hargreaves (1961), using the electric organ of the electric eel, described a purification procedure that employs adsorption on tricalcium phosphate gel and diethylaminoethyl cellulose. However, the specific activity of his preparation was lower than that obtained by Rothenberg and Nachmansohn or by Lawler.

Kremzner and Wilson (1963), using the electric organ of the electric eel as starting material, described a chromatographic method which produces a relatively pure and stable enzyme preparation in good yield.

Gordon and Rutland (1967) succeeded in solubilizing bovine erythrocytes AChE by ultrasonic vibration. Evidence is reported to prove that the enzyme is really in solution.

In 1967, the results of a large scale purification of AChE from the electric organ of the electric eel were reported. Leuzinger and Baker claimed to have developed a readily reproducible method. The preparation obtained appears to be a homogeneous protein, as tested by disk electrophoresis and high speed centrifugation. The preparation has a specific activity of 12.3×10^4 units per mg of protein. This is by far the most active preparation. The molecular weight was estimated to be in the order of 2.5×10^5. This value is in good agreement with the values obtained previously by Lawler and Kremzner & Wilson. In 1968, Leuzinger and Baker described the growth of AChE crystals using the enzyme preparation previously reported.

It is evident, from a very brief study of the data reported in the literature, that a great deal of work is still required on the extraction and purification of AChE. When faced with the prospect of adopting a method for starting the protein extraction, there was obviously very little choice. Since the source was to be erythrocytes, by far the best available procedure seemed to be that of Cohen and Warringa (1953). The more recent procedure could not be used due to the unavailability of a pancreatic extract (trade name: Cotazym) which would be responsible for breaking down the lipid materials present on the erythrocyte membranes. The 1953 procedure was followed with changes being made only in the preparation of the stromata. Later, when the "Cotazym" finally

became available, work was started on the more recent method. Half-way through the procedure, it became obvious that the "Cotazym" was not only breaking down lipids but proteins as well. The breaking down of the protein was due to the presence of trypsin.

Another attempt was then made using the method of Leuzinger and Baker, even though the starting material was different. This time the work did not advance very far. Now the need for a measure of the enzyme activity after each step was greater. An entirely new procedure was being adapted for erythrocytes and it was very difficult to know exactly where the enzyme would end up after each major step. The search for a new method to measure enzyme activity began; this method had to be quick, accurate and use only a very small enzyme sample.

C. Assay Methods

1. General Principles

The rate of hydrolysis of acetylcholine is a direct measure of the purity and/or the amount of AChE present in a given enzyme preparation. The determination of the activity of AChE, like all measures of enzymatic activity, consists in measuring the amount of substrate (acetylcholine or some other choline ester) hydrolyzed as a function of time by a known amount of enzyme. The medium for the measurement of AChE activity should contain at least 0.01M $MgCl_2$. Although purified enzyme does not require Mg^{2+}, impure preparations are frequently greatly activated by Mg^{2+} ions (Nachmansohn & Wilson, 1955). A suitable medium contains 0.1M NaCl and 0.04M $MgCl_2$, in addition to any buffer required. The optimum substrate concentration is between 0.004M and 0.007M. The optimum pH is between 7.5 and 9.0 (Wilson & Bergman, 1950).

The activity of AChE may be determined using three basic methods: (1) the determination of unreacted substrate, (2) the determination of choline or thiocholine produced from the hydrolysis of choline esters, (3) the determination of the acid produced from the hydrolysis of choline esters.

Before going into the details of individual methods, it is necessary to specify exactly the requirements of a method for the particular work in progress. During the purification of the enzyme, a need arose for a method which would be sensitive to micro quantities of enzyme. This limits the reaction volume, and five ml was set as the most suitable volume. The method was not to be time consuming in terms of operator's time. As 1-15 or more samples had to be assayed daily, the method should not require any involved standardizing procedures. A solution needed in connection with a run had to be stable for a reasonable length of time. The involved experimental procedures of some methods should be avoided. The units of the method should be readily convertible into standard units. Once the purification was completed, the same technique was to be used to study rates of reaction. To be precise, initial rates of reactions were to be measured; therefore, a rapid response mechanism for detection purposes has to be used. The method was not to be unduly limited by pH, ionic strength of the medium, and types of buffers. Temperature control also became necessary due to possible wide fluctuations in room temperature.

2. Methods Based Upon Chemical Determination of Unreacted Acetylcholine

a) Colorimetric Method

The colorimetric method, developed by Hestrin (1949), was primarily described for the estimation of acetylcholine synthesis by choline acetylase. The method has also proved convenient for the estimation of the rate of hydrolysis of ACh by AChE. It is also suited to the determination

of ester synthesis in the presence of an excess of products of ester hydrolysis. The method is based on the interaction of ACh with hydroxylamine. Hydroxylamine, at an alkaline pH in water, rapidly converts ACh stoichiometrically to acethydroxamic acid throughout a wide range of ester concentration. Hydroxamic acids give a red purple colour with ferric ion. The intensity of the colour at 520 to 540 mμ is then easily related to the rate of disappearance of acetylcholine. The colorimetric method is very convenient because, unlike manometric methods, it is not limited by a fixed pH or bicarbonate buffer system. It is, therefore, very impractical for routine assays of AChE samples. First, the colour of the ferric-acethydroxamic acid complex fades on standing. Second, one of the reagents is fairly unstable; it must be freshly prepared and used within three hours. This method is probably more convenient than some titration methods. However, it is less accurate, since it measures the remaining ester, and the activity is obtained by difference. For our own needs this would be a most detrimental factor. It was mentioned earlier that we were only interested in initial rates of reaction. The velocity is only linear very early in the reaction. It would, therefore, be practically impossible to determine small changes in substrate concentration when the total concentration of substrate is large.

b) Electrochemical Method

An ion selective electrode was used by Baum (1970) to determine the activity of AChE. A continuous determination of the rate of change of substrate concentration in the presence of active enzyme is made, using a liquid membrane electrode which has a high selectivity for acetylcholine over choline. The results obtained using this new procedure, have been compared with the spectrophotometric method of Hestrin. It appears that further work is needed with this technique to increase the precision of

the results.

3. Methods Based Upon Choline (Thiocholine) Production

a) Colorimetric Methods

Although methods based upon choline production have been developed, their accuracy is so low that they are of no practical use. The use of thiocholine esters has improved results, since the free SH group may be determined by various techniques.

The thioester analog of choline, acetylthiocholine, was first used by Koelle in 1949 as an artificial substrate for AChE in histochemical studies. In practice, a tissue preparation is incubated for 10 to 60 minutes in a medium containing copper glycinate saturated with copper thiocholine in a substrate of acetylthiocholine. After this treatment, the tissue sections are rinsed with saturated copper thiocholine solution, and then immersed in an ammonium sulfide solution. The latter converts the white precipitate of copper thiocholine to a dark brown deposit of copper sulfide. Koelle improved the initial methods when serious difficulties were encountered with other tissues. Another method, published in 1956 by McOsker and Daniel, was a micromethod in which the production of the sulfhydryl groups, as determined by the nitroprusside reaction, served as a measure of enzyme activity. This method compares well with manometric methods but is still unsatisfactory in many respects. The results have been found to vary with pH and are best in the acid range where the enzyme activity is markedly poorer. The types of tissues used also affects the pH dependance. Up to the present time no quantitative evaluation of this technique has been attempted.

Ellman, in 1962, also used acetylthiocholine to measure the activity of AChE. This method examines at 540 mμ a colour product of the

reaction between the SH group of thiocholine and 5,5-dithiobis-2-nitrobenzoate. A major drawback of this method is that, in the assay of biological preparations, other colored substances absorb at the wavelength employed, and may interfere.

b) Polarographic Methods

In 1962, Fiserova-Bergerova published some kinetic data associated with the use of polarography to follow the enzyme catalyzed hydrolysis of acetylthiocholine. Thiocholine gives an anodic polarographic wave typical of SH substances, while acetylthiocholine is polarographically inactive. The reaction rate of the enzymatic hydrolysis of acetylthiocholine is then directly related to the increase in the anodic wave of thiocholine. In 1964, Fiserova-Bergerova described a most useful clinical application for this technique. A year later, Ridgway and Mark determined the validity of the polarographic method, by comparing the rate of production of thiocholine obtained polarographically with the results obtained spectrophotometrically, by Ellman (1962). In addition, they also reported kinetic data for the inhibition reaction with neostigmine, which compared well with the values reported in the literature. The polarographic method is accurate and simple, its main advantage being that it can follow moderately rapid reactions. In fact, data can be obtained ten seconds after mixing of the solutions.

At the time of our investigation to find a good method for measuring the activity of AChE preparations, the polarographic method seemed to be the answer. Attempts were made to duplicate Fiserova-Bergerova's results as published in 1964. Unfortunately, all efforts failed, due to the difficulty in securing an anodic polarographic wave for the glutathione, used as a standard in place of thiocholine. Thiocholine is not commercially

available. The amount of time devoted to this problem was probably not sufficient. In fact, at a later date, the results of the 1964 publication were duplicated by Dick and Zinger, (unpublished data). This method would have been an ideal answer to the needs of the present research. However, by the time the results were duplicated, another method had already been taken up.

4. Methods Based Upon Acid Production From Choline Esters

Of the methods which measure the activity of AChE by determining the amount of acetic acid produced by the hydrolysis of ACh, the manometric and the titrimetric methods are, by far, the most common methods. It is only since 1960 that new methods, using polarography, radiometry and nuclear magnetic resonance as instrumental techniques, have been developed.

a) Manometric Methods

First developed by Ammon, (1933), this method is very common, due to its sensitivity and the fact that it allows determinations to be conducted in the presence of very low quantities of substrate (0.0006M ACh). The reaction takes place in a bicarbonate-CO_2 buffered solution in a Warburg flask. The method involves the measurement of the amount of carbon dioxide evolved due to the action of the acetic acid, liberated during the hydrolysis, on the bicarbonate buffer. This method is limited by a fixed pH. The greatest draw-back of this technique is in terms of the operator's time needed. In addition, the equipment needed is not readily available in most laboratories.

b) Radiometric Methods

In 1962, Winteringham and Disney first published a radiometric assay method for AChE. Their method involved the incubation of blood and tissues with C^{14}-acetylcholine and the measurement of unhydrolyzed substrate

with a Geiger-Muller tube after removal of volatile C^{14}-acetate in a vacuum desiccator. Reed et al., in 1966, used the same basic procedure, but they measured the reaction product, acetate, after removal of the substrate with an ion exchange resin. Enzyme samples as low as 0.1mg of brain tissue were used by Potter, in a modified procedure published in 1966. The most significant change in this new procedure is the fact that the labelled acetate, produced by the enzymatic hydrolysis of ACh, was extracted into an organic solvent and was counted by liquid scintillation spectrometry. In general, the radiometric method offers little advantage over existing methods for the study of AChE in micro quantities. There are serious limitations to the method, such as the obvious inconvenience for routine determinations of using radioactive materials. The procedures outlined are also very involved and time consuming.

c) Nuclear Magnetic Resonance Methods

The technique of nuclear magnetic resonance (NMR) spectroscopy was used in 1968 by Kato to study the hydrolysis of acetylcholine by horse serum cholinesterase. Since the liberation of acetate is a measure of the amount of hydrolysis of ACh, then the changes in acetate peaks can be related to enzyme activity. There is a distinct change in the chemical shift, in the NMR spectrum, of the acetate protons formed during the hydrolysis reaction from the acetate protons of acetylcholine. This method makes possible the continuous observation of the reaction; the rate constants obtained with this technique compare well with the values reported in the literature.

d) Titrimetric and Change in pH Methods

Of all the methods ever employed to measure the activity of AChE, these methods have been most widely used, since they are, as a whole,

relatively simple, precise and accurate. The acetic acid formed by the hydrolysis is titrated with standardized alkali, little or no buffer being required; or the change in pH is directly related to the amount of acetic acid produced, again little buffering action being required. There are at present, various procedures available, which are only slight variations of the two basic types.

The indicator method is basically a volumetric titration method in which neutralization of the acid takes place in the presence of a colored indicator. The original method goes back to Easson and Stedman, who, in 1937, measured the activity of AChE by titrating, with 0.02N NaOH, the amount of acid formed over a period of twenty minutes. Bromthymol blue, phenol red and cresol red have been used as indicators in the several modifications of this technique. The amount of hydrolysis has been found to be directly proportional to time.

In the change in color of an indicator method, the variation of color of a buffered solution is studied as a function of time, in the presence of an indicator, and in a very short time interval. The original method goes back to Croxatto et al. (1939). They used m-nitrophenol as indicator, and measurements were made photometrically. Lowry et al. described a similar method in 1954; they used small amounts of brain, five to ten μg, as enzyme source. Phenol red has been also used as an indicator in conjunction with a barbital buffer. This method has a limited range of activity. However, the greatest difficulty lies in the choice of a suitable indicator, whose color change must occur at a pH very close to the pKa of the buffering system.

Two general types of methods, employing the glass electrode, have been developed. The principle of the first method consists in following the neutralization of the acid formed with a pH meter, over a fixed

time, or in a given pH interval. At about the same time as Easson & Stedman, Glick, and Alles & Hawes employed this technique for the determination of AChE activity. They measured the rate of formation of acetic acid, from the catalyzed hydrolysis of ACh, by titrating with dilute alkali to a constant pH.

In the second method, developed by Michel in 1949, the enzymatic hydrolysis of ACh is allowed to occur in a standard buffer. The decrease in the pH of the buffer caused by the acetic acid formation serves as a measure of enzymatic activity. These last two methods are particularly useful for non-routine analysis of a small number of samples, because individual analysis can be accomplished with ease.

In general, these methods are simple, and conditions for an optimum pH are easily controlled. However, volumetric analyses are always very "tricky" in the case of an organic acid, since the color change at the end point is far from being clearly defined. Although the electrometric methods have been modified, they are still somewhat lacking in precision and sensitivity. All these methods, also call for the addition of foreign substances, which undoubtedly interfere with the enzyme reaction.

The ideal method would involve measurement of the effects of the interaction of enzyme and substrate, in a solution of these two constituents, by a process which would introduce no foreign substances. The electrolytic method seems at this point to be the ideal answer.

In the electrolytic titration method, a constant pH is maintained by removing hydrogen ions electrolytically, at the same rate as they are formed by the enzyme reaction. This method does not utilize buffers, is temperature and atmospherically controlled, has easily calculated units which are interconvertible with most other methods and is rapid and simple to run. In addition, the substrate and enzyme concentrations

can be adjusted and maintained at levels which allow optimal enzyme activity.

The electrolytic titration method is, essentially, controlled current coulometry, with a few variations, to adjust to the enzymatic reaction, and also, to enable the study of the kinetics of the reaction. The coulometric generation of reactants is one of the most sensitive and accurate methods for the micro determination of a substance in solution. It is also a most practical method, since there is no need for standardization of reactants, and no volume increase on addition of the titrant or reactant.

The principle of the method is that the quantity of electrolysis product formed at each electrode is governed by Faraday's law. Several methods of analysis based on this principle have been described. In 1938, Szebelledy and Somogyi reported a method for coulometric analysis in which a constant current, passed through a cell by means of platinum electrodes, produced electrolysis products which reacted with the material to be analyzed. The color change of an indicator was used for the detection of the end point, and the quantity of electricity was determined from the deposition of silver, in a silver weight coulometer, which had been connected in series with the electrolysis cell. This method, even though it is accurate and precise, had the disadvantage of being slow, and not suitable for small quantities. Furthermore, indicators had to be selected which were not destroyed by electrolysis. In 1947, Epstein et al. described a modification of Szebelledy and Somogyi's method. They determined the end point potentiometrically. The advantage of this method over the original one is in the fact that the quantity of electricity was determined by measuring the electrolysis time by means of a stopwatch, and the current by a milliammeter.

In 1949, Lingane used a feed-back system to maintain the potential of a working electrode constant during electrolysis. The instrument could be used as the controlling element in automatic potentiometric titrations, by means of electrolysis, at constant total applied voltage, or with constant current. The instrument was also suited for coulometric titrations, with an electrolytically generated titrant, according to the techniques of Epstein et al. (1947).

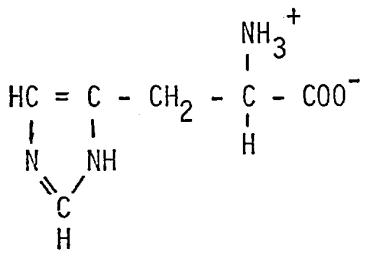
In 1956, Farrington and Sawyer applied the automatic coulometric titration technique to determine the rate of hydrolysis of aliphatic esters. Liberti and Ciavatta, also in 1956, studied the rate of hydrolysis of several alkyl esters with a similar set up. The first practical application of this principle, to the study of the reaction kinetics of an enzyme system, also appeared in 1956, due to the work of Einsel et al. Their method was designed to measure pseudo-zero order reactions. Under normal conditions, the enzymatic hydrolysis of AChE causes a decrease in the pH of the solution as the reaction proceeds. However, if the acid formed is neutralized electrolytically, at the same rate as it is formed, the pH of the solution remains constant. The reaction is then "pseudo" zero order when the concentration of ACh is in excess. The principal features of the method of Einsel et al. were used in the present investigation, in an attempt to study the enzymatic reaction on a much more sensitive scale.

D. Polarographic Determination of Serine and Histidine

As the final object of the present research was the study of DFP inhibited reactions, which would eventually provide a greater insight into the nature of the active sites on the AChE molecule, an attempt was

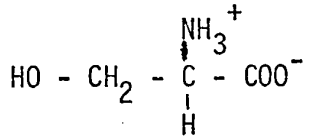
made at detecting serine and histidine polarographically. It has been previously mentioned that at one time, during the course of the present investigation, the prospect of using polarography, as a technique to study rates of reactions, seemed a good one. Unfortunately, our efforts failed. However, the little information which was obtained with serine, might be worth mentioning.

The behaviour of the active site of AChE has been analyzed in terms of two sub-sites. One site seems to be largely concerned with specificity, while the other is concerned with the hydrolytic process. Of course, the elucidation of the exact chemical identity of the hydrolytic group would be invaluable. It was established by Wilson (1950) that organophosphate inhibition of AChE involves phosphorylation. Evidence from different sources suggest that the primary phosphorylation is of histidine:



The first suggestion that the phosphorylation site might be an imidazole group was made on the basis of finding, from kinetic measurements, a pKa of 6.5 for the active site (Bergmann et al., 1950). The imidazole group was also found to have very weak catalytic properties with easily hydrolyzed substrates. One other observation does not fit the imidazole hypothesis; enzymes which are inhibited with alkyl phosphates consistently yield phosphorylserine. It seems, then, that serine might also be involved

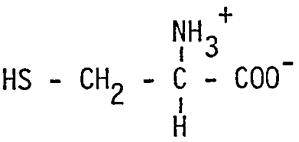
at the active site (Schaffer et al., 1953).



The evidence, at the present time, does not allow any conclusions to be drawn. The exact chemical nature of the active sites of AChE is still a matter for investigation.

Polarography has been successfully applied in following the hydrolysis of acetylthiocholine in the presence of AChE (Fiserova-Bergerova, 1962). If serine and histidine could be detected individually, using polarography, then, maybe their presence in the intact protein could be investigated by carefully planned reactions with DFP.

By analogy with cysteine, whose side chain contains a sulfydryl group



which has been successfully detected polarographically, the hydroxyl group of serine could probably be detected (Kolthoff and Barnum, 1940, 1941). It would be of little use to obtain a polarographic wave for the amino acid groups, because these groups will not be free in the intact protein. On the other hand, the hydroxyl group in serine, and the imidazole ring in histidine, could be reactive groups on the enzyme and, therefore, the determination of their presence and reactivity would be of interest. These groups must also be detected in very low quantities, because the concentration of the enzyme will eventually be very low, and serine and histidine

are but a small percent of the total amino acids in the protein.

Alcohol groups have been detected polarographically. However, it is difficult due to the extremely negative voltages needed. In all these cases it is reported that a water free medium must be used; this is hardly possible in the case of amino acids which are only slightly soluble in anything other than water. Even if amino acids could be detected in a solvent other than water, it is unthinkable to exclude water completely since the object is eventually to study the enzymatic reaction in as normal a physiological environment as possible. The information gathered using serine is reported but no serious attempts were made with histidine as this particular point did not warrant extensive investigation at the time.

II. RESEARCH OBJECTIVE

The present work is part of a major project whose aim is as follows :

To elucidate the mode of action of AChE by studying the interaction between AChE and DFP in the presence of substrate. Such a study requires first, a reliable source of pure enzyme, and second, a method for studying reaction rates. The minimum purity of the enzyme preparation is to be suitable for kinetic studies, i.e. no other cholinesterases present and also no compounds interfering with DFP binding or interfering with the analytical procedures. The ideal would be a crystalline enzyme preparation electrophoretically and ultracentrifugally pure. As no commercial enzyme preparation fulfils these requirements, a possible starting point would be the extraction of the enzyme followed by the purification of the preparation. However, a review of all the available methods for measuring AChE in a given sample indicated that none of these methods would be suitable for both a study of rates of reaction and following the progress in the purification. It was then decided to make the development of a suitable method the object of this work.

III. MATERIALS AND METHODS

A. Preparation and Purification of Acetylcholine Hydrolase from Human Erythrocytes

The only deviation from Cohen & Warringa (1953) is in the preparation of the stromata (Roelofsen et al., 1964). The erythrocytes were washed three times with an isotonic solution of NaCl, 0.9% by weight. Hemolysis was effected by dilution with 20 volumes of distilled water saturated with CO_2 at a temperature of 5°. After storage overnight, two layers were formed: the bottom layer consisting of the hemoglobin free cells and the top layer consisting of hemoglobin. The top layer was then removed by aspiration. Traces of hemoglobin adhering to the stromata were removed by washing with water, saturated with CO_2 at room temperature, until the water remained colorless. Normally two liters of erythrocytes were used as starting material and an average of eight washings was needed to remove all of the hemoglobin. After each washing the stromata were allowed to settle overnight. The method of Cohen & Warringa (1953) was used from hereon.

Electrophoresis runs were made on the final AChE preparations. The procedure was essentially that outlined in the Beckman manual. The membranes were scanned using a Densicord according to the procedure given in the instruction manual.

All chemicals used in these preparations were A.C.S. reagent grade.

Small amounts of materials were centrifuged in a cold room using a Servall superspeed centrifuge Model SS-1.

Large amounts of materials were centrifuged using the refrigerated centrifuge Model PR-6.

Phosphate buffers were prepared according to the method described by Gomori (1955).

A vertis Manifold Style Freeze-Dryer was used for all freeze drying procedures.

B. Determination of the Activity of Acetylcholine Hydrolase Using an Automatic Electrolytic Titrator.

The coulometric generation of chemical reagents constitutes one of the most sensitive and accurate methods for the microdetermination of a substance in solution. This is exactly the principle on which the present method, for measuring AChE activity, is based. Modifications have been made to the design of Einsel et al. (1956) to adjust to the particular needs of the research in progress.

As the limiting reactions in sodium chloride solutions at platinum electrodes are :

$$H_2O + e^- \rightarrow 1/2H_2 + OH^- \text{ cathode}$$

$$Cl^- - e^- \rightarrow 1/2\ Cl_2 \text{ (gas) anode}$$

acids can be titrated by cathodic electrogeneration of hydroxide ion. The electrolyte used in the present work was 0.5 M NaCl. The auxiliary Pt electrode is in the anode compartment which is separated from the cathode compartment by means of a 'Visking' semi-permeable dialysis membrane. Hydrogen ions and acetate ions will be produced in the cathode compartment due to the ester hydrolysis. The hydrogen ions will be neutralized by the electrically generated hydroxyl ions. Charge flow through the semi-permeable membrane may be chloride or acetate ion from the cathode

to the anode compartment, with the higher mobility of chloride ion favouring this as the transfer ion. The sodium ion concentration will be the same in both compartments and as such there will be no unbalanced movement of these ions across the membrane.

The pH electrode is in the cathode compartment where the chemical generation of the acid occurs. An initial shift in pH, due to the production of acid from the hydrolysis reaction, activates the mechanism by which an increasing voltage is applied between the two electrodes. Electrolysis occurs with the resulting generation of hydroxide ions in the cathode compartment. This allows the gradual removal of hydrogen ions until such a time when the amount of hydroxide ions produced is exactly equal to the amount of hydrogen ions produced. At this point the pH stays constant and the current in this steady state condition is directly proportional to the hydroxide ions produced or the hydrogen ions produced by the hydrolysis of ACh or any other ester.

A block diagram of the experimental equipment is shown in Fig.1. A separate diagram is given for the cell in order to show more details in Fig. 2. The electrolytic cell was glass - blown into two cylindrical compartments connected laterally by a ground glass joint. It was thermostated by means of an outer jacket with water from a constant temperature bath. The cell openings were sealed with paraffin, the tubes and the electrodes were introduced by means of holes through the paraffin. The anode compartment of the cell had the platinum electrode for electrolysis plus two polyethylene tubes: one used as nitrogen gas inlet, the other as outlet. The outlet tube was also used for the injection of material into the cell. Stirring in the anode compartment was by the stream of nitrogen flowing through the liquid. The cathode compartment had, in addition to

Figure 1

Block diagram of automatic electrolytic titrator

R_1 10,000 ohms, 0.5 watt

R_2 Helipot, 10-turn, 1000 ohms

R_3 Potentiometer, 10,000 ohms, 3 turns

R_4 20,000 ohms, 5 watts

R_5 100,000 ohms, 0.5 watt

M_1 Multirange milliammeter 0-50, 0-150 μa

M_2 Milliammeter, 15 ma

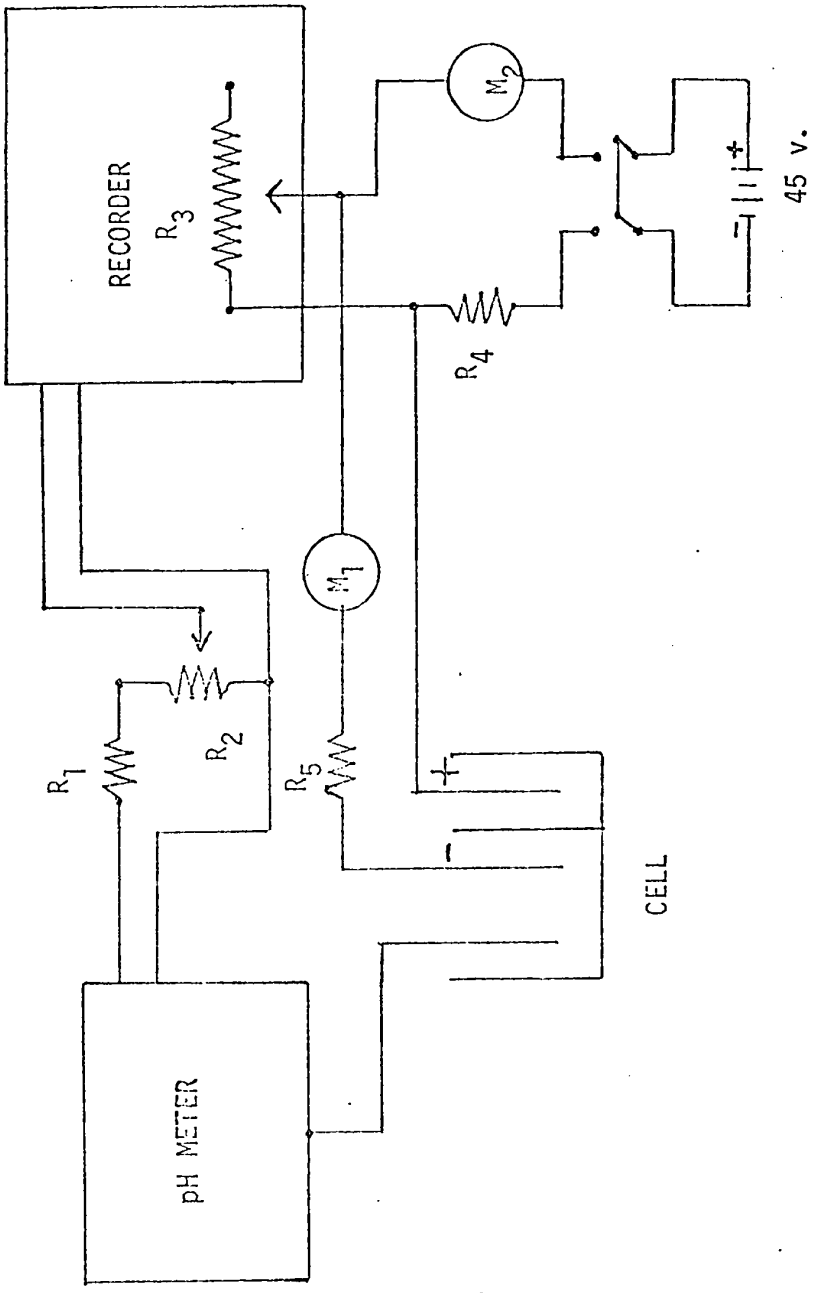


Figure 1

Block diagram of automatic electrolytic titrator

Figure 2

Electrolytic Cell

A. Nitrogen gas inlet

B. Platinum electrodes

C. pH Combination electrode

D. Nitrogen gas outlet

E. Stirrer

F. Semipermeable membrane

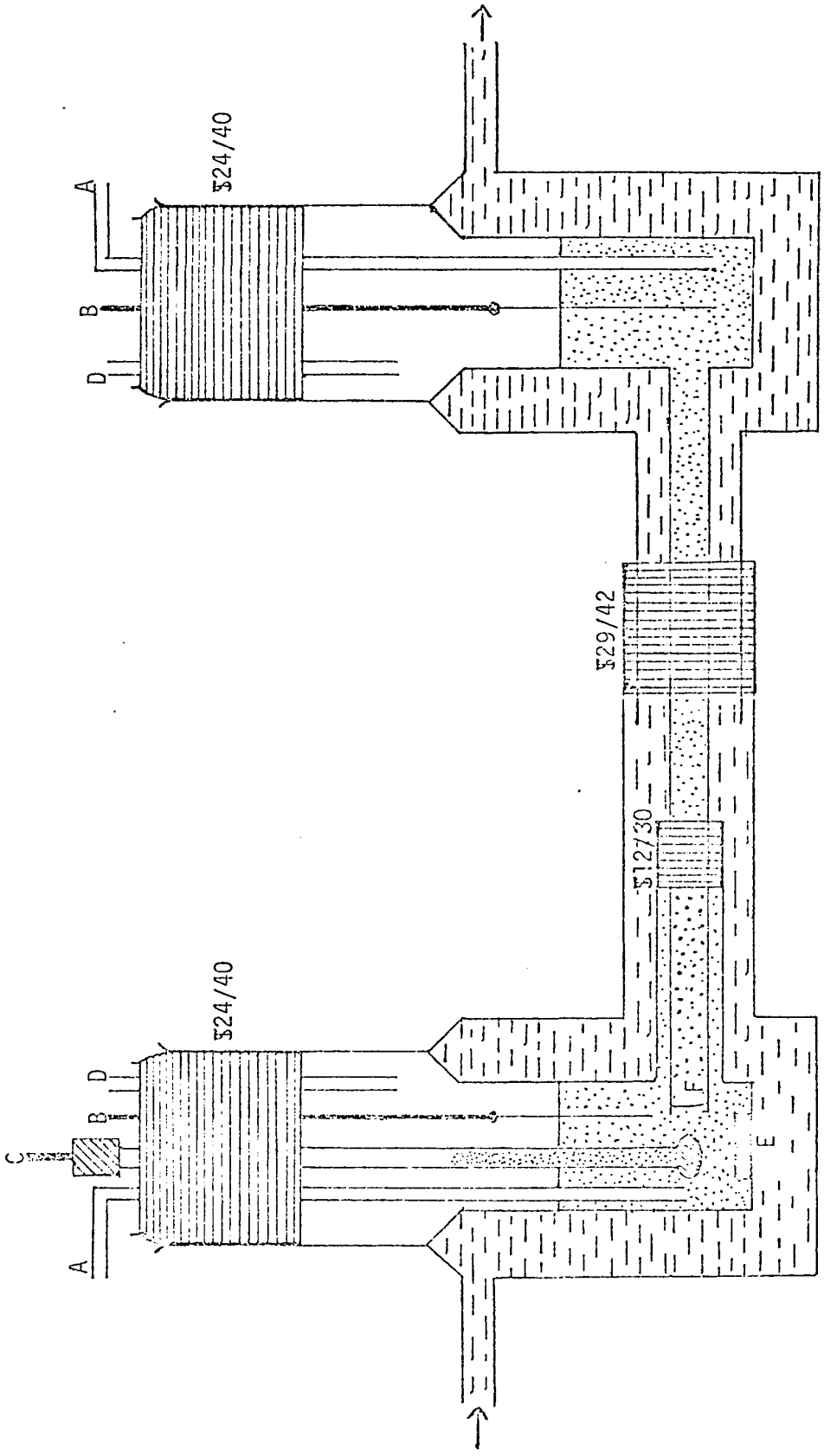


Figure 2

Electrolytic Cell

the platinum electrode, nitrogen inlet and outlet tubes, the pH combination electrode. Stirring in the cathode compartment was accomplished by a one centimeter long magnetic stirrer.

The chemical reaction takes place in the cathode compartment where the alkalinization is accomplished by the following reactions:

$$H^+ + e^- \rightarrow 1/2\ H_2$$

$$H_2O + e^- \rightarrow OH^- + 1/2\ H_2$$

If needed the anode and the cathode could be switched and then hydrogen ions would be discharged in the reaction compartment. In the cathode compartment where the hydrolysis of the ester occurred, the reaction volume could not be lower than six ml due to the particular cell measurements. In the anode compartment approximately ten ml of electrolyte were used. The volume in this case was not critical. The pH electrode used was a combination microelectrode for there were limitations as to the type of electrode that could be used due to the small volume of the reaction cell.

Ordinary platinum wire, 0.316 mm in diameter, was used as the platinum electrodes. The Pt wire was soldered directly to the electrical wire. Since it is extremely important that a very small surface of the Pt wire be in contact with the solution, the wires were protected by a polyethylene tube plus a layer of epoxy glue. The hardened glue was filed off at the tip of the wire to expose a very small surface.

The reaction medium consisted of 0.5M NaCl and 0.01M $MgCl_2 \cdot 6H_2O$. Concentrated solutions of NaCl (5M) and $MgCl_2 \cdot 6H_2O$ (2M) were originally prepared and then diluted to the desired molarity to prepare four liters of the electrolyte as it was needed. Great care must be taken in the preparation of all solutions in order to keep CO_2 absorption from the atmosphere

to the minimum. The electrolyte was stored in a four liter aspirator bottle, the liquid was removed by means of a 10ml syringe through a bunsen valve. A carbon dioxide trap (ascarite) was connected to the bottle. The original pH of the electrolyte was between 6.5 and 7.0, it was then adjusted to 8.0 using a carbonate free 0.1N NaOH solution.

For a practical run each side of the cell was washed twice with distilled water and three times with five ml volumes of electrolyte. Both compartments were then sealed and nitrogen was allowed to flow through the empty cell for one minute. Ten ml of the electrolyte were then added to the anode and 5.4 ml to the cathode. Nitrogen was bubbled through the solution for at least three minutes before the pH meter was turned on, followed by the recorder and the battery circuit. Before going through the solution the nitrogen gas passed through a 20% KOH solution to make sure that no carbon dioxide was present in the gas.

When the pH meter was turned on, the needle was in the center position corresponding to pH 8.0. The voltage drop existing across the meter was utilized to activate the recorder pen in such a way that the recorder pen followed the movement of the pH meter needle as it went from pH 8.0 to pH 7.0. The pH meter was used in the expanded scale mode which has a span of two pH units, therefore pH 8.0 to 7.0 corresponds to one side only of the pH meter.

The feed back system consisted of a potentiometer coupled mechanically to the pen drive of the recorder in such a way that an increasing voltage was applied to the electrolytic cell as the pen moved to the left. After the addition of 0.6ml of the acetylcholine bromide solution spontaneous hydrolysis caused a decrease in pH which resulted in the leftward movement of the recorder pen. A voltage appeared at the Pt

electrodes resulting in electrolytic removal of hydrogen ions from the cathode compartment. The pH continued to decrease and the voltage to increase until the removal of hydrogen ions was just as fast as the production of hydrogen ions. At this point the pH remained constant and the current, I'μa, registered was a direct measure of the hydrogen ions produced by the spontaneous hydrolysis of the ester. As this steady state was reached, about one to three minutes, the enzyme solution was added and the same process was repeated. After about four minutes a new steady state current, I"μa was obtained. (I"-I')μa was taken as a measure of the rate of the enzymatic hydrolysis of the ester. Multiplying the current in microamperes by 0.621 nanoequivalents per μampmin gave the reaction rate in nanoequivalents per minute.

Calibration of the instrument was accomplished by flowing standardized HCl into the cell by means of a motor driven syringe. The acid was added to the cathode compartment at measured rates. The current necessary to keep the pH constant was recorded and compared with the rate of acid addition. The experimental data for the calibration will be reported in Appendix 'B'.

Materials

Constant temperature water bath - Model ED

Platinum wire, 0.361mm in diameter.

Beckman Expandomatic pH meter. Range 0-14 pH or any 2 pH span.

Accuracy: Standard 14 pH scale $\pm$ 0.05 pH

Expanded 2 pH scale $\pm$ 0.01 pH

Repeatability: Standard 14 pH scale - 0.015 pH

Expanded 2 pH scale - 0.003 pH

Sensor: Fisher combination electrode. Model 13-641-586

Presentation: Terminals provided for use with either potentiometric or current recorder.

Temperature compensation: Automatically over 0° to 100° range

Ambient temperature range: -10° to 40°

Heathkit Ammeter: Range 0-50μa Uncertainty $\pm$ 0.3μa.

Fisher Recordall, Catalog No. 13-940-100

Chart Speed: two inches per hour. A zero shifting device is also connected to shift the electrical zero point of the recorder. Catalog No. 13-940-140.

Anhydrous Acetylcholine Bromide M.Wt. 226.1

Purchased from Sigma Chemical Company, Lot No.106-B-1980.

0.2261gm in 25ml electrolyte solution.

Molarity = $(4.000 \pm 0.008) \times 10^{-2}$ Stock solution.

Acetylcholinesterase: From Bovine Erythrocytes.

Contents: Cholinesterase plus Gelatin, NaCl and Sodium Phosphate Buffer, pH 7.6. Purchased from Sigma Chemical Co. Lot No.107-B 0450. 2.6 μMolar units/mg.

By definition: One μMolar unit of AChE hydrolyzes one μmole of Acetylcholine per min. at pH 8.0 at 37°.

C. Polarographic Detection of Serine

The polarographic cell used had a reaction volume of 40ml. In an attempt to keep the amount of water to a minimum, the following concentrations of reagents were found to be the best, in the sense that both serine and tetraethylammonium iodide stayed in solution.

5 ml of 1 x 10^{-2}M Serine in distilled water.

20ml of 1M Tetraethylammonium iodide in 95% Ethyl alcohol.

15ml of 95% Ethyl alcohol.

0.5ml of 0.2% Triton X in Ethyl alcohol.

Serine = 1.25 x 10^{-3} M

Tetraethylammonium iodide = 0.5 M

Ethyl alcohol = 92%

Triton X = 0.0025%

For the measurements the following were used:

Polarecord Model E261. Maximum sensitivity 0.025 a full scale, and capable of accepting both standard and rapid drop signals.

The dropping mercury equipment involved a Metrohom polarographic stand, Model E354, capable of standard and rapid drop polarography.

A reaction vessel with a capacity of 40 ml.

An Ag/AgCl reference electrode was used for all applied potential measurements. The potentials involved are therefore, except as noted, referred to this electrode.

All solutions examined polarographically were deoxygenated by bubbling nitrogen gas through the solution (for three min.) prior to examination.

Reagent grade 95% ethyl alcohol was used in preparing all solutions except the serine and histidine solutions.

1 M solution of reagent grade Tetraethylammonium iodide: 36.938 g made up to 100 ml with 95% ethyl alcohol.

0.2% Triton-X 100 was used as maxima suppressor.

1×10^{-2}M Serine M. Wt. 105.09

0.10509 g made up to 100 ml with distilled water.

1×10^{-2}M Histidine M. Wt. 155.16

0.15516 g made up to 100 ml with distilled water.

IV. RESULTS

A. Preparation and Purification of Acetylcholine Hydrolase from Human Erythrocytes

Two liters of packed human erythrocytes were used each time as starting material. Out of four different batches: A, B, C, D, 20ml (A) and 31ml (D) solutions of supposedly purified AChE were obtained. Nothing was obtained from B and C due to the lack of precipitate in one of the very last steps. The amount of enzyme present in both A and D is unknown. The activity of the enzyme in solution was not measured using the old techniques since the new method was to be set up. Table II summarizes the results obtained for a series of microzone electrophoretic runs on the AChE preparations. The negative results obtained with both A and D seem to indicate that the enzyme must either be present in very minute quantities or not present at all. The membranes of Runs 3 and 10 were scanned using a Densicord. The resolution obtained for the serum membrane was very poor even though four bands were visible with the naked eye. Nothing was obtained when scanning the membrane of run number three.

TABLE II

Experimental Results Obtained For A Series Of Microzone Electrophoresis Runs

Run No.	Sample	Sample Application	Results
1	AChE Solution A	0.25µl Four parallel runs	Negative
2	Egg Albumin	0.25µl Four parallel runs	Negative
3	Serum	0.25µl Four parallel runs	Four bands visible
4	AChE solution A conc. 10:3	0.25µl Four parallel runs	Negative
5	"	0.25µl Two parallel runs, longer straining time.	Negative
6	"	0.75µl Two parallel runs	Negative
7	"	1.25µl Two parallel runs	Negative
8	AChE Solution D	0.25µl Two runs 0.50µl Two runs 0.75µl Two runs	Negative Negative Negative
9	"	1.25µl One run	Negative
10	"	1.50µl One run	A very faint trace visible

B. Hydrolysis of Acetylcholine Bromide by Acetylcholine Hydrolase

TABLE III

Enzymatic Hydrolysis of AChBr (0.004M) at 37°

No. of Runs	E Vol µl	Nanomolar Enzyme Units	Moles/min x 10^{-9} ± 0.2	Current µa ± 0.3	Dev. µa ±	St. Dev. (s) µa	Rel. St. Dev.	Rel. % error	pH ± 0.01
5	5	2.6 ± 0.2	3.2	5	1	$1.^{3}$	0.2^{6}	23	7.59
8	10	5.2 ± 0.3	5.7	9	1.2	$1.^{5}$	0.1^{7}	9.6	7.59
6	15	7.8 ± 0.4	7.2	12	0.5	0.7^{8}	0.06^{4}	7.6	7.54
* 21	20	10.4 ± 0.5	10.4	17	0.5	0.7^{1}	0.04^{2}	-	7.42
7	25	13.0 ± 0.6	13.4	22	1	$1.^{5}$	0.06^{8}	3.0	7.34
4	30	16 ± 1	16.8	27	1.5	$1.^{8}$	0.06^{7}	5.0	7.37
4	40	21 ± 1	20.0	33	0.8	$1.^{3}$	0.03^{9}	-4.8	7.29

*20µl samples were used as standard to observe day to day variations until results were found to be comparable.

Figure 3

Enzymatic hydrolysis of AChBr (0.004M) at 37°

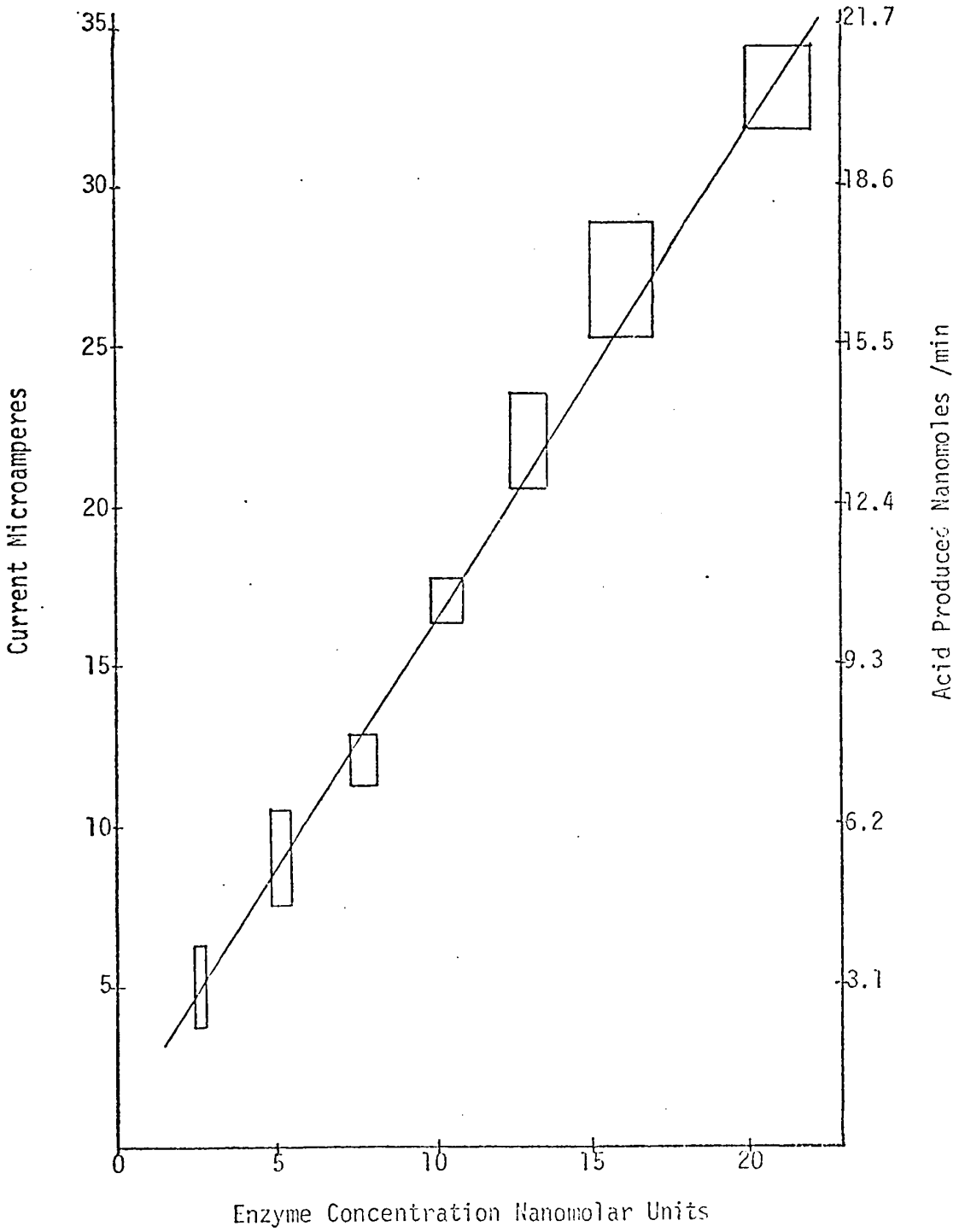


TABLE IV

Enzymatic Hydrolysis of AChBr (0.004M) at 25°

No. of Runs	E Vol µl	Nanomolar Enzyme Units	Moles/min x 10^{-9} ± 0.2	Current µa ± 0.3	Dev. µa ±	St. Dev. (s) µa	Rel. St. Dev.	Rel. % error	pH ± 0.01
4	10	5.2 ± 0.3	5.3	8	0.5	0.8^{2}	0.1^{0}	1.9	7.64
4	20	7.8 ± 0.4	8.1	13	1	$1.^{4}$	0.1^{1}	-22	7.58
4	30	16 ± 1	14.4	23	0.8	$1.^{4}$	0.06^{1}	-10	7.43
4	40	21 ± 1	19.9	32	1	$1.^{2}$	0.03^{8}	- 5.2	7.34

Figure 4

Enzymatic hydrolysis of AChBr (0.004M) at 25°

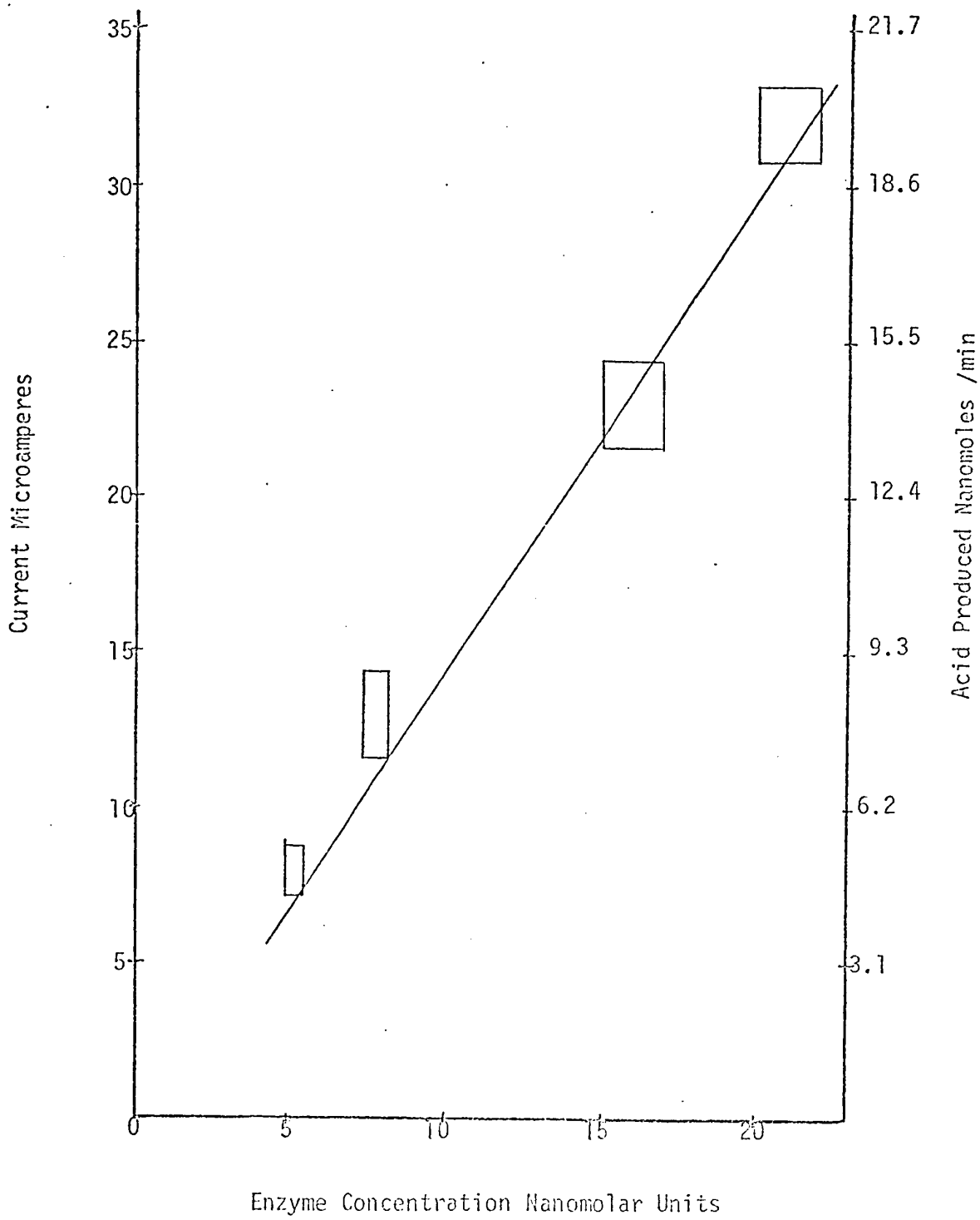


TABLE V

Enzymatic Hydrolysis of AChBr (0.004M) at 30°

No. of Runs	E Vol µl	Nanomolar Enzyme Units	Moles/min x 10^{-9} ± 0.2	Current µa ± 0.3	Dev. µa ±	St. Dev. (s) µa	Rel. St. Dev.	Rel. % error	pH ± 0.01
4	10	5.2 ± 0.3	5.9	10	0.5	0.8^{2}	0.08^{2}	13	7.65
4	15	7.8 ± 0.4	10.2	16	2	$2.^{4}$	0.1^{5}	31	7.55
4	20	10.4 ± 0.5	13.7	22	0.2	0.2^{5}	0.01^{0}	31	7.49
4	25	13.0 ± 0.6	15.3	25	1.6	$1.^{9}$	0.07^{6}	18	7.46
4	30	16 ± 1	18.6	30	1	$1.^{4}$	0.04^{7}	16	7.34

Figure 5

Enzymatic hydrolysis of AChBr (0.004M) at 30°

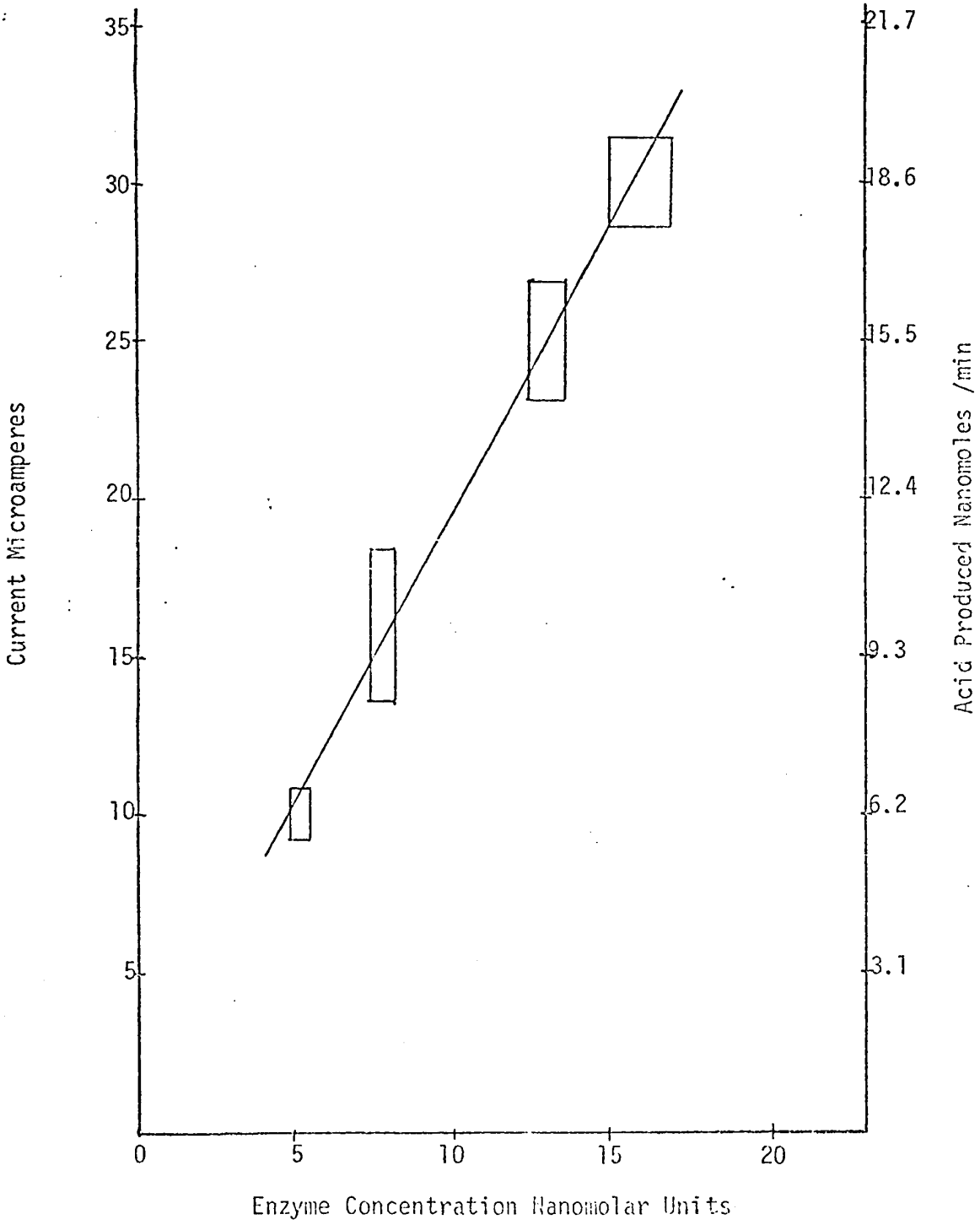


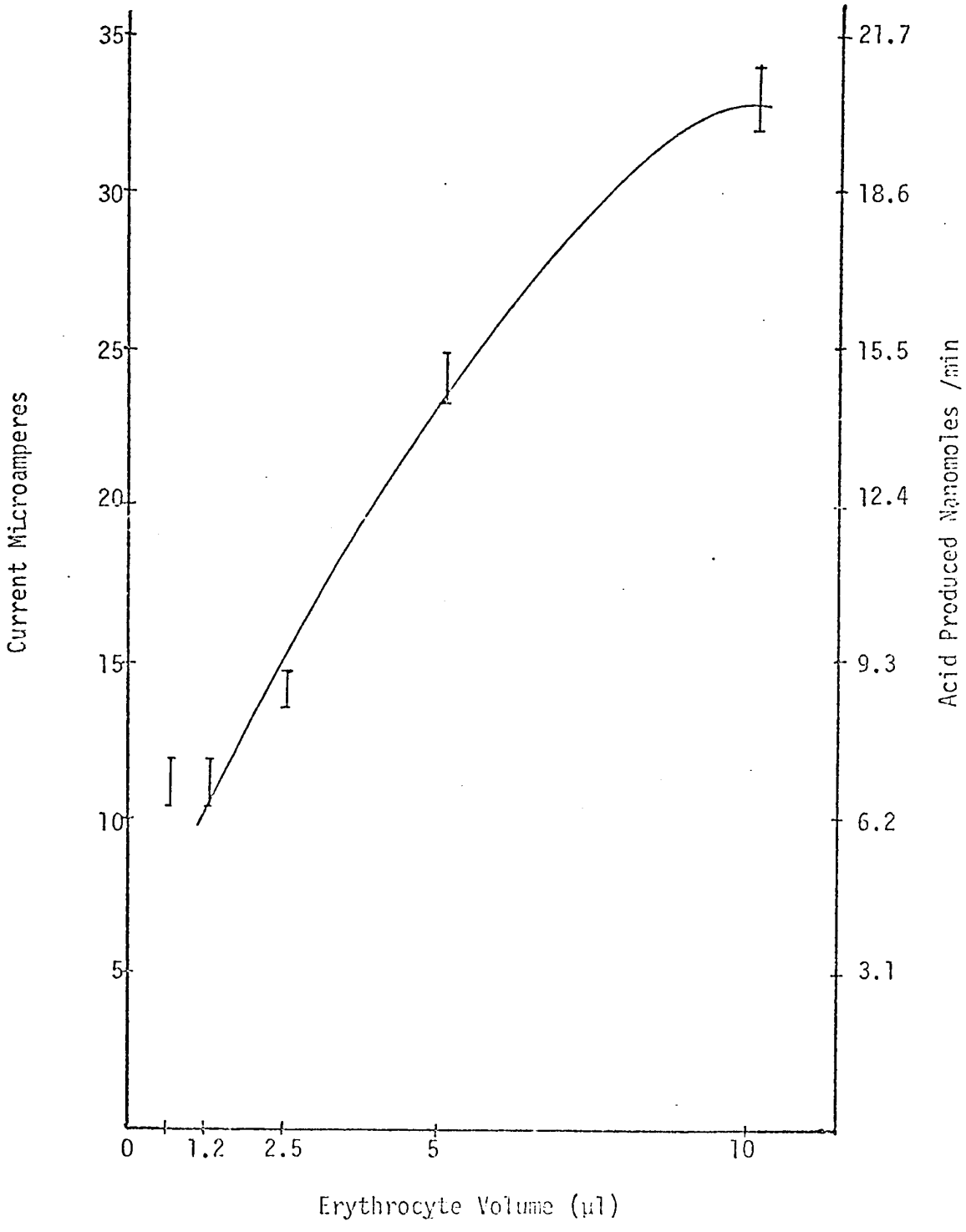
TABLE VI

Hydrolysis of AChBr (0.004M) by Rat Erythrocytes at 37°

No of Runs	Erythrocytes Vol µl	Moles/min x 10^{-9} ± 0.2	Current µa ± 0.3	Dev µa ±	St. Dev. (s) µa	Rel St Dev.	pH ± 0.01
5	10.0 ± 0.1	20.5	33	0.8	$1.^{0}$	0.03^{3}	7.30
4	5.00 ± 0.06	14.9	24	0.0	-	-	7.34
4	2.50 ± 0.04	8.8	14	0.2	0.2^{5}	0.01^{8}	7.39
4	1.25 ± 0.02	6.8	11	0.0	-	-	7.43
4	0.62 ± 0.01	6.8	11	0.5	0.8^{2}	0.07^{4}	7.48

Figure 6

Hydrolysis of AChBr (0.004M) by rat erythrocytes at 37°



C. Polarographic Detection of Serine

The following provides general information pertinent to the polarograms for serine:

DL Serine obtained from Nutritional Biochemicals Co. Lot No.2086

Rapid drop polarography, drop life 0.28 seconds

Full scale setting of 500 μa

Applied potential scan from 0.000v to -3.00v vs Ag/AgCl

Test temperature for all solutions was room temperature

Damping factor = 4

When serine was replaced by histidine no results were obtained. No further attempt was made with histidine since this particular point did not warrant extensive investigation at this time.

Polarogram 1 : No IR Compensator

1. $E_{1/2}$ = -0.228 v vs. Ag/AgCl

   = -0.273 v vs. S.C.E.

   Plateau Potential = - 1.16 v

   Current at $E_{1/2}$ = 76 μa

2. $E_{1/2}$ = -2.030 v vs. Ag/AgCl

   = -2.075 v vs. S.C.E.

   Plateau Potential = - 2.25 v

   Current at $E_{1/2}$ = 188 μa

Polarogram 2 : IR Compensator

1. $E_{1/2}$ = -0.105 v vs. Ag/AgCl

   = -0.150 v vs. S.C.E.

   Plateau Potential = - 1.06 v

   Current at $E_{1/2}$ = 92 μa

2. $E_{1/2}$ = -1.920 v vs. Ag/AgCl

   = -1.965 v vs. S.C.E.

   Plateau Potential = - 2.10 v

   Current at $E_{1/2}$ = 223 μa

V. DISCUSSION

As indicated previously the elucidation of the mode of action of AChE is the original goal to which this work contributes. A rapid, accurate and simple method for the determination of AChE was necessary prior to any attempts at enzyme preparation. Thus only preliminary attempts were made in the extraction of the enzyme. The analytical method to be used during purification was to be the same as the one to be used later to study rates of reaction with DFP. These uses immediately imposed certain limits upon the choice of a method.

The number of different techniques available, with all the modifications, might seem sufficient in providing us with a wide choice. The development of techniques is not an end in itself; each time a new method is perfected, it is the answer to a specific need on the part of the author of the method. In the present investigation it became obvious from the start that only two of all the available methods might be suitable.

The polarographic method is the first of these two methods which appeared as the answer to our needs. The simplicity, accuracy and ability of this method to follow initial rates of reaction make it almost ideal.

Unfortunately, as it has already been mentioned, all our efforts with this technique failed. Our temporary interest with this method also explains the work done on the polarography of serine and histidine. This particular aspect of the research was left incomplete at the time. Since then the polarographic technique for measuring AChE activity has been successfully applied and as such the possibility of further use of this work is not excluded.

The second method, which is the one discussed and improved in this work, is based on the automatic electrolytic titrator method of Einsel et al. (1956). This method is, in principle, ideal in almost all of its aspects. However, we were trying to avoid a mechanical connection between the two circuits. This connection, if not functioning smoothly, can introduce a very significant delay in the system response. The original intention was to make the system entirely electronic by the use of an operational amplifier for the feed back mechanism. This would result in a much more efficient electrolytic titrator in addition to a greater simplification in terms of the size of the equipment. A presumably suitable circuit was secured which did not serve our purpose. A great deal of time and effort were spent in modifying the circuit to no avail. It was then decided to go back to the original work of Einsel et al. (1956) which promised to give results.

Due to the requirements of our project the cell design had to be changed. For some time the cell itself became the most important aspect of the work. The first cell was made out of lucite: two separate compartments held together by bolts. Leakage was the major problem with this cell. The second cell was moulded again out of lucite. The two compartments were separated by a membrane, which was held in place by two sheets of

thick polyethylene. Leakage between the compartments was again the problem. When vacuum grease was used to keep the membrane in place, it was found to interfere with the determination. The absorption of carbon dioxide seemed to increase considerably in the presence of silicone grease. Other types of grease were used with same effect. In all cases the cell was sealed; however, at this time nitrogen gas was only bubbled through one compartment. In addition to these problems there was the possibility of the protein being held on to the cell wall even after repeated washings of the cell. At this point a new glass cell was designed. An outer jacket was needed for temperature control. Fig.2 is a diagram of the cell only. Several attempts were needed before a successful cell was completed. Countless problems were encountered in connection with the leakage of water from the outer jacket into the cell compartments. At the present time the cell is still extremely fragile due to the strain on the glass.

The absorption of atmospheric carbon dioxide created a real problem when trying to detect very small shifts in pH. Due to the very small reaction volume, the change in pH due to the carbon dioxide became so significant that it obscured all other effects. It became necessary to seal the two cell compartments and to maintain a constant nitrogen pressure above the liquid in both sides of the cell during the reaction. In addition all solutions had to be prepared with carbon dioxide-free water and stored under nitrogen. This applies naturally to the electrolyte solution which was prepared in units of four liters. The substrate and enzyme solutions were prepared daily. The presence of any type of vacuum grease seemed to increase carbon dioxide absorption greatly even with this type of cell. For this reason the amount of grease on the inside

junction of the cell had to be kept to the minimum. One of the additional requirements of the cell was that it had to come apart easily to change the membrane. As the membrane was changed every few days it was important that this procedure be accomplished quickly.

In calibrating the instrument with standard hydrochloric acid, Appendix B, the error ranged from 5.5 to -6.6 percent. The major problem with this type of calibration was the fact that the acid was added at one specific spot in the reaction cell. On the contrary, during the enzymatic reaction this problem does not exist since the acid is produced throughout the solution. The results show that the instrument is basically working.

Measurement of the amount of hydrolysis of ACh in the presence of known amounts of enzyme is also a form of instrument calibration. The activity of the enzyme preparation used is reported by the manufacturers at 37° and pH 8.0.

Table III gives the experimental data for the enzymatic hydrolysis at 37°. As it was mentioned in the procedure, an equilibrium current was obtained after the addition of the AChBr, a second equilibrium after the addition of the enzyme. It is the difference between these two values that is reported as the enzymatic hydrolysis. The values for the spontaneous hydrolysis of AChBr, as reported in Table C.IV cannot be compared to each other due to large differences between them. It could be said that the amount of spontaneous hydrolysis registered is no indication of the real spontaneous hydrolysis. Even though the cell was washed carefully after each run, it seems that some enzyme might still adhere to the cell wall and contribute to the hydrolysis of AChBr. These fluctuations in AChBr spontaneous hydrolysis do not affect the enzymatic hydrolysis since the

value for the enzymatic hydrolysis is obtained by difference.

The instrument was also extremely sensitive to the surroundings. Grounding helped only to a limited extent since all the wiring was exposed. In the future it will probably be required to ground the cell itself. This could be done by coating the cell walls with some type of metal and grounding the outside directly. Most of the difficulties encountered in this work were not encountered by the authors of the original design. We are working at a much lower level of activity and, therefore, are more sensitive to electronic noise and interferences. When the slope for graphs 3-5 is obtained the activity of the enzyme seems to reach a peak at 30°. It is difficult to explain such behaviour and this point needs further investigation. As with any enzyme one would expect the highest reactivity at physiological conditions. At temperatures higher than 37° a decrease in activity would be expected and could be explained in terms of protein denaturation. Body temperature is 37°, however, the actual temperature of blood could be slightly lower than 37°. If this is so then the increased activity at 30° might be explained as really being the ideal temperature for erythrocyte AChE. Our data is certainly not sufficient to make any speculation. When our data was secured there was no intention of studying temperature dependance. The aim at the time was to prove that the instrument was responding and that the change in activity with respect to enzyme concentration was linear over a given range. The changes between 25° and 30° might be apparent, in fact they are within the experimental error at each point. There is practically no change in activity between 25° and 30°.

Before studying any temperature dependance or ionic strength dependance with the present instrumental design a little more work will

be required in order to avoid all interfering disturbances. The electrolytic value has been found to be higher than theoretical in almost all cases. The absolute error may be attributed, at least in part, to the platinum electrodes. In the original work the authors mentioned that ordinary platinum wire had been found to be unsatisfactory unless the exposed surface is kept down to a minimum. They used Beckman platinum electrodes (No.281). Great care has been taken to minimize the exposed surface of the electrodes used in this work.

In Table VI the results for the hydrolysis of AChE in the presence of rat erythrocytes are given. Fig.6 is a plot of nanomoles of acid produced per min. with respect to volume of erythrocytes. It is possible that a linear relationship exists in the range 2.5 to 10 µl of erythrocytes. There are not enough points to be able to make a definite statement. At very low concentration of erythrocytes it was extremely difficult to determine when the steady state was reached. There was a steady but very slow increase in current. Addition of 0.62 µl of erythrocytes to the electrolyte solution (no substrate present) resulted in the production of current. Since we are dealing with intact cells, the acid does not necessarily have to result from the hydrolysis of ACh. Breakdown of glucose would also result in the production of acid. To be able to detect only the acid produced by the action of ACh many other reactions might have to be inhibited. Ultrasonic treatment of cells might be the answer, in the future, to the problem of interferences. After ultrasonic treatment the AChE activity of the erythrocyte samples should also increase as the enzyme will no longer be membrane bound. Of course, we are at a very sensitive level where every small change becomes significant. Too many interfering influences are still present in this instrumental set-up,

this makes it impossible at times to know whether the observed effect is real or apparent. When dealing only with the purified enzyme it was easier to detect changes. In the presence of red blood cells many complications arose.

VI. SUGGESTIONS FOR FUTURE WORK

As has already been mentioned, the development of a new method to measure the activity of an enzyme is never an end in itself. The modifications of an established technique, as described in this thesis, provided a more sensitive method of measuring the activity of AChE.

1. The present instrumental design still requires a few modifications in order to facilitate any future work. The electrolysis cell is fragile and, as such, not very practical to work with in routine analyses. The instrument is subject to environmental changes: the build up of static electricity can, at times, completely obscure the experimental results.

2. Once the above mentioned inconveniences are eliminated, the use of this method in conjunction with the polarographic method is suggested. Since these two techniques make use of two different substrates, namely, acetylcholine and acetylthiocholine, a comparison of the reaction rates should provide some useful insight into the mode of action of the enzyme.

3. The original work on the extraction and purification of AChE could also be resumed since two techniques for activity measurements are available.

4. After obtaining a relatively pure enzyme sample the effect of change in temperature and ionic strength of the medium could be investigated. From the experimental data reported there seems to be an apparent change in AChE activity with temperature. Our data is not sufficient to make any conclusive statement.

5. The study of DFP inhibited reactions for a better insight

into the chemical nature of the active sites, would be the next step. As serine has been detected polarographically, further attempts could be made in investigating the presence of serine on the enzyme molecule with the aid of DFP as inhibitor.

6. Because of the high sensitivity of the present technique, additional work should be done using the intact red blood cells as the enzyme source. If conditions could be set up, maybe by the use of inhibitors, to control the various metabolic reactions still taking place within the cells, then it might be possible to obtain some suggestion into the role of AChE in red blood cells. At present the function of AChE in blood is completely unknown.

VII. APPENDIX A

Preparation of Solutions

1. The electrolyte solution: 0.5 M NaCl and 0.01 M $MgCl_2 \cdot 6H_2O$ at pH 8.0, ionic strength 0.55 was prepared using carbon dioxide free water

2. The Acetylcholine Bromide (AChBr) solution was prepared as a $(4.00 \pm 0.008) \times 10^{-2}$M solution in the electrolyte solution. 25ml of this solution were prepared freshly at the beginning of a series of runs.

3. The AChE stock solutions were prepared in the electrolyte solution.

a. Stock solution : 1 mg/ml using a 5 ml volumetric flask.

$$\frac{(0.0050 \pm 0.00002)\text{g} \times 2.6\ \mu\text{Munits}}{(5.000 \pm 0.005) \times 10^{-3}\ \text{l}}$$

2.6 ± 0.1 µMunits/ml

The uncertainty is an indication of the maximum probable error.

b. Working solution : 1 ml stock solution diluted to 5 ml.

1 ml transfer pipette and 5 ml volumetric flask are used.

$$\frac{(1.0 \pm 0.002)\ \text{ml} \times (2.6 \pm 0.1)\ \mu\text{Munits/ml}}{(5.000 \pm 0.005)\ \text{ml}}$$

0.52 ± 0.02 µMunits/ml or

0.52 ± 0.02 nanoMunits/µl

Solution No. 1

5.40 ± 0.04 ml of electrolyte solution.

0.600 ± 0.002 ml of (4.000 ± 0.008) x 10^{-2}M AChBr solution.

5.0 ± 0.2 ul of AChE stock solution (0.52 ± 0.02 nano Molar units/µl)

AChBr = 24.00 ± 0.05 µMoles/6.00 ml

AChE = 2.6 ± 0.2 nano Molar units/ ml

Note: 10.00 ± 0.04 ml syringe was used to measure electrolyte solution

1.000 ± 0.002 ml syringe was used to measure AChBr solution

50.0 ± 0.2 µl syringe was used to measure AChE solution

TABLE A. I

Concentration of AChE in Six ml Reaction Volume

Solution No.	AChE volume in µl	nMolar units ± m.p.e.
1	5.0 ± 0.2	2.6 ± 0.2
2	10.0 ± 0.2	5.2 ± 0.3
3	15.0 ± 0.2	7.8 ± 0.4
4	20.0 ± 0.2	10.4 ± 0.5
5	25.0 ± 0.2	13.0 ± 0.6
6	30.0 ± 0.2	16 ± 1
7	40.0 ± 0.2	21 ± 1

4. Carbonate-free base

To adjust the pH of the electrolyte solution the following carbonate free base was prepared:

4.0 to 4.5 g of NaOH were dissolved in 400 ml of distilled water, 10 ml of 0.25 M barium chloride were then added. The solution was well-mixed and allowed to stand overnight so that the barium carbonate could settle out. The solution was then decanted from the solid into a clean bottle and diluted to one liter. The solution was stored in a polyethylene aspirator bottle. Ascarite was used as a carbon dioxide absorbent.

5. Erythrocyte Solution

Fresh blood was obtained from a live rat as needed. The serum was separated from the erythrocytes by centrifugation. The erythrocytes were then washed three times with isotonic (0.9%) NaCl solution and the suspension was centrifuged between each washing. The packed cells were then taken up in an equal volume of isotonic NaCl solution (first dilution). 1 ml transfer pipettes were used for all subsequent dilutions. 20 µl sample volumes of the cell suspension were used for each individual determination.

VIII. APPENDIX B

Instrument Calibration with Standardized Hydrochloric Acid

1. A standard NaOH solution was first prepared, N = 1.06 $\pm$ 0.01

2. Standard HCl was prepared by titrating against NaOH.HCl solution, N = 0.70 $\pm$ 0.01

3. The following HCl solutions were then prepared by dilution.

1 ml pipette Uncertainty $\pm$ 0.002 ml

100 ml volumetric flask $\pm$ 0.04 ml

Solution 1

0.140 $\pm$ 0.002 ml of 0.70 $\pm$ 0.01 N HCl was diluted to 100 ml.

$$N = \frac{(0.140 \pm 0.002)\ \text{ml} \times (0.70 \pm 0.01)\ N}{(100.00 \pm 0.04)\ \text{ml}}$$

$$N = (0.98 \pm 0.03) \times 10^{-3}$$

Solution 2

0.210 $\pm$ 0.002 ml of 0.70 $\pm$ 0.01 N HCl was diluted to 100 ml.

$$N = \frac{(0.210 \pm 0.002)\ \text{ml} \times (0.70 \pm 0.01)\ N}{(100.00 \pm 0.04)\ \text{ml}}$$

$$N = (1.47 \pm 0.03) \times 10^{-3}$$

Solution 3

0.290 $\pm$ 0.002 ml of 0.70 $\pm$ 0.01 N HCl was diluted to 100 ml.

$$N = \frac{(0.290 \pm 0.002\ \text{ml}) \times (0.70 \pm 0.01)\ N}{(100.00 \pm 0.04)\ \text{ml}}$$

$$N = (2.03 \pm 0.03) \times 10^{-3}$$

Solution 4

0.360 ± 0.002 ml of 0.70 ± 0.01 N HCl was diluted to 100 ml.

$$N = \frac{(0.360 \pm 0.002)\ \text{ml} \times (0.70 \pm 0.01)\ \text{N}}{(\ 100.00 \pm 0.04\ )\ \text{ml}}$$

$$N = (2.52 \pm 0.03) \times 10^{-3}$$

4. A peristaltic pump (LKB ReCyChrom, Type 4912A) was used to flow HCl into the cell.

TABLE B.I

Rates of Acid Flow Into Reaction Cell

Flow rate ml/min	Acid Solution: HCl Normality	μmoles of HCl per min
0.009	$(2.03 \pm 0.03) \times 10^{-3}$	$0.01^{8} \pm 0.00^{1}$
0.009	$(2.52 \pm 0.03) \times 10^{-3}$	$0.02^{3} \pm 0.00^{1}$
0.013	$(2.03 \pm 0.03) \times 10^{-3}$	$0.02^{7} \pm 0.00^{1}$
0.018	$(0.98 \pm 0.03) \times 10^{-3}$	$0.01^{7} \pm 0.00^{1}$
0.018	$(1.47 \pm 0.03) \times 10^{-3}$	$0.02^{6} \pm 0.00^{1}$
0.018	$(2.03 \pm 0.03) \times 10^{-3}$	$0.03^{6} \pm 0.00^{1}$
0.018	$(2.52 \pm 0.03) \times 10^{-3}$	$0.04^{5} \pm 0.00^{1}$

TABLE B.II

Experimental Data For The Calibration Of The Instrument With Acid

Run No.	HCl µmoles/min ±0.00[1]	Current µa ±0.3	Dev. µa	moles/min ±0.00[2]	Rel. % error	pH ±0.01
1		30.5	-0.6			7.24
2		31.5	0.4			7.29
3		31.0	-0.1			7.29
4		31.5	0.4			7.28
Av.	0.01[8]	31.1	±0.4	0.01[9]	5.5	7.28
1		35.0	-0.2			7.28
2		35.0	-0.2			7.28
3		35.2	0			7.26
4		35.0	-0.2			7.25
Av.	0.02[3]	35.2	±1.5	0.02[2]	-4.3	7.27
1		31.2	3.1			7.24
2		26.0	-2.1			7.29
3		26.0	-2.1			7.29
4		29.4	1.3			7.24
5		28.0	-0.1			7.26
Av.	0.01[7]	28.1	±1.7	0.01[7]	-	7.26
1		42.5	0.3			7.11
2		41.2	-1.0			7.13
3		41.4	-0.8			7.13
4		43.0	0.8			7.10
5		43.0	0.8			7.10
Av.	0.02[6]	42.2	±0.7	0.02[6]	-	7.11
1		45.0	1.2			7.18
2		43.5	-0.3			7.13
3		44.2	0.4			7.29
4		43.5	-0.3			7.24
5		43.0	-0.3			7.28
Av.	0.02[7]	43.8	±0.6	0.02[7]	-	7.22

TABLE B.II (con't)

Run No.	HCl μmoles/min ±0.00[1]	Current μa ±0.3	Dev. μa	moles/min ±0.00[2]	Rel. % error	pH ±0.01
1		54.5	-0.1			6.96
2		53.0	-1.6			6.99
3		60.0	5.4			6.89
4		50.0	-4.6			7.02
5		57.0	2.4			6.95
6		52.0	-2.6			6.99
7		55.0	0.4			6.96
8		55.0	0.4			6.96
9		55.0	0.4			6.98
Av.	0.03[6]	54.6	±2.0	0.03[4]	-5.5	6.96
1		69.0	0.6			<6.80
2		68.5	0.1			<6.80
3		68.5	0.1			<6.80
4		69.0	0.6			<6.80
5		67.0	-1.4			<6.80
Av.	0.04[5]	68.4	±0.6	0.04[2]	-6.6	<6.80

IX. APPENDIX C

Experimental Data for the Hydrolysis of Acetylcholine Bromide

TABLE C. I

Enzymatic Hydrolysis of AChBr (0.004M) at 37°

Run No	E vol µl	Current µa ± 0.3	Dev. µa	pH ± 0.01
1	5	7	2	7.60
2	5	4	-1	7.58
3	5	5	0	7.56
4	5	6	1	7.62
5	5	4	-1	7.57
Av.	5	5	±1	7.59
1	10	8	-1	7.58
2	10	9	0	7.68
3	10	8	-1	7.55
4	10	7	-2	7.60
5	10	10	1	7.58
6	10	12	3	7.56
7	10	8	-1	7.61
8	10	10	1	7.59
Av.	10	9	±1.2	7.59
1	15	12	0	7.57
2	15	12	0	7.50
3	15	11	-1	7.55
4	15	11	-1	7.54
5	15	12	0	7.53
6	15	11	-1	7.52
Av.	15	12	±0.5	7.54
1	20	16	-1	7.50
2	20	17	0	7.48
3	20	18	1	7.49
4	20	16	-1	7.55
5	20	16	-1	7.50
6	20	16	-1	7.42
7	20	17	0	7.48
8	20	17	0	7.50
9	20	17	0	7.50
10	20	18	1	7.49
11	20	17	0	7.51
12	20	16	-1	7.42
13	20	18	1	7.47
14	20	16	-1	7.50

TABLE C. I (con't)

Run No	E vol µl	Current µa ± 0.3	Dev. µa	pH ± 0.01
15	20	17	0	7.48
16	20	16	-1	7.51
17	20	17	0	7.46
18	20	17	0	7.45
19	20	17	0	7.48
20	20	17	0	7.47
21	20	17	0	7.47
Av.	20	17	±0.5	7.42
1	25	22	0	7.36
2	25	22	0	7.35
3	25	21	-1	7.42
4	25	24	2	7.36
5	25	22	0	7.30
6	25	20	-2	7.32
7	25	20	-2	7.30
Av.	25	22	±1	7.34
1	30	26	-1	7.41
2	30	28	1	7.36
3	30	25	-2	7.40
4	30	29	2	7.32
Av.	30	27	±1.5	7.37
1	40	32	1	7.30
2	40	33	0	7.28
3	40	31	-2	7.33
4	40	33	0	7.27
Av.	40	33	±0.8	7.29

TABLE C. II

Enzymatic Hydrolysis of AChBr (0.004M) at 25°

Run No	E vol µl	Current µa ± 0.3	Dev. µa	pH ± 0.01
1	0	15	0	7.75
2	0	16	1	7.74
3	0	14	-1	7.76
4	0	15	0	7.75
Av.	0	15	±0.5	7.75
1	10	9	1	7.63
2	10	8	0	7.65
3	10	9	1	7.64
4	10	8	0	7.65
Av.	10	8	±0.5	7.64
1	20	14	1	7.57
2	20	11	-2	7.59
3	20	13	0	7.58
4	20	14	1	7.56
Av.	20	13	±1	7.58
1	30	23	0	7.43
2	30	22	-1	7.43
3	30	24	1	7.43
4	30	24	1	7.43
Av.	30	23	±0.8	7.43
1	40	33	1	7.35
2	40	31	-1	7.33
3	40	31	-1	7.32
4	40	33	1	7.35
Av.	40	32	±1	7.34

TABLE C. III

Enzymatic Hydrolysis of AChBr (0.004M) at 30°

Run No	E vol µl	Current µa ± 0.3	Dev. µa	pH ± 0.01
1	0	16	2	7.73
2	0	14	0	7.76
3	0	13	-1	7.78
4	0	13	-1	7.78
Av.	0	14	±1	7.76
1	10	9	-1	7.67
2	10	10	0	7.66
3	10	9	-1	7.65
4	10	10	0	7.61
Av.	10	10	±0.5	7.65
1	15	15	-1	7.57
2	15	14	-2	7.57
3	15	19	3	7.53
4	15	18	2	7.53
Av.	15	16	±2	7.55
1	20	21	-1	7.50
2	20	22	0	7.49
3	20	22	0	7.49
4	20	22	0	7.49
Av.	20	22	±0.2	7.49
1	25	26	1	7.44
2	25	27	2	7.40
3	25	24	-1	7.48
4	25	23	-2	7.48
5	25	23	-2	7.48
Av.	25	25	±1.6	7.46
1	30	30	0	7.38
2	30	29	-1	7.35
3	30	29	-1	7.33
4	30	32	2	7.29
Av.	30	30	±1	7.34

TABLE C. IV

Hydrolysis of AChBr (0.004M) by Rat Erythrocytes at 37°

Run No	Erythrocytes µl	Current µa ± 0.3	Dev. µa	pH ± 0.01
1	10.0 ± 0.1	32	-1	7.35
2	10.0 ± 0.1	34	1	7.32
3	10.0 ± 0.1	34	1	7.30
4	10.0 ± 0.1	33	0	7.28
5	10.0 ± 0.1	32	-1	7.26
Av.	10.0 ± 0.1	33	±0.8	7.30
1	5.00 ± 0.06	24	-	7.34
2	5.00 ± 0.06	24	-	7.33
3	5.00 ± 0.06	24	-	7.40
4	5.00 ± 0.06	24	-	7.29
Av.	5.00 ± 0.06	24	-	7.34
1	2.50 ± 0.04	15	1	7.40
2	2.50 ± 0.04	14	0	7.37
3	2.50 ± 0.04	14	0	7.35
4	2.50 ± 0.04	14	0	7.45
Av.	2.50 ± 0.04	14	±0.2	7.40
1	1.25 ± 0.02	11	-	7.45
2	1.25 ± 0.02	11	-	7.44
3	1.25 ± 0.02	11	-	7.42
4	1.25 ± 0.02	11	-	7.42
Av.	1.25 ± 0.02	11	-	7.43
1	0.62 ± 0.01	11	0	7.47
2	0.62 ± 0.01	11	0	7.47
3	0.62 ± 0.01	12	1	7.50
4	0.62 ± 0.01	10	-1	7.47
Av.	0.62 ± 0.01	11	±0.5	7.48

TABLE C. V

Spontaneous Hydrolysis of AChBr (0.004M) at 37°

Date	Runs	Current μa ± 0.3	Dev. μa	pH ± 0.01
18-2-71	1	29	8	7.57
	2	15	-6	7.70
	3	15	-6	7.70
	4	22	1	7.68
	5	22	1	7.66
	6	24	3	7.66
	7	22	1	7.64
	8	22	1	7.68
	Av.	21	±3.4	
2-3-71	1	13	-1	7.66
	2	13	-1	7.66
	3	11	-3	7.68
	4	12	-2	7.68
	5	15	1	7.66
	6	15	1	7.66
	7	17	3	7.65
	8	12	-2	7.73
	9	15	1	7.68
	10	14	0	7.68
	11	17	3	7.66
	Av.	14	±1.6	
3-3-71	1	17	-1	7.64
	2	17	-1	7.65
	3	15	-3	7.67
	4	18	0	7.64
	5	17	-1	7.65
	6	21	3	7.66
	Av.	18	±1.5	
8-3-71	1	16	1	7.66
	2	15	0	7.68
	3	16	1	7.63
	4	13	-2	7.69
	5	15	0	7.67
	6	14	-1	7.65
	7	13	-2	7.66
	8	16	1	7.64
	Av.	15	±1.0	

TABLE C. V (con't)

Date	Runs	Current μa ± 0.3	Dev. μa	pH ± 0.01
9-3-71	1	16	-5	7.67
	2	22	1	7.61
	3	22	1	7.61
	4	17	-4	7.68
	5	21	0	7.63
	6	19	-2	7.66
	7	19	-2	7.66
	8	22	1	7.63
	9	21	0	7.64
	10	22	1	7.64
	11	19	-2	7.65
	12	25	4	7.58
	13	24	3	7.59
	14	19	-2	7.64
	15	21	0	7.64
	Av.	21	±1.9	
11-3-71	1	23	-5	7.62
	2	23	-5	7.62
	3	21	-7	7.62
	4	26	-2	7.58
	5	26	-2	7.58
	6	26	-2	7.58
	7	27	-1	7.58
	8	27	-1	7.59
	9	30	2	7.54
	10	30	2	7.54
	11	35	7	7.51
	12	35	7	7.53
	13	30	2	7.58
	14	31	3	7.55
	15	30	2	7.58
	16	30	2	7.58
	17	31	3	7.57
	18	31	3	7.57
	19	29	1	7.58
	20	29	1	7.61
	21	26	-2	7.59
	22	29	1	7.57
	Av.	28	±3.0	

TABLE C. V (con't)

Date	Runs	Current μa ± 0.3	Dev. μa	pH ± 0.01
8-4-71	1	18	-	7.71
	2	18	-	7.71
	3	18	-	7.71
	4	18	-	7.72
	Av.	18	-	

TABLE C. VI

Summary Table for the Spontaneous Hydrolysis of AChBr (0.004M) at 37°

Date	Number of Runs	Current μa ± 0.3	Dev. μa ±	St. Dev. (s) μa	Rel. St. Dev.
18-2-71	8	21	$3.^{4}$	$4.^{6}$	0.2^{2}
2-3-71	11	14	$1.^{6}$	$2.^{0}$	0.1^{4}
3-3-71	6	18	$1.^{5}$	$2.^{0}$	0.1^{1}
8-3-71	8	15	$1.^{0}$	$1.^{3}$	0.08^{7}
9-3-71	15	21	$1.^{9}$	$2.^{6}$	0.1^{2}
11-3-71	22	28	$3.^{0}$	$3.^{6}$	0.1^{3}
8-4-71	4	18	0	0	0

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