Advisory Board:

S. BERGSTROM, University of Lund, Sweden
A. M. BRUES, Argonne National Laboratory, Lemont, Illinois
G. O. BURR, Experiment Station, Hawaiian Sugar Planters' Association, Honolulu
R. CONSDEN, The Canadian Red Cross Memorial Hospital, Taplow, Maidenhead, Berkshire, England
A. B. HASTINGS, Harvard Medical School, Boston
H. HOLTER, Carlsberg Laboratory, Copenhagen, Denmark
R. D. HOTCHKISS, The Rockefeller Institute for Medical Research, New York
J. K. N. JONES, Queen's University, Kingston, Ontario, Canada
H. A. LARDY, University of Wisconsin, Madison
H. C. LICHSTEIN, University of Minnesota, Minneapolis
G. F. MARRIAN, University of Edinburgh, Scotland
B. L. OSER, Food Research Laboratories, New York
J. ROCHE, Collège de France, Paris
W. C. ROSE, University of Illinois, Urbana
A. TISELIUS, University of Uppsala, Sweden
D. D. VAN SLYKE, Brookhaven National Laboratory, Upton, Long Island, New York
Preface to the Series

Annual review volumes dealing with many different fields of science have proved their value repeatedly and are now widely used and well established. These reviews have been concerned primarily with the results of the developing fields, rather than with the techniques and methods employed, and they have served to keep the ever expanding scene within the view of the investigator, the applier, the teacher, and the student.

It is particularly important that review services of this nature should now be extended to cover methods and techniques, because it is becoming increasingly difficult to keep abreast of the manifold experimental innovations and improvements which constitute the limiting factor in many cases for the growth of the experimental sciences. Concepts and vision of creative scientists far outrun that which can actually be attained in present practice. Therefore an emphasis on methodology and instrumentation is a fundamental need for material achievement to keep in sight of the advance of useful ideas.

The current volume is the first of a series which is designed to try to meet this need in the field of biochemical analysis. The topics to be included are chemical, physical, microbiological and, if necessary, animal assays, as well as basic techniques and instrumentation for the determination of enzymes, vitamins, hormones, lipids, carbohydrates, proteins and their products, minerals, antimetabolites, etc.

Certain chapters will deal with well established methods or techniques which have undergone sufficient improvement to merit recapitulation, reappraisal, and new recommendations. Other chapters will be concerned with essentially new approaches which bear promise of great usefulness. Relatively few subjects can be included in any single volume, but as they accumulate these volumes should comprise a self-modernizing encyclopedia of methods of biochemical analysis. By judicious selection of topics it is planned that most subjects of current importance will receive treatment in these volumes.
The general plan followed in the organization of the individual chapters is a discussion of the background and previous work, a critical evaluation of the various approaches, and a presentation of the procedural details of the method or methods recommended by the author. The presentation of the experimental details is to be given in a manner that will furnish the laboratory worker with the complete information required to carry out the analyses.

Within this comprehensive scheme the reader may note that the treatments vary widely with respect to taste, style, and point of view. It is the editor's policy to encourage individual expression in these presentations because it is stifling to originality and justifiably annoying to many authors to submerge themselves in a standard mold. Scientific writing need not be as dull and uniform as it too often is. In certain technical details a consistent pattern is followed for the sake of convenience, as in the form used for reference citations and indexing.

The success of the treatment of any topic will depend primarily on the experience, critical ability, and capacity to communicate of the author. Those invited to prepare the respective chapters are scientists who have either originated the methods they discuss or have had intimate personal experience with them.

It is the wish of the Advisory Board and the editor to make this series of volumes as useful as possible and to this end suggestions will always be welcome.

David Glick

Minneapolis, Minnesota
January, 1954
CONTRIBUTORS

Klas-Bertil Augustinsson, Institute of Organic Chemistry and Biochemistry, University of Stockholm, Stockholm, Sweden

W. S. Bauld, Metabolism Department, The Montreal General Hospital, Montreal, Canada

R. M. Greenway, Metabolism Department, The Montreal General Hospital, Montreal, Canada

J. H. Humphrey, Department of Biological Standards, National Institute for Medical Research, London, England


D. A. Long, Department of Biological Standards, National Institute for Medical Research, London, England

B. A. Loveridge, Atomic Energy Research Establishment, Harwell, Didcot, Berks, England

William J. P. Neish, Cancer Research Unit, University of Sheffield, Western Bank, Sheffield, England (formerly Department of Biochemistry, University of Edinburgh, Edinburgh, Scotland)

W. L. M. Perry, Department of Biological Standards, National Institute for Medical Research, London, England

Harris Rosenkrantz, The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts


A. A. Smales, Atomic Energy Research Establishment, Harwell, Didcot, Berks, England

Ralph E. Thiers, Biophysics Research Laboratory of the Department of Medicine, Harvard Medical School, and Peter Bent Brigham Hospital, Boston, Massachusetts

vii
CONTENTS

Assay Methods for Cholinesterases. By Klas-Bertil Augustinson


α-Keto Acid Determinations. By William J. P. Neish

Microdetermination of Cobalt in Biological Materials. By Bernard E. Saltzman and Robert G. Keenan

Activation Analysis and Its Application in Biochemistry. By B. A. Loveridge and A. A. Smales

Contamination in Trace Element Analysis and Its Control. By Ralph E. Thiers

Chemical Determination of Estrogens in Human Urine. By W. S. Bauld and R. M. Greenway

The Infrared Analysis of Vitamins, Hormones, and Coenzymes. By Harris Rosenkrantz

Author Index

Subject Index

Cumulative Index
Assay Methods for
CHOLINESTERASES

KLAS-BERTIL AUGUSTINSSON, University of Stockholm

I. Introduction ........................................... 2
II. General Principles .................................... 4
   1. Choice of Substrate ................................ 4
   2. Enzyme Preparations ............................... 4
      A. Crude Enzyme Preparations ................. 4
      B. Purified Enzyme Preparations ........ 7
   3. Choice of Assay Method ......................... 10
   4. Definition of Units ................................ 12
III. Biological Methods ................................. 14
IV. Methods Based upon Acid Production from Choline Esters ........................................ 14
   1. Gasometric Methods ................................ 14
      A. Warburg Technique ........................... 14
      B. Barcroft Differential Technique ........ 19
      C. Van Slyke Technique ....................... 19
      D. Cartesian Diver Technique ............... 20
      E. Simplified Gasometric Technique .... 20
   2. Change in pH ....................................... 21
      A. Electrometric Measurement ................. 21
      B. Change in Color of an Indicator .......... 29
      C. Nephelometric Method ...................... 33
   3. Titrimetric Methods ................................ 34
      A. Indicator Methods ........................... 34
      B. Electrometric Methods ..................... 38
      C. Conductometric Methods ................... 39
   4. Colorimetric Methods ............................. 39
      A. Ferric Chloride Test for Acetic Acid .. 39
      B. Carbonaphthoxycholine as Substrate ... 40
   5. Ultraviolet Spectrophotometric Method ........ 41
V. Methods Based upon Choline (Thiocholine) Production ........................................ 42
VI. Methods Based upon Chemical Determination of Unreacted Acetylcholine .................. 43
VII. Methods Based upon the Use of Non-Choline Esters ........................................ 47
VIII. Methods for the Differentiation of Various Cholinesterases ............................. 51
      1. Specific Substrates ............................ 51
      2. Selective Inhibitors ........................... 54
         A. Selective Inhibition of Acetylcholinesterases .. 54
         B. Selective Inhibition of Cholinesterases of Group II ... 55
References ................................................ 56
I. INTRODUCTION

Cholinesterases (ChE)* are defined simply as enzymes which catalyze the hydrolysis of choline esters, the most important of which is acetylcholine (ACh). There has been considerable discussion during the last 15 years about the specificity of these enzymes, their role in physiological systems, the pharmacological importance of ChE inhibitors, etc. (14,16,18). Everybody taking part in this discussion on the biochemical level will probably agree with the following: There exist enzymes in the animal body which split ACh and other choline esters at a very high rate. None of these enzymes so far studied splits only esters of choline; non-choline esters are hydrolyzed as well but at a lower rate. The specificity of ChE therefore is not an absolute one and "true" ChE do not exist as far as the specificity is concerned.

The specificity patterns of various ChE studied from different origins differ greatly. This is also true for kinetic properties, substrate concentration patterns, and sensitivity to certain esterase inhibitors. It will be noted, however, that most data so far collected on these enzymes have been obtained with more or less crude enzyme preparations. A final picture, as far as specificity and kinetic properties are concerned, has to wait for a critical and comprehensive study of isolated systems from various sources.

The most useful feature for distinguishing ChE from "simple" esterases (ali-esterases, aromatic esterases, etc.) is probably the effect of eserine (physostigmine). Esterases which are not inhibited by 10^{-6}M eserine should not be designated as ChE. It is far easier to establish whether an esterase is "eserine-sensitive" (and thus a ChE) or "eserine-resistant" (other esterases) than to find out its specificity pattern. The ChE may then be regarded as a family of enzymes with many divergent properties. Within this family the various activities can be classified in at least two groups (I and II). These groups show differences in many respects, but overlapping of properties exists. It will also be understood that in each group esterases exist which show differences in certain properties when various animal species

* ChE is the abbreviation used in this survey for cholinesterases irrespective of the type of enzyme. AChE (acetylcholinesterases) is used to designate Group I cholinesterases. Owing to the heterogeneity and unknown physiological role of other types of ChE (Group II), specific names for these enzymes are for the time being best left open.
CHOLINESTERASES

are compared (15, 125, 130, 173). In each case, the designation of these enzymes according to their source is important. Methods for the differentiation of various ChE will be discussed later (Section VIII).

Group I cholinesterases, in the following termed acetylcholinesterases (AChE; other equivalent terms proposed: specific ChE, true ChE, e-ChE, aceto-ChE), have the physiological function of splitting ACh (23). These enzymes are inhibited by high ACh concentrations (optimum substrate concentration, $3-5 \times 10^{-4} M$) and split ACh at a higher rate than butyrylcholine, which is split at a very low rate or not at all. The activity substrate concentration pattern for various types of AChE differs when other choline esters are used. Thus, acetyl-$\alpha$-methylcholine, the $\beta$-isomer of which is a useful and more or less specific substrate for AChE, shows various relationships in this respect depending upon the source of enzyme used (14, 118). Among non-choline esters hydrolyzed by these esterases, acetates are split preferentially (2, 187).

Group II cholinesterases consist of all those eserine-sensitive esterases which do not have the properties and known function of the AChE. They have been designated using various terms (non-specific or unspecific ChE, pseudo-ChE, s-ChE, butyro- or butyryl-ChE, propiono-ChE, X-ChE; cf. footnote on page 2) and their physiological function is still unknown. Butyrylcholine is split at a higher rate than ACh (15) and may be useful as a specific (not absolute) substrate. Benzoylcholine has been considered a specific substrate, but it is also hydrolyzed by certain eserine-resistant esterases, and its use has “led to incorrect conclusions concerning the occurrence of pseudocholinesterase in the tissues of ruminants” (114). Some of these ChE split propionylcholine at a higher rate than butyrylcholine (and ACh) (125, 131). Among non-choline esters, butyrates (or propionates) are split preferably by esterases belonging to this group (2, 187).

AChE are present in nervous tissues, in the erythrocytes of most animals, and in the electric organs. Similar types of esterase are found in cobra venom, Helix blood, and other animal material. Other ChE, belonging to Group II, are present in the blood plasma of most vertebrate animals (not in the ruminant plasma), in the liver, pancreas, and in various smaller amounts together with AChE in nervous and other tissues.
II. GENERAL PRINCIPLES

1. Choice of Substrate

It is obvious from what is said in the preceding about the specificity of ChE that the choice of substrate in ChE studies is a delicate problem. In order to be sure that the activity measured is due to a ChE, activity measurements should always be made with a choline ester (preferably ACh) and the eserine-sensitivity of the esterase has to be proved. If the activity is measured irrespective of type of ChE, ACh is the substrate of choice; either of its available water soluble salts can be used (anions in moderate concentrations have no effect on the activity), but the iodide or bromide is probably to be preferred because they are more convenient to handle (non-hygroscopic compared with the highly hygroscopic chloride). The ACh concentration should be close to the optimum concentration when the enzyme is an AChE; when other types of ChE are studied (e.g., human serum ChE) a higher ACh is recommended (to obtain maximum activity).

The choice of substrate except ACh is obviously dependent on the purpose of the study. It is wise to find out the activity substrate concentration relationship in each case in order to obtain the substrate concentration which gives optimum or maximum activity. This is especially important because various substrates show differences in this respect. Butyrylcholine is the best substrate for human serum ChE and esterases with similar properties when these enzymes are mixed with other esterases (including AChE), e.g., whole human blood. Benzoylcholine has to be used with care because it is hydrolyzed both by ChE and eserine-resistant esterases. Acetyl-β-methylcholine can be used to measure AChE in crude preparations containing various esterases but should not be regarded uncritically as a "specific" substrate for AChE. In clinical work this ester is of value in measuring erythrocyte AChE activity in whole human blood.

The use of non-choline esters as substrates for ChE should be avoided (see Section VII). Such esters, however, may be of value in particular cases, e.g., in histochemical detection of ChE.

2. Enzyme Preparations

A. CRUDE ENZYME PREPARATIONS

(1) Tissue Homogenates. Whole homogenates of fresh tissue can be used in ChE studies and prepared by generally accepted proce-
If not used immediately, an organ may be stored after rapid freezing (in solid CO₂) without loss of ChE activity. In an homogenate prepared by the Potter-Elvehjem technique, the cell disruption is complete but the destruction of nuclei, mitochondria, and other cell particulates is minimized. The choice of medium for the homogenates depends on the uses intended, the method to be used for esterase assay, stability and solubility of the enzyme. The buffer solution recommended for the assay method is preferably used as the medium for the homogenate. Whole tissue homogenates should be used if the solubility of the esterase studied is unknown; centrifugation is not recommended in this case. Many ChE, especially the erythrocyte and nerve tissue AChE, are very difficult to get into solution. They may be extracted first after the disruption of the cell membrane and dissociation of the protein (lipoprotein) complex with which the enzymes seem to be associated. A number of procedures for extracting AChE from such cell material have been described, e.g., extraction with ammonia, chloroform, lysolecithin, chlorophyllin, butanol (see a recent review by Morton, 124). Other ChE, e.g., those of vertebrate blood plasma, cobra venom, Helix blood, are in natural true solution. Still other ChE, especially those belonging to Group II, may be regarded as being in true solution in the cytoplasm and therefore remain in the supernatant after complete removal of all particulate components of an homogenate.

The presence of endogenous salts in tissue homogenates is of importance for ChE activity (166) (see Section IV.1.A). When comparing the activities of various homogenates the salt concentration must therefore be known. The homogenates are preferably dialyzed against the buffer solution used in the assay procedure.

When the activity of intact cells is assayed it should be remembered that the quaternary ammonium compounds (as ACh) do not penetrate the cell membrane, a fact which will give results different from those obtained with an homogenate.

(2) Blood Sampling. Whole blood of mammals is best taken up in heparinized tubes, prepared by wetting the walls with a heparin solution (5 to 10 mg. per ml.) and subsequent slow drying in a gentle air stream to leave a coating of anticoagulant. Citrate, oxalate, and fluoride must be avoided because these ions form complexes with certain divalent metallic ions (e.g., Ca⁺⁺) which activate ChE activity. Serum and plasma show the same ChE activity, not influenced by heparin. The blood is centrifuged and the red cells washed
three times with physiological NaCl solution; the cells are then hemolyzed with distilled water to the same volume as the original blood volume.

Special techniques for blood sampling have been described, mainly for use in clinical studies on blood ChE. The following procedures are useful for certain assay methods described below.

(a) Micro Sample Method (76,110,191,206,208). Blood from a clear, dry finger tip is allowed to flow into heparinized capillary tubes (2 x 100 mm. in size) until the tubes are approximately 3/4 full, leaving 2.5 to 3 cm. free space at one end to permit flame-sealing of the tip of the tube in a Bunsen burner without overheating the blood sample. The other end of the capillary is plugged with solid paraffin (room-temperature). The capillary is now labeled with an adhesive-tape tag and then centrifuged (3000 r.p.m. for 50 min.) as soon as possible. So treated, the sample may be stored for a week or more (in a refrigerator) without significant change in ChE activity, or shipped (if necessary).

For analysis, the capillary is cut in sections (from the sealed end) with a sharp ampule file: 0-5 mm. (discarded), 5-35 mm. (packed cells section to be used), 35-45 mm. (interface discarded), 45-75 mm. (plasma section to be used). From the appropriate sections 0.02 ml. is drawn directly into a Sahli-type hemoglobin pipette. The erythrocyte sample in the pipette is discharged into 1.0 ml. of 0.01% saponin solution in a microbeaker, and the pipette is rinsed (three times) into the solution. The plasma sample is similarly discharged into 1.0 ml. of distilled water or buffer solution, the Sahli pipette being rinsed into the solution (three times). The samples so treated can then be used directly for the Michel electrometric method (Section IV.2.A) or any other suitable assay method.

(b) Sample Dried on Filter Paper (20,22). Exactly 0.05 ml. of whole blood is collected with a heparinized blood pipette from a punctured finger and then discharged on to a filter paper (5-7 cm. diameter; Munktell No. 3). The sample is carefully dried in air at room temperature (approximately 30 min.). For each complete analysis 4 blood samples (0.05 ml. each applied on separate papers) are used. If kept under dry conditions in the refrigerator the samples can be stored for one to two months without any significant loss of ChE activity. Simultaneous with the blood sampling, blood is taken for hematocrit. The blood spots are cut out, extracted with buffer, and used in the manometric assay method (20) (Section IV.1.A) or any other suitable technique. In this case the human erythrocyte esterase activity is obtained with acetyl-β-methylcholine as substrate and the plasma esterase activity with butyrylcholine as substrate.

(3) Stable Preparations as Standards of Cholinesterase Activity. Partly purified preparations can be stabilized by mixing with certain
albumins, bovine serum albumin being especially favorable. Certain amounts of the enzyme solution mixed with the stabilizer are placed on filter paper and air-dried; stored in the cold over a desiccant, these preparations are stable for months. The technique has been described for the electric tissue AChE (21) and erythrocyte AChE (57).

Solutions (0.2–1.0%) of purified AChE (from erythrocytes or electric tissue) are prepared in 0.3 M KCl, 3–4% serum albumin, and 0.008 M phosphate buffer (pH 7.4); for erythrocyte AChE the solution is recommended to contain also 0.5% hemoglobin (57). 0.02–0.04 ml. drops are placed on filter paper (Whatman No. 31 or Munktell No. 3) and then dried 30 minutes and stored over a desiccant in the cold. Cut disks are eluted with the buffer solution suitable for the assay method to be used.

B. PURIFIED ENZYME PREPARATIONS

(1) Acetylcholinesterases (AChE; Group I). Among favorable sources for obtaining highly active and purified AChE preparations (Table I) the electric organs are the most important (21). Electrophorus electricus (from the Amazon and Orinoco rivers) and Torpedo marmorata and T. ocellata (from the Mediterranean and the Atlantic coast of U.S.A.) are the most suitable species. Actually, the most active and purest AChE preparation obtained so far is that prepared by Rothenberg and Nachmansohn (127; see Table I, k) using Electrophorus electric organs. Mucin, present in considerable amounts and a distributing factor in the process of purification, is first removed and then the enzyme is fractionated with ammonium sulfate at various pH. The best preparation described had a specific activity of about 400 millimoles ACh (split per hour per mg. of protein) and was shown to contain one component both in electrophoresis and in ultracentrifuge runs. The enzyme shows comparatively great stability, also demonstrated for the Torpedo AChE (21).

The nucleus caudatus in the brain of mammals has a very high AChE activity and can be obtained in large amounts (e.g., from ox brain) for purification studies. No satisfactory procedure of purification, however, has yet been worked out.

A variety of methods have been reported for purifying AChE from the erythrocytes. The difficulty with this material is to get the enzyme in solution. The most successful procedure is probably that described by Cohen and Warringa (Table I, h). Frozen-dried stromata are extracted with butanol (in the cold), desiccated in vacuum, treated with
### TABLE I

<table>
<thead>
<tr>
<th>Type of ChE present in tissue</th>
<th>Principle of method used for purification</th>
<th>Degree of purification <em>X</em> fold</th>
<th>Yield per cent protein</th>
<th>Sp. activity split per hr.</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes</td>
<td>Adsorption on infusorial earth</td>
<td>20-30</td>
<td>—</td>
<td>—</td>
<td>Ref. b</td>
</tr>
<tr>
<td>Brain</td>
<td>Extn. with NH₄OH, pH 8.3</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>Ref. c</td>
</tr>
<tr>
<td>Brain</td>
<td>Extn. with CH₄Cl₄, adsorption on Ca₃(P₄O₁₀)₄</td>
<td>80</td>
<td>—</td>
<td>—</td>
<td>Ref. d</td>
</tr>
<tr>
<td>Brain</td>
<td>Ptn. of stroma with HAc, fractionation with (NH₄)₂SO₄, stabilization with serum or egg white</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Ref. e</td>
</tr>
<tr>
<td>Brain</td>
<td>Adsorption on infusorial earth, elution with NaHCO₃</td>
<td>23</td>
<td>—</td>
<td>—</td>
<td>Ref. f</td>
</tr>
<tr>
<td>Brain</td>
<td>Ptn. with Cd-acetate, solubilization with Tween 20 and toluene, electrophoresis, ultracentrifugation, gradient extraction</td>
<td>100</td>
<td>23</td>
<td>100</td>
<td>Ref. g</td>
</tr>
<tr>
<td>Brain</td>
<td>Extn. with butanol, etc.</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Ref. h</td>
</tr>
<tr>
<td>Brain</td>
<td>Fractionation with (NH₄)₂SO₄, use of surface active agents</td>
<td>260-400</td>
<td>23</td>
<td>250</td>
<td>Ref. i</td>
</tr>
<tr>
<td>Brain</td>
<td>Stable fractions described</td>
<td>3-4</td>
<td>—</td>
<td>—</td>
<td>Ref. j</td>
</tr>
<tr>
<td>Brain</td>
<td>Mucin free prepns., fractionated with (NH₄)₂SO₄ at various pH</td>
<td>120,000</td>
<td>—</td>
<td>15</td>
<td>Ref. k</td>
</tr>
<tr>
<td>Brain</td>
<td>Mucins not removed, otherwise according to Rothenberg and Nachmansohn</td>
<td>6,000</td>
<td>—</td>
<td>20</td>
<td>Ref. l</td>
</tr>
<tr>
<td>Rat</td>
<td>I (and II) Active fractions</td>
<td>20</td>
<td>20</td>
<td>24</td>
<td>Ref. m</td>
</tr>
<tr>
<td>Pig</td>
<td>I (and II) Stable fractions described</td>
<td>24</td>
<td>—</td>
<td>24</td>
<td>Ref. n</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Adsorption on infusorial earth</td>
<td>20-30</td>
<td>—</td>
<td>—</td>
<td>Ref. o</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Extn. with NH₄OH, pH 8.3</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>Ref. p</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Extn. with CH₄Cl₄, adsorption on Ca₃(P₄O₁₀)₄</td>
<td>80</td>
<td>—</td>
<td>—</td>
<td>Ref. q</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Ptn. of stroma with HAc, fractionation with (NH₄)₂SO₄, stabilization with serum or egg white</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Ref. r</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Adsorption on infusorial earth, elution with NaHCO₃</td>
<td>23</td>
<td>—</td>
<td>—</td>
<td>Ref. s</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Ptn. with Cd-acetate, solubilization with Tween 20 and toluene, electrophoresis, ultracentrifugation, gradient extraction</td>
<td>100</td>
<td>23</td>
<td>100</td>
<td>Ref. t</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Extn. with butanol, etc.</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>Ref. u</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Fractionation with (NH₄)₂SO₄, use of surface active agents</td>
<td>260-400</td>
<td>23</td>
<td>250</td>
<td>Ref. v</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Stable fractions described</td>
<td>3-4</td>
<td>—</td>
<td>—</td>
<td>Ref. w</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Mucin free prepns., fractionated with (NH₄)₂SO₄ at various pH</td>
<td>120,000</td>
<td>—</td>
<td>15</td>
<td>Ref. x</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Mucins not removed, otherwise according to Rothenberg and Nachmansohn</td>
<td>6,000</td>
<td>—</td>
<td>20</td>
<td>Ref. y</td>
</tr>
<tr>
<td>Man, ox</td>
<td>Fractionation with sulfates, electrophoresis and adsorption</td>
<td>20</td>
<td>20</td>
<td>24</td>
<td>Ref. z</td>
</tr>
<tr>
<td>Source</td>
<td>Horse</td>
<td>Man, horse, rabbit</td>
<td>Horse</td>
<td>Man</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>------------------------</td>
<td>--------------------</td>
<td>-------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pptn. with Pb-acetate</td>
<td>100-150</td>
<td>10-15</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Electrophoresis</td>
<td>10-15</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5,000</td>
<td>360</td>
<td>360</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fractionation with (NH₄)₂SO₄</td>
<td>5,000</td>
<td>2,100</td>
<td>2,100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol fractionation at low temp.</td>
<td>3,400</td>
<td>9,000</td>
<td>9,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ether fractionation</td>
<td>48</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol fractionation at low temp.</td>
<td>3,400</td>
<td>9,000</td>
<td>9,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethanol fractionation and combined chromatography on Cu₃(PO₄)₂ and Dowex 2</td>
<td></td>
<td>20,000</td>
<td>20,000</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>Dog</th>
<th>Pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>Fractionation with (NH₄)₂SO₄, adsorption on infusorial earth</td>
<td>Fractionation with (NH₄)₂SO₄, adsorption on infusorial earth</td>
</tr>
<tr>
<td></td>
<td>2,000</td>
<td>2,000</td>
</tr>
</tbody>
</table>

* Specific activity values have to be taken with caution because activity was measured under different conditions of pH and temperature, and by methods not comparable.

phosphate buffer (pH 8), and centrifuged at 4000 r.p.m. The supernatant, containing the enzyme in solution, is then fractionated using ammonium sulfate. Another method employs precipitation with cadmium acetate and solubilization of the enzyme with polyoxy-methylene sorbitan monolaurate (Tween 20) and toluene.

(2) Other Cholinesterases (Group II). The most active preparations of human serum ChE are those obtained by Strelitz and by Surgenor and Ellis (Table I). The latter preparation (Fraction IV-6-4), purified according to the well-known ethanol fractionation method, is probably the most suitable one for large-scale production of starting material for further purification. Such studies by Boman et al. (Table I, t) using Fraction IV-6-4 and combined chromatography on columns containing calcium phosphate and Dowex 2 have given successful results.

A highly active ChE preparation of dog pancreas has also been described.

3. Choice of Assay Method

The choice of assay method for ChE is mainly dependent on the purpose of the study, available laboratory equipment, required precision, and number of assays to be performed. In the following, a comprehensive review of the principles of various methods will be discussed, and, in addition, the details will be described for six procedures found to be the most useful in a variety of enzymatic studies (Table II).

The existence of reliable and accurate chemical methods for assaying ChE makes the far less accurate biological methods outdated. The most reliable and also most commonly employed method is the Warburg manometric technique. It can be applied in almost any study and with all types of ChE preparations under different experimental conditions. Measurements can be performed in series. The disadvantage of this technique is the fixed pH of the medium, and the rather complicated and expensive apparatus. Other all-round methods are the hydroxylamine-ferric chloride test and the titrimetric method. The former method is very convenient and may be run at any pH. The accuracy is not as good as with the Warburg technique or the titrimetric method, since the activity is obtained by difference (30% of the ester must be hydrolyzed). The titrimetric method is less convenient, but preferably it should be run with an automatic titrator for keeping the pH constant (by addition of alkali). In this technique,
**TABLE II. Methods Recommended for the Assay of Cholinesterases**

<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Technique</th>
<th>Special equipment needed</th>
<th>Medium</th>
<th>Particularly suitable studies</th>
<th>Precision</th>
<th>Expression of units of ChE commonly used</th>
<th>Reference to Section</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CO₂ production</strong></td>
<td>Manometric</td>
<td>Warburg app.</td>
<td>Bicarbonate buffer, pH 7.4</td>
<td>Routine assays; kinetic and other studies with great possibility for variation of exp. conditions at fixed pH</td>
<td>High</td>
<td>µl.CO₂/30 min.</td>
<td>IV.1.A</td>
<td></td>
</tr>
<tr>
<td><strong>Change in pH</strong></td>
<td>Electrometric</td>
<td>pH meter, preferably calibrated</td>
<td>Barbital buffer</td>
<td>Routine assays, especially in clinical work with blood</td>
<td>High to moderate</td>
<td>ΔpH/hr. (or µmoles of acetic acid)</td>
<td>IV.2.A</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Color change of indicator</td>
<td>Colorimeter, preferably calibrated</td>
<td>None</td>
<td>Field studies of human whole blood ChE</td>
<td>Low</td>
<td>Time for fixed color to occur</td>
<td>IV.2.B(2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Color change of indicator</td>
<td>None</td>
<td>No buffer</td>
<td>Rapid detn. with a variety of substrates and enzyme prepns., at various pH and other exp. conditions</td>
<td>High</td>
<td>ml. of 0.01 M NaOH/20 min.</td>
<td>IV.3.B</td>
<td></td>
</tr>
<tr>
<td><strong>Continuous titration to fixed pH</strong></td>
<td>Titrimetric</td>
<td>Automatic recording devices recommended</td>
<td>Little or no buffer required</td>
<td>Restricted to serum ChE in clinical work</td>
<td>Moderate</td>
<td>mg. of β-naphthol liberated</td>
<td>IV.4.B</td>
<td></td>
</tr>
<tr>
<td><strong>β-Carbonophth-oxocholine as substrate</strong></td>
<td>Colorimetric</td>
<td>Colorimeter</td>
<td>Barbital buffer, pH 7.4</td>
<td>Convenient under widely different exp. conditions</td>
<td>Moderate</td>
<td>µmoles of ACh/hr.</td>
<td>VI</td>
<td></td>
</tr>
<tr>
<td><strong>Formation of hydroxamic acid from unreacted ACh</strong></td>
<td>Colorimetric</td>
<td>Colorimeter</td>
<td>Phosphate buffer, pH 7.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
little or no buffer is required, in contrast to the gasometric and colorimetric methods.

The electrometric method, making use of the change in pH during ester hydrolysis, has been found very reliable and useful for assaying ChE in blood. It is therefore recommended either as in its original description or in a modification making use of a recorder for simultaneous registration of six enzyme reactions. The disadvantage of this method is the relative complexity and high cost of apparatus. The colorimetric modification of this technique is also recommended, either in a form useful in standard laboratories or in a form found satisfactory and rapid as a screening test. Finally, the use of carbonaphthoxycholine as substrate for human serum ChE or similar Group II ChE may be found convenient in some cases. The experimental temperature of choice for any of the methods preferred is 25°C. or 37.5°C., the former generally being the most convenient.

Review articles on assay methods for ChE have recently been published by Pochet (207) and Stumpf (209).

4. Definition of Units

It would be of great value if the results obtained using various methods could be made comparable. However, even if the results are expressed in the same activity units, a direct comparison will be of little value. This is primarily due to the different experimental conditions used in each method (temperature, pH, composition of medium, substrate concentration). It is recommended, however, that activity should always be expressed in absolute values, such as μmoles of ACh hydrolyzed and μl. CO₂ evolved. This is particularly advisable for those methods in which the activity values so far reported are impossible to recalculate in the form of absolute values, e.g., changes in pH and light absorption. Moreover, the expression of ChE activity on an arbitrarily chosen amount of enzyme (e.g., disks of dried blood on paper) is objectionable.

Whenever possible, the initial reaction velocity should be used in any quantitative assay of ChE. Depending on the method used, the activity can be expressed in various ways (Table II). Specific ChE activity is expressed best in μmoles of ACh hydrolyzed in one hour per mg. of protein. In other cases, the activity is expressed in units per ml. enzyme solution (e.g., blood plasma) or per mg. dry weight (after dialysis of enzyme preparation used).
<table>
<thead>
<tr>
<th>Test object</th>
<th>Investigation</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frog heart (Straub's cannula)</td>
<td>Hydrolysis of &quot;Vagusstoff&quot; and ACh by various tissues</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Blood plasma and erythrocytes; kinetics</td>
<td>b</td>
</tr>
<tr>
<td></td>
<td>Effect of narcotics on blood ChE</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td>ChE in various organs of rabbit, cat. dog</td>
<td>d</td>
</tr>
<tr>
<td></td>
<td>ChE in various nervous tissues</td>
<td>e</td>
</tr>
<tr>
<td>Guinea-pig intestine</td>
<td>Hydrolysis and synthesis of ACh by small intestine</td>
<td>f</td>
</tr>
<tr>
<td></td>
<td>Effect of anesthetics on rabbit blood ChE</td>
<td>g</td>
</tr>
<tr>
<td></td>
<td>ChE activity as function of serum and ACh concentration</td>
<td>h</td>
</tr>
<tr>
<td>Mouse intestine</td>
<td>Blood ChE in disease in pediatrics and adult medicine</td>
<td>i</td>
</tr>
<tr>
<td>Leech muscle</td>
<td>Mainly pharmacological; introduction of new method</td>
<td>j</td>
</tr>
<tr>
<td></td>
<td>ACh and ChE in portal blood</td>
<td>k</td>
</tr>
<tr>
<td>Frog rectus abdominis</td>
<td>Mainly pharmacological; introduction of new method</td>
<td>l</td>
</tr>
<tr>
<td></td>
<td>ChE in blood and tissues of invertebrates</td>
<td>m</td>
</tr>
<tr>
<td></td>
<td>Effect of denervation on muscle ChE</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Graphical method for assay of serum ChE</td>
<td>o</td>
</tr>
<tr>
<td></td>
<td>Method described for clinical assay of serum ChE</td>
<td>p</td>
</tr>
<tr>
<td></td>
<td>ACh and ChE in frog tissues</td>
<td>q</td>
</tr>
<tr>
<td>Cat's blood pressure</td>
<td>Inhibiting activity of drugs on tissue ChE</td>
<td>r</td>
</tr>
</tbody>
</table>

*Loewi, O., and Navratil, E., Pflügers Arch. ges. Physiol., 214, 678 (1926).*
*Galehr, O., and Plattner, F., Pflügers Arch. ges. Physiol., 218, 488, 506 (1928); Koderer, Y., ibid., 219, 181 (1928).*
*Plattner, F., and Galehr, O., Pflügers Arch. ges. Physiol., 220, 606 (1928).*
*Plattner, F., and Hintner, H., Pflügers Arch. ges. Physiol., 225, 19 (1930).*
*Hellauer, H., Pflügers Arch. ges. Physiol., 242, 382 (1939).*
*Abderhalden, E., and Paffrath, H., Fermentforschung, 8, 299 (1926).*
*Villanueva Novoa, A. M., Rev. fac. farm. y bioqutm., Univ. nac. mayor San Marcos (Lima, Peru), 10, 72 (1949).*
*Ströder, J., and Stüttgen, G., Z. Kinderheilk., 65, 179 (1947).*
*Minz, B., Naunyn-Schmiedebergs Arch. exp. Pathol. Pharmacol., 168, 292 (1932).*
*Feldberg, W., and Rosenfeld, P., Pflügers Arch. ges. Physiol., 232, 212 (1933).*
*Chang, H. C., and Gaddum, J. H., J. Physiol. (London), 79, 255 (1934).*
*Baci, Z. M., Arch. intern. physiol., 42, 47 (1935); Ann. physiol. physicochim. biol., 12, 663 (1936).*
*Scheiner, H., Compt. rend. soc. biol., 130, 748, 752 (1939).*
*Crivetz, D., Bull. acad. méd. Roumanie, 17, No. 4/6, 25 (1945).*
*Danielopolu, D., and Popesco, M., Bull. acad. méd. Roumanie, 18, No. 1/3, 95 (1946).*
The results obtained for human blood ChE with various assay methods have recently been discussed (20).

III. BIOLOGICAL METHODS

In the biological methods a known amount of ACh is incubated with the enzyme preparation. After a certain period of time the enzymatic ACh hydrolysis is stopped by enzyme inactivation (e.g., with a ChE inhibitor). The residual ACh is estimated pharmacologically by comparing its action with that of known ACh amounts on isolated organs (frog heart, guinea-pig and mouse intestine, leech muscle, frog rectus abdominis) or on the blood pressure of the cat.

Biological methods for the assay of ChE activities were chiefly used in the early years by the pioneer workers in this field. These methods are, however, less accurate and more cumbersome than the chemical methods. Biological methods are not recommended, and details of such methods will therefore not be given. The principal differences between biological and chemical methods are the high enzyme and low substrate concentrations used in the former methods compared with the excess of substrate and the dilute enzyme solution employed in the chemical methods. Owing to these and other differences the results obtained with the two types of assay methods are not comparable (53, 107).

The best-known investigations on ChE in which biological methods were employed are listed in Table III.

IV. METHODS BASED UPON ACID PRODUCTION FROM CHOLINE ESTERS

1. Gasometric Methods

The enzymatic hydrolysis of the ester proceeds in a bicarbonate buffered system, and through the acid production CO₂ is evolved in equivalent amounts and estimated manometrically.

A. WARBURG TECHNIQUE

Principle. The most frequently used method of assaying ChE activity is to apply the Warburg manometric technique. It was
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>NaHCO₃</th>
<th>Mg²⁺ or Ca²⁺</th>
<th>Other ions</th>
<th>Temp., vol., zyme, Substrate, Total Enzyme, mol.</th>
<th>Buffer soln., saturated with 5% CO₂, final Mg⁺ or Ca⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammon</td>
<td>1933</td>
<td>0.0336</td>
<td>0.0012</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Goldstein</td>
<td>1944</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Nachmansohn and Rothenberg</td>
<td>1951</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Dulbois and Mangan</td>
<td>1947</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Augustinsson</td>
<td>1948</td>
<td>0.034</td>
<td>0.0013 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Metcalf and March</td>
<td>1949</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Mendel and Hawkins</td>
<td>1950</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Aldridge</td>
<td>1951</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Callaway et al.</td>
<td>1952</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Myers</td>
<td>1953</td>
<td>0.025</td>
<td>0.04 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
<tr>
<td>Augustinsson</td>
<td>1950</td>
<td>0.034</td>
<td>0.0013 Mg⁺</td>
<td>Na⁺</td>
<td>0.115 Na⁺, 0.0012 Ca⁺, 0.0024 Mg⁺, 0.04 Mg⁺, 0.15 Na⁺, 0.15 Na⁺</td>
<td>pH 7.4-7.7, depending on the concn. of bicarbonate.</td>
</tr>
</tbody>
</table>
first used for this purpose by Ammon (11) and has since been described in a large number of modifications (Table IV). In most methods described, the medium (pH ~7.5) contains not only bicarbonate but also Ca²⁺ or Mg²⁺ as ChE activators, and Na⁺ and K⁺ depending on the type of ChE studies. This medium is used to dissolve the substrate and dilute (and/or dissolve) the enzyme preparation. The Warburg flasks have the standard size and shape; in some cases bigger flasks or flasks with special shape have been used. The total volume of the reaction mixture is generally kept to 2 or 3 ml., in some special cases a larger volume (e.g., 5 ml.) has been found useful (112). The enzyme is either placed in the main compartment of the flask or in the side bulb; the substrate is placed separated from the enzyme, with which it is mixed at zero time. Readings of the manometers are then made after various periods of time.

The procedures described below are essentially those used regularly in the laboratory of the present author for 15 years (14,20). This method has recently been used for the assay of bovine blood ChE (198).

**Apparatus.**

*Warburg Apparatus.* Total capacity of the flask should be approximately 20 ml. and preferably without center tube. Thermostat temperature, 25°C.

**Reagents.**

*Bicarbonate Ringer's solution* (*R₉₀*), pH 7.4. The following aqueous solutions are mixed: 100.0 ml. of 0.90% NaCl, 30.0 ml. of 1.26% NaHCO₃, and 2.0 ml. of 1.76% MgCl₂·6H₂O. The solution is saturated with a 95% N₂-5% CO₂ gas mixture (by volume). A fresh solution is made up each 2nd or 3rd day.

*Substrates.* Stable stock solutions of choline esters are prepared by dissolving the pure salts (chloride, iodide, bromide, etc.) in diluted HCl of pH 4.0. Immediately before use these stock solutions are diluted with four parts of bicarbonate buffer. The following concentrations are suggested for the most useful substrates:

<table>
<thead>
<tr>
<th>Ester</th>
<th>Stock soln., %</th>
<th>Final molar concn.: 0.4 ml. dild. soln. used, Total vol. of reaction, 3.00 ml.</th>
<th>G. of substr. equiv. to 1 µl. CO₂</th>
<th>Spont. hydrol. µl. CO₂ per 30 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine iodide</td>
<td>7.52</td>
<td>7.33 × 10⁻³</td>
<td>12.20</td>
<td>4.5</td>
</tr>
<tr>
<td>Butyrylcholine iodide</td>
<td>7.30</td>
<td>6.47 × 10⁻³</td>
<td>13.42</td>
<td>3</td>
</tr>
<tr>
<td>Acetyl-β-methylcholine iodide</td>
<td>7.15</td>
<td>6.64 × 10⁻³</td>
<td>8.75</td>
<td>4</td>
</tr>
</tbody>
</table>
**Enzyme Solutions.** Crude or purified enzyme preparations are used in bicarbonate buffer solution. If possible the concentration of the enzyme should be chosen so that the amount of CO₂ released during 30 minutes is 100-150 μl.

When the blood ChE activity is assayed for clinical routine purposes, spots of 0.05 ml. of whole blood applied and dried on filter paper are used (see Section II.2.A).

**Procedure.**

1. 1.60 (or 2.60) ml. of the enzyme solution is placed in the main compartment of the flask and 0.40 ml. of the substrate solution in the side bulb. For whole blood ChE determinations, the dried spot (corresponding to 0.05 ml. of blood) is cut out from the paper, placed in small pieces into the main compartment of the Warburg flask, and 2.60 ml. of bicarbonate buffer is added. Duplicate runs are always carried out.

2. The flasks are connected to manometers and the gas mixture (95% N₂ + 5% CO₂) bubbled through.

3. Before the contents of the flasks are mixed, temperature equilibrium is attained by shaking in the water thermostat (25°C.) for about 15 minutes.

4. The first manometer is read immediately before enzyme and substrate are mixed at zero time. At 1 or 0.5 minute intervals thereafter the other manometers are read and the contents mixed. Each manometer is read every 5-10 minutes for 35-40 minutes. In all determinations a thermobarometer (filled with 2.00 ml. of water) is used for corrections due to alterations in temperature and atmospheric pressure.

**Calculation.**

The amount of CO₂ expressed in μl. is plotted against time in minutes. The slope of the straight line thus obtained is used as a measure of enzyme activity. The extrapolated 30-minute value (= aₚ) minus the amount of CO₂ evolved during the same period of time by spontaneous (non-enzymatic) hydrolysis of the substrates (see above) is used as the unit in expressing the ChE activity and symbolized by bₚ ( = aₚ - spontaneous hydrolysis value). The bₚ values are easily converted to μmoles of substrate by using the correction factor 1/22.4, i.e., bₚ/22.4 μmoles of substrate hydrolyzed enzymatically during 30 minutes. The values thus obtained are then corrected to give ChE activity per mg. of protein, 0.1 ml. of blood, cell counts (176), etc.

**Comment.** The Warburg manometric technique is probably the most reliable method available of assaying ChE activity. It is useful in all types of experiments, including those with inhibitors and activators; it lends itself readily to the study of esterase activity in any crude tissue extract, body fluid, or purified preparation, irrespective of whether it is highly colored or turbid. The only disadvantage of the technique is that the pH of the medium cannot be
altered. The variation in pH over a relatively small range made possible by altering the bicarbonate concentration and the partial pressure of CO₂ is not recommended in studies on the pH dependence of ChE activity; in these cases other methods must be used.

The many modifications of the general technique can be applied as well. The only important difference of the various techniques described by various authors (see Table IV) is the composition of the buffer solution. The importance of various ions, especially of K⁺ and Na⁺, for various ChE is still a subject of controversy. The problem has been studied recently by Smallman and Wolfe (166), who compared the manometric and titrimetric techniques (Section IV.3). The latter method permits the ChE activity to be measured in water alone. It was demonstrated that both the relative activation by added salts (e.g., NaCl) and the absolute activity of ChE were reduced by the presence of NaHCO₃. Agreement between the two methods was obtained when the activity was measured in the presence of both NaHCO₃ and NaCl. It was also pointed out that the activation of ChE by added salts varies with homogenate concentration and that this effect is attributed to the presence of endogenous salts in tissue homogenates. Generally, the medium should have an ionic strength of about 0.1 to 0.2. It will be pointed out that the enzyme activity has to be measured if possible under optimum conditions even with regard to the presence of various metallic ions. To assay ChE activity in the absence of Mg²⁺ or Ca²⁺ for instance, especially in impure preparations, is not advisable because Mg²⁺ (or Ca²⁺) seems to be essential for catalytic activity and may be involved in the elementary processes (61).

One of the most careful workers using the Warburg technique in ChE studies is Hardegg who, together with Schaefer (77), has made a detailed study of the reaction using serum and erythrocyte enzymes. They also described a modification of the apparatus making possible the mixing of enzyme and substrate in the water bath thus avoiding a disturbance of the temperature equilibrium in the beginning of the reaction. Similar careful application of the Warburg technique to ChE studies is characteristic of the investigations published by Maier (108).

The commonly employed Warburg apparatus measures reactions occurring in a closed system of known and constant volume. At constant temperature the readings of pressure changes are made on the open side of the manometer after leveling the fluid on the vessel