of Cohn and Carter,[10] with the chief impurity in each case consisting of adenosine diphosphate (and an equivalent quantity of inorganic phosphate). The Sigma sample analyzed for 71% of dibarium adenosine triphosphate by dry weight, while that from Nutritional Biochemicals Corp. gave an 86% analytical figure. Kinetic dephosphorylation runs on these two samples under the same conditions (49.53°, *p*H 1.35) gave values of the respective rate constants (1.26 ± 0.08, $1.27 \pm 0.10 \times 10^{-5}$ sec.$^{-1}$) identical within experimental error, pointing to the relative hydrolytic inertness of the varying amounts of adenosine diphosphate found in these materials.

Triply distilled water was used in all the work, and the other reagents employed were of analytical grade.

Procedure for Kinetic Runs.—In a representative rate determination, a weighed sample of the dry barium salt was treated in a 50-ml. beaker with 20 ml. of water and the desired volume of a standard 1.3 *M* solution of hydrochloric acid. After stirring to effect solution of the salt, the barium ion was precipitated by the slow addition, with continual stirring, of the required amount of sodium sulfate. The mixture was allowed to settle for about 10 minutes at 5°, and then filtered through a prewashed analytical filter paper directly into the 50-ml. volumetric flask to be used as the reaction vessel. In a run employing added sodium chloride, a preweighed quantity of salt had already been transferred to the reaction flask before the filtration procedure. About 15 ml. of water was used to aid the filtration and in washing the precipitated barium sulfate. The solution was then made to about 48 ml., placed in a water thermostat held constant to ± 0.02° or better (as measured using Beckmann thermometers calibrated with an N.B.S. certified thermometer), and allowed to come to temperature during an approximately 15-minute interval. It was finally made to volume with water already at the bath temperature, and the first 2-ml. aliquot taken for analysis after about 20 minutes.

Aliquots of reaction mixture were quenched by addition to a large volume of water containing sufficient sodium acetate to neutralize all free acid, and the resulting solutions made to the proper volume for colorimetric estimation of orthophosphate present, using the method of Lowry and Lopez.[11] ATP concentrations at times *t* were evaluated from the initial weights of samples of known purity and the inorganic phosphate concentrations at *t*, assuming one orthophosphate ion to be derived from one hydrolyzed ATP molecule. The observed phosphate ion concentrations in the reaction mixture aliquots were corrected for the blank phosphate content of the initial samples.

First-order rate constants were calculated analytically in the standard manner, and were found to be reproducible within the limits of accuracy of the constants themselves (*i.e.*, about 5% on the average). Occasionally, in scattered runs made at the lower temperatures, some erratic behavior in phosphate production was noted at the start of a given run. In such a case, the rate constant was calculated from the steady portion of the rate curve following cessation of the abnormal initial behavior. Such rate constants were generally verified by duplicate runs of more normal character.

The *p*H values of reaction mixtures were determined on samples withdrawn from solution and cooled to room temperature, using a Beckman *p*H meter.

Acknowledgment.—The technical assistance of Mr. James A. Robinson in some of the kinetic determinations is gratefully acknowledged.

(10) W. E. Cohn and C. E. Carter, THIS JOURNAL, **72**, 4273 (1950).

(11) O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).

BETHESDA, MD.

[CONTRIBUTION FROM THE VENEREAL DISEASE EXPERIMENTAL LABORATORY, U. S. PUBLIC HEALTH SERVICE, UNIVERSITY OF NORTH CAROLINA, SCHOOL OF PUBLIC HEALTH]

A Method for the Preparation of a Stable Brain Fraction Containing Acetylcholinesterase[1]

BY HENRY TAUBER

RECEIVED AUGUST 22, 1952

A dried fraction containing acetylcholinesterase activity has been prepared from frozen pig brain. The dried material is stable and can be readily redissolved. Although not free of impurities, such preparation represents a convenient source of reproducible enzyme preparations having acetylcholinesterase activity. A few anticholinesterase experiments are presented to indicate the activity and specificity of the enzyme.

Introduction

The preparation of a stable mammalian brain acetylcholinesterase has not been reported previously. Without exception, fresh homogenates or supernatants of homogenates of mammalian brain tissue have been used as the source of this enzyme. Such preparations have obvious limitations as to purity, reproducibility and availability.

There is here reported a rapid, convenient and inexpensive method for the preparation of a stable fraction containing dry, soluble acetylcholinesterase from frozen pig brain. Such a method, while not offering a highly active product, provides a convenient source of the enzyme for *in vitro* studies of anticholinesterases. This is of particular value in the study of new anticholinesterase compounds, which have assumed considerable pharmacological and insecticidal importance. Experiments concerning the enzymic properties of this material are presented to demonstrate its activity as an acetylcholinesterase as opposed to a cholinesterase.

Materials and Solutions

Activator Buffer–Salt Solution.—For full activity the pig brain acetylcholinesterase requires bivalent ions and sodium chloride. The following activator buffer solution was employed: 5.8 g. of NaCl and 0.202 g. of $MgCl_2 \cdot 6H_2O$ were dissolved in 495 ml. of 0.2 *M* phosphate buffer of *p*H 7.0. The solution was readjusted to *p*H 7.0 with 3.5 *N* NaOH, and brought to a final volume of 500 ml. with distilled water.[2a,b] We found the salt concentrations as used in these experiments (final concentration of $MgCl_2$ 0.00027 *M* and NaCl 0.025 *M*) to be optimal for our enzyme preparation.

Substrates.—The acetylcholine bromide and the other substrates were dissolved in 0.1 *M* phosphate buffer at *p*H 7.0. The final substrate concentrations in the 4-ml. digests were 0.004 *M*. The acetylcholine concentration used in these investigations is optimal for acetylcholinesterase of conductive tissue (electric eel, mammalian brain).[2a,b,3]

(1) Presented before the Division of Biological Chemistry of the National Meeting of the American Chemical Society at Atlantic City, N. J., September 14–19, 1952.

(2) (a) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **185**, 479 (1950); (b) D. Nachmansohn and M. A. Rothenberg, *ibid.*, **158**, 653 (1945).

(3) K.-B. Augustinsson, *Nature*, **161**, 194 (1948).

Human Plasma Cholinesterase.—This enzyme was prepared from Cohn's Fraction IV-6 by Cutter Laboratories. The enzyme was dissolved in 0.6% sodium chloride. A trace of insoluble matter was removed by filtration. 0.28 mg. of this cholinesterase caused 50% hydrolysis of acetylcholine under the conditions of our experiments.

Sand.—Sea sand (washed and ignited; Fisher Scientific Company).

Experimental

Preparation of Soluble Pig Brain Acetylcholinesterase.—In this method autolysis was found to be essential, since without autolysis the mixture cannot be filtered or centrifuged. The presence of a fair quantity of toluene greatly facilitates autolysis and filtration.

Four hundred and fifty grams of washed, frozen pig brain was ground up into a uniform paste with 150 g. of sand. The ground material was placed into 250 ml. of 0.05 *M* sodium acetate–acetic acid buffer of *p*H 5.4, containing 1 ml. of Tween 21 (Atlas Powder Co.) and 15 ml. of toluene, and was stirred for 5 minutes with an electric stirrer. Then 6 g. of Hyflo was added and stirring was continued for 5 minutes. If the frozen tissue is first minced, the Waring Blendor instead of sand may be used for the disintegration and the extraction of the enzyme. This one-step procedure may be carried out within a few minutes and requires much less effort. The mixture was placed in a 1-l. erlenmeyer flask, and stoppered with a cork. The tissue was allowed to autolyze at 37°. During the first 4 hours the mixture was shaken occasionally. After 20 hours the mixture was filtered by gravity through a folded filter (Whatman No. 12). Usually 155 ml. of filtrate was obtained. The pink or tan filtrate was placed in 4 cellophane tubings and dialyzed at 4° for 36 hours against 10 liters of distilled water containing 5 ml. of toluene. The water was occasionally changed. During dialysis the volume of the enzyme solution increased to 175 ml.

As judged by the disappearance of color and turbidity, considerable purification was accomplished by the following procedure: to the combined dialyzed enzyme solution was added, per 100 ml., in small quantities and while stirring, a mixture containing 0.8 g. of bentonite, 0.8 g. of $Ca_3(PO_4)_2$ and 0.4 g. of Hyflo. Stirring was continued for 10 minutes. Then the mixture was filtered by gravity through a folded Whatman No. 12 filter. The solid may be removed in a centrifuge if desired. The solid portion was discarded. The filtrate contained practically all of the acetylcholinesterase originally present in the first filtrate. The filtrate was clear but slightly yellow. Removal of all the pigment by using excessive quantities of adsorbents resulted in an inactive solution. The solution was lyophilized. The yield was 3 mg. of enzyme preparation per ml. of treated filtrate. The lyophilized product is an almost white non-hygroscopic powder which is quite soluble in distilled water.

After dialysis, however, the enzyme solution need not be lyophilized. It may be kept in the refrigerator for many weeks provided a small quantity of toluene is added in order to prevent bacterial growth.

Proportionality between Enzyme Concentration and Activity.—The results shown in Fig. 1 demonstrate that there exists fairly good proportionality between enzyme concentration and brain acetylcholinesterase (BAChE) activity as measured by Hestrin's colorimetric (hydroxylamine) procedure.[4] In this graph 410 Klett units[4] represent the substrate without enzyme; 364 units represent 11%; 315, 22%; 273, 29%; and 230, 44% hydrolysis. The Klett–Summerson photoelectric colorimeter with green filter No. 54 was employed as recommended by Hestrin.[4] The digests contained 1 ml. of acetylcholine bromide (0.016 *M*), 0.5 ml. of activator–buffer solution of *p*H 7.0, 0.25 ml. to 1 ml. enzyme solution and distilled water to make 4 ml.; final acetylcholine concentration 0.004 *M*. The time was 30 minutes, the temperature 23°.

Specificity of Soluble Pig Brain Acetylcholinesterase.—This enzyme, as shown in Table I, splits acetylcholine 3 times faster than the enzyme splits (Me) mecholyl (acetyl-β-methylcholine bromide). Benzoylcholine chloride which is well known to be readily attacked by human plasma cholinesterase is not hydrolyzed by this acetylcholinesterase. These results confirm those of other investigators using brain tissue homogenates of certain mammals and are interpreted as showing that the preparation here described contains acetylcholinesterase rather than cholinesterase.[3,5] The properties of these enzymes have been recently reviewed in an excellent monograph.[5] All investigators appear to agree on the existence of a specific enzyme in the brain tissue (and the electric tissue of certain fish) which is different from the plasma cholinesterase and other esterases but which is probably identical or closely related to the erythrocyte acetylcholinesterase. It should be noted that cholinesterases of different species differ considerably in activity.

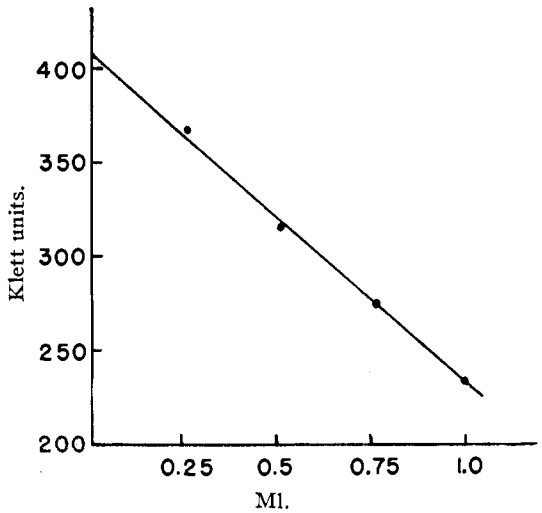

Fig. 1.—Proportionality between pig brain acetylcholinesterase concentration and activity.

TABLE I

ACTION OF SOLUBLE PIG BRAIN AChE ON VARIOUS SUBSTRATES

The digests contained 1 ml. (3 mg.) of enzyme solution, 1 ml. of substrate, 1.5 ml. of distilled water and 0.5 ml. of NaCl–$MgCl_2$–phosphate buffer of *p*H 7.0. Temperature 23°; time 30 minutes after addition of substrate; final substrate concentration 0.004 *M*. When 1 ml. of the digest, containing AChBr without enzyme, was tested by Hestrin's method, it gave a reading of 430 Klett units. Thus, 100 Klett units represent close to 0.001 *M* of acetylcholine. AChBr = Acetylcholine bromide; Me = Mecholyl (acetyl-β-methylcholine bromide); BeCHCl = Benzoylcholine chloride.

No.	Substrate	Klett units[1,4] Reading	Difference from zero time
1	AChBr	222	208
2	Me	370	65
3	BeChCl	620	None

Inhibition of Brain Acetylcholinesterase by Prostigmine and by Nu 683.—In Fig. 2 inhibition curves of prostigmine and Nu 683 (Dimethylcarbamate of (2-hydroxy-5-phenyl)-trimethylammonium bromide) are shown. Here, the % inhibition of the enzyme (BAChE) is plotted against the negative log of the molar concentration of inhibitors (*p*I). Prior to the addition of the acetylcholine, enzyme and inhibitors were kept at 23° for 20 minutes. One ml. of enzyme solution was employed; the other conditions were as in Table I.

It may be seen that prostigmine bromide caused 50% inhibition at a molarity of 3.6×10^{-7}, whereas Nu 683 showed 50% inhibition at a molarity of 4.4×10^{-7}.

Inhibition of Brain Acetylcholinesterase and Plasma Cholinesterase by Tetraethyl Pyrophosphate.—In Fig. 3 are recorded results on the inhibition of brain acetylcholinesterase (BAChE) and human plasma cholinesterase (PChE) by TEPP (Tetraethyl pyrophosphate, Monsanto, 40% pure). The brain enzyme was inhibited 50% by 5.3×10^{-8} *M* TEPP, whereas the plasma enzyme required 5×10^{-9} *M*

(4) S. Hestrin, *Nature*, **180**, 249 (1949).

(5) K.-B. Augustinsson, *Acta Physiol. Scand.*, **15**, Suppl. 52 (1948).

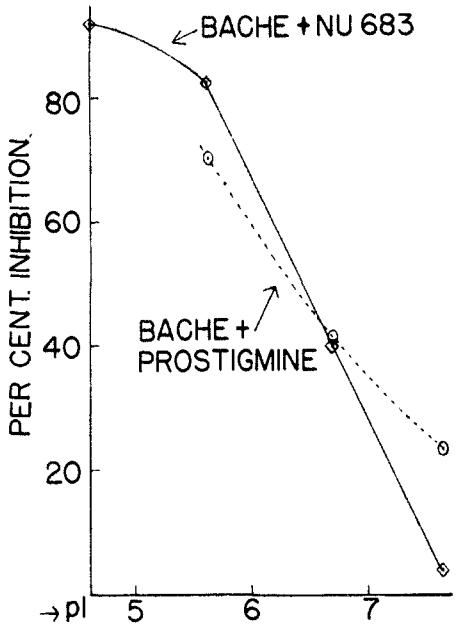

Fig. 2.—Inhibition of pig brain acetylcholinesterase by prostigmine and Nu 683.

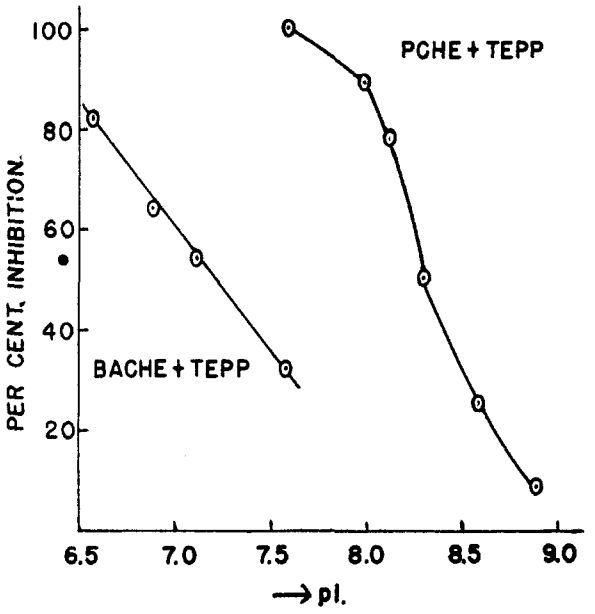

Fig. 3.—Inhibition of pig brain acetylcholinesterase and human plasma cholinesterase by TEPP.

of the inhibitor. This finding also confirms the acetylcholinesterase properties of the present preparation.

Activity of Pig Brain Homogenate.—Frozen pig brain (200 g.) was minced, mixed with activator-buffer salt solution (200 ml.) and homogenized for 3 minutes in a Waring Blendor. One-tenth ml. (50 mg.) of homogenate brought about 50% hydrolysis of acetylcholine (0.004 *M*) in 30 minutes at 23°. Thus, it appears that this quantity (50 mg.) of brain tissue is equal in activity to 3 mg. of our purified enzyme. There appears to be only a 3- to 4-fold purification over the homogenate (dry basis). It is well known, however, that homogenates of brain tissue contain several esterases capable of hydrolyzing esters of choline (reviewed in ref. 5).

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CHAPEL HILL, NORTH CAROLINA

[CONTRIBUTION FROM THE EASTERN REGIONAL RESEARCH LABORATORY[1]]

Isolation of Crystalline α-Lactalbumin from Milk[2]

BY WILLIAM G. GORDON AND WILLIAM F. SEMMETT

RECEIVED AUGUST 25, 1952

A protein, previously named "crystalline insoluble substance," has been isolated in crystalline form from the albumin fraction of bovine milk whey. The crystalline protein is homogeneous both in the electrophoresis apparatus and in the ultracentrifuge. Its sedimentation and diffusion constants check well with those reported for α-lactalbumin (Kekwick's lactalbumin). There is little doubt that the substances are identical and it is proposed that the protein in milk whey of approximate molecular weight 16,000 and electrophoretic mobility −4.2 at *p*H 8.5 be called α-lactalbumin. This protein comprises about 12% of the total proteins of whey.

Several investigators have reported the isolation of crystalline proteins from the so-called albumin fraction of bovine milk whey.[3] Except for Palmer's β-lactoglobulin and the crystalline albumin isolated by Polis, *et al.*,[4] none of these proteins has been characterized adequately and it has been difficult, if not impossible, to repeat the isolations as originally described. Svedberg and Pedersen in reviewing their studies of whey proteins in the ultracentrifuge[5] attribute the α-peak, one of the three major components in the sedimentation diagram of whey, to a lactalbumin isolated by Kekwick (unpublished). This protein, with constants $s_{20} = 1.9 \times 10^{-13}$, $D_{20} = 10.6 \times 10^{-7}$ and $M_s = 17{,}400$, was referred to as α-lactalbumin.

In 1939, Sørensen and Sørensen prepared from the albumin fraction of whey a crystalline protein which they designated "crystalline insoluble substance"[6] on the basis of its insolubility in water

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) A preliminary report of this work was presented at the 1952 Meeting of the American Society of Biological Chemists (*Federation Proc.*, **11**, 220 (1952)).

(3) T. L. McMeekin and B. D. Polis, "Advances in Protein Chemistry," Vol. V, Academic Press, Inc., New York, N. Y., 1949, p. 202.

(4) This protein isolated in trace amounts by B. D. Polis, H. W. Shmukler and J. H. Custer (*J. Biol. Chem.*, **187**, 349 (1950)) was shown to be identical with crystalline bovine serum albumin.

(5) T. Svedberg, *Nature*, **139**, 1051 (1937); T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford University Press, London, 1940, p. 379.

(6) M. Sørensen and S. P. L. Sørensen, *Compt. rend. trav. lab. Carlsberg, Sec. chim.*, **23**, 55 (1939).