hydrate (10 mg.) and 2-methyl-1,3-dioxolane (10 ml.), which was boiled as in A for 18 hours and worked up as in A, gave from methanol (1 drop of pyridine) 3-ethylenedioxyetiocholan-17β-ol acetate (0.92 g., 81%, m.p. 120°).

(G) **Attempted Dioxolanation with 1,3-Dioxolane.**—Cholestanone (1.0 g.) was dissolved in 1,3-dioxolane (10 ml.), *p*-toluenesulfonic acid monohydrate (10 mg.) added and the mixture boiled under reflux and anhydrous conditions for 5 hours. The usual work-up as in A gave only non-crystalline polymeric material[11] (3 g.).

Transformation of Ethylenedioxysteroids. Reduction of 3-Ethylenedioxy-Δ⁵-cholestene. 3-Ethylenedioxycholestane.—3-Ethylenedioxy-Δ⁵-cholestene (430 mg.) in absolute ethanol (20 ml.) was added to pre-hydrogenated 5% palladium–barium sulfate catalyst (40 mg.) suspended in absolute ethanol (15 ml.) and hydrogenated at atmospheric pressure. The theoretical amount of hydrogen (24.4 ml. at 24°, 760 mm.; 1.00 equiv.) was absorbed in 1.5 hours. The filtered solution on concentration under reduced pressure and cooling deposited 3-ethylenedioxycholestane (380 mg., 88%, m.p. 111–113°, $[\alpha]^{25}D$ +20.2°), identical with the product prepared directly from cholestanone.

Conversion of Δ⁴-Androstene-3,17-dione into Testosterone. 3-Ethylenedioxy-Δ⁵-androsten-17-one (VI).—A solution of Δ⁴-androstene-3,17-dione (1.0 g.) and *p*-toluenesulfonic acid monohydrate (15 mg.) in pure, carefully fractionated[8,21] 2-methyl-2-ethyl-1,3-dioxolane (16 ml.) was distilled slowly through a glass helices-packed column for 5.5 hours (10 ml. of distillate collected). Work-up as in A followed by crystallization from methanol (1 drop of pyridine) gave the 3-monodioxolane product, 3-ethylenedioxy-Δ⁵-androsten-17-one (840 mg., 74%, m.p. 197–198°, $[\alpha]^{24}D$ +15.4°; reported m.p. 199°[5b] and double m.p. of 194° and 202°[5c]). No attempt was made to recover androstenedione by acid hydrolysis of mother liquors.[18]

3-Ethylenedioxy-Δ⁵-androsten-17β-ol (VII).—3-Ethylenedioxy-Δ⁵-androsten-17-one (535 mg.) in absolute ethanol (40 ml.) was added to prehydrogenated W-4 Raney nickel catalyst[29] (0.5 g.) in absolute ethanol (25 ml.) and hydrogenation at atmospheric pressure ceased in 1 hour after the absorption of one equivalent (39.8 ml. at 27°, 760 mm.; 1.00 equiv.). Concentration of the filtered solution, after addition of 2-methyl-2-ethyl-1,3-dioxolane (5 ml.), under reduced pressure to remove the ethanol followed by refrigeration overnight yielded 3-ethylenedioxy-Δ⁵-androsten-17β-ol (472 mg., 90%, m.p. 181–182°).[30] The twice recrystallized (from methanol, 1 drop of pyridine) product (m.p. 183–184°, $[\alpha]^{24}D$ −41.7°) was identical with authentic testosterone dioxolane (m.p. and m.m.p. 182–183°, $[\alpha]^{24}D$ −43.1°) prepared by exchange dioxolanation.

Testosterone (VIII).—3-Ethylenedioxy-Δ⁵-androsten-17β-ol (1.0 g.) was dissolved in anhydrous acetone (50 ml.), *p*-toluenesulfonic acid monohydrate (50 mg.) added and the mixture boiled under reflux for 14 hours. Concentration of the resultant solution to a small volume (10 ml.) and precipitation with water gave a quantitative yield of slightly impure testosterone (0.87 g., 100%, m.p. 147–151°). Recrystallization from ether furnished the pure product (m.p. 152–154°, $[\alpha]^{24}D$ +109°), identical in all respects with authentic testosterone.

3-Ethylenedioxy-Δ⁵-androsten-17β-ol 17-Propionate.—A mixture of 3-ethylenedioxy-Δ⁵-androsten-17β-ol (500 mg.), pyridine (3.5 ml.) and propionic anhydride (1.5 ml.) was heated at 95° for 14 hours. Dimethylaniline (3 ml.) was added, the solution concentrated under reduced pressure almost to dryness and treated with methanol (5 ml.). Refrigeration overnight gave 3-ethylenedioxy-Δ⁵-androsten-17β-ol 17-propionate (505 mg., 86%, m.p. 196–198°); the once recrystallized (from methanol, 1 drop of pyridine) product (m.p. and m.m.p. 201–202°, $[\alpha]^{23}D$ −47.7°) was identical with a sample prepared by exchange dioxolanation of testosterone propionate.

Acknowledgments.—We are indebted to Dr. Georg Rosenkranz and Dr. Carl Djerassi of Syntex, S.A., to Dr. Caesar Scholz of Ciba Pharmaceutical Co., to Dr. E. B. Hershberg of Schering Corp., and to Nyegaard and Co. A.-S., Oslo, Norway, for samples of most of the steroids used in these studies.

(29) A. Pavlic and H. Adkins, THIS JOURNAL, **68**, 1471 (1946).

(30) The same yield was obtained when the reduction was carried out by lithium aluminum hydride in tetrahydrofuran solution.

SEATTLE 5, WASHINGTON

[CONTRIBUTION FROM THE U. S. NAVAL MEDICAL RESEARCH INSTITUTE]

Nature of the Acetyl Cholinesterase Surface. I. Some Potent Competitive Inhibitors of the Enzyme[1a,b]

BY S. L. FRIESS AND W. J. MCCARVILLE

RECEIVED AUGUST 19, 1953

Highly purified acetyl cholinesterase from electric eel tissue has been used in a test of the theory that one class of reversible, competitive inhibitors of the enzyme has as an essential feature of structure a locus of high electron density separated by roughly a $-CH_2CH_2-$ unit's distance from a polymethylated nitrogen atom (preferably quaternary). Specific compounds tested in this category include the N-(β-trimethylammonium)-ethyl derivatives of pyrrolidine and piperidine, as well as the corresponding dimethylamino compounds. These materials were found to inhibit competitively and to possess an anticholinesterase activity equal to or greater than that shown by eserine or prostigmine, as measured by the dissociation constants of the enzyme–inhibitor complexes. These data are discussed in terms of the corresponding fine structure of the enzyme surface. It has also been observed that the enzymatic hydrolysis rates for acetyl choline are virtually independent of ionic strength over the range 0.18–0.56, at a substrate concentration yielding the maximum rate, and that phosphate ions are not essential to enzymatic activity. Solution constituents which complex Mg(II) ion markedly reduce the activity.

Introduction

As part of a program designed to investigate the surface configuration of the enzyme acetyl cholinesterase (AChE), and ultimately to unravel details of the mechanism involved in its catalysis of the hydrolysis of acetylcholine (AC) in intact nervous tissue, the present study deals with the relative effects of some powerful inhibitors of the hydrolysis reaction. Working from the structures and the approximate distances of charge separation in these competitive inhibitors, and the assumption that these quantities are not perturbed on adsorption by the enzyme, certain tentative conclusions may be drawn as to the complementary fine structure of the enzymatic surface. These results constitute a logical extension of the excellent studies of Nachmansohn, Wilson and collaborators[2] leading to their postulation of the existence of two active sites per

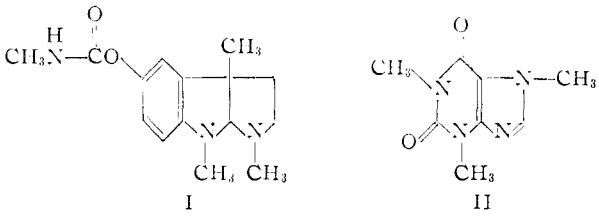
(1) (a) The opinions in this paper are those of the authors, and do not necessarily reflect the views of the Navy Department. (b) Presented in part before the Division of Biological Chemistry, The American Chemical Society, Chicago, Illinois, September 6–11, 1953.

(2) For a detailed review of their work in this field, see D. Nachmansohn and I. B. Wilson, *Advances in Enzymol.*, **12**, 259 (1951).

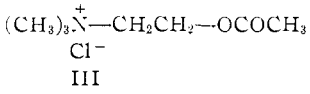
catalytic unit of the enzyme, the so-called "anionic" and "esteratic" sites.

To obtain accurate data on effective competition between the substrate molecule acetylcholine and the inhibitors selected, it was desirable to have as nearly pure and highly active an enzyme preparation as possible. Consequently, the AChE was prepared from the electric organ of the Brazilian eel *Electrophorus electricus* (Linnaeus) essentially according to the procedure of Rothenberg and Nachmansohn.[3]

Potential inhibitor structures were selected by means of the following considerations. In two representative cholinesterase inhibitors of the reversible, competitive type, namely, eserine (I) and caffeine (II)



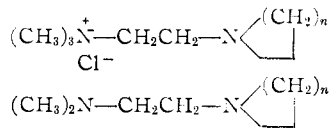
I II



III

which are to compete with AC (III) for sites of adsorption on the enzyme, several structural features can be taken as roughly comparable to those found in the substrate III. Both I and II possess a region of high electron density (—OCO— and —C=O functions, respectively) situated at nearly a —CH_2-CH_2— group's distance from a tertiary amine center.[4] In III, the corresponding groups at a similar distance of separation might be inferred to be the —OCO— ester function (with *over-all* high electron density possibly leading to attraction at the esteratic site on the enzyme) and the quaternized amine function. Consequently, it is conceivable that the effective skeletal portions of I and II for anticholinesterase activity might simply include a region of localized high electron density and a polymethylated nitrogen (tertiary or quaternary) separated by the approximate distance represented by a single —CH_2CH_2— unit.

In test of this hypothesis, the series of compounds shown below was synthesized and subjected to kinetic tests for the strength of enzymatic inhibition that they might display.



Compound	n
IV	2
V	3
VI	2
VII	3

Variation in the size of the ring containing the N function designed to serve as the site of localized high electron density could also serve to outline within rough limits the steric requirements of the enzymatic site adsorbing this particular functional part of the molecule, with the strength of inhibition of enzymatic activity being taken as a measure of the binding power between enzyme and inhibitor.

Also included in the kinetic work using this purified enzyme preparation were experiments showing the catalytic activity of the enzyme as a function of the total ionic strength of the medium, and as a function of the nature of the buffering constituents present.

Results

Inhibitor Studies.—All of the kinetic runs in this series were carried out at 25.12°, and in the phosphate buffer of *p*H 7.4 containing 0.1 *M* NaCl and 0.01 *M* $MgCl_2$ as described by Nachmansohn.[2] Plots of time *vs.* the volume of 0.0127 *N* base required to keep constant *p*H were quite linear, and initial velocities of reaction were obtained from these graphs. Initial substrate concentrations were held constant at 3.33×10^{-3} *M*, which is about the optimum value for the enzyme concentration employed. Inhibitor concentrations in the final reaction mixtures were held to the region of $1\text{–}10 \times 10^{-7}$ *M*. Representative rate curves for an uninhibited (blank) catalyzed hydrolysis of AC and for one inhibited by a 5.79×10^{-7} *M* concentration of compound VII are shown in Fig. 1.

Tests for the competitive nature of the inhibition were carried out using the equation of Wilson[5] given as 1 below.

$$\frac{v}{v_I} = 1 + \frac{K_m}{K_I}\left(\frac{[I]}{K_m + [S]}\right) \qquad (1)$$

Here, v is the initial velocity for the reaction with no added inhibitor, v_I that with inhibitor present, K_m the Michaelis constant for the uninhibited reaction under the given conditions, K_I the dissociation constant for the reversibly formed enzyme–inhibitor complex, and [I] and [S] the initial concentrations of inhibitor and substrate, respectively. At constant concentrations of enzyme and AC initially, a plot of v/v_I as a function of [I] should be linear and show an intercept of 1 on the v/v_I axis if the inhibition is of the competitive type, with AC and inhibitor at equilibrium with respect to the same catalytic sites on the enzyme. Such plots were linear over the same tenfold range in concentration for each of the inhibitors, and possessed intercepts of unity. Each gave a least-squares fit of the data well within the limits of precision of the individual v determinations. A representative plot is given in Fig. 2 below for compound VII.

Having satisfied the conditions above for compe-

(3) M. A. Rothenberg and D. Nachmansohn, *J. Biol. Chem.*, **168**, 223 (1947).

(4) A qualitative comparison of the internuclear separation between the O and N atoms para to each other in the benzenoid ring of I, for example, and between the N and O atoms attached to the —CH_2CH_2— unit in III can be made by use of data on covalent bond distances. Using the convenient tabulation of G. W. Wheland, "The Theory of Resonance," John Wiley and Sons, Inc., New York, N. Y., 1944, p. 102, the separation distance in I is of the order of 5.5 Å., while that in III at maximum extension of the molecule approximates 4.4 Å. The corresponding *mean* separation distance of these atoms in the normal molecule III is only about 2.3 Å. though, using the Eyring formulation for a flexible chain, which raises the possibility that an extended configuration of III is a prerequisite for adsorption on the catalytic sites also adsorbing I. However, this possibility and the matching of enzyme and inhibitor distances in general again rest on the presumptions that the enzyme presents a fixed matrix to the entity to be adsorbed and that solvent molecules do not form part of the bonding structure.

(5) P. W. Wilson, "Respiratory Enzymes," H. A. Lardy, ed., Burgess, Minneapolis, Minn., 1949, p. 24.

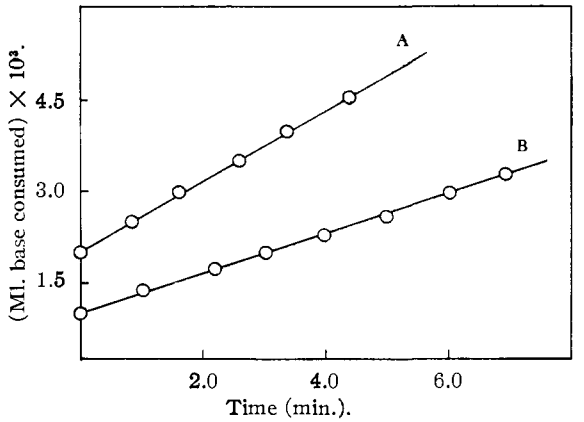


Fig. 1.—A, rate curve for the catalyzed hydrolysis of 3.33×10^{-3} M AC in the absence of inhibitor; B, rate run in the presence of a 5.79×10^{-7} M concentration of VII.

titive inhibition, these plots were then used to calculate K_I values for each of the inhibitors tested. First, the slopes of the best least-squares lines through the experimental points were determined analytically, with each slope yielding a unique $K_m/[K_I(K_m + [S])]$ value. The value of K_m was taken from the data of Wilson and Bergmann[6] as 2.6×10^{-4}, which then permitted the calculation of K_I values for the inhibitor–enzyme complexes. These values and their mean deviations are listed in Table I, together with the corresponding values[2] for the potent inhibitors eserine and prostigmine at neutral pH. The mean deviations shown are derived from the least-squares fitting of the v/v_I *vs.* [I] plots, and do not reflect the absolute accuracy of the K_I values. The latter depend directly on the accuracy of the K_m value employed in the calculation. However, even a systematic error in K_I values arising from this source does not materially affect their subsequent comparison.

TABLE I

DISSOCIATION CONSTANTS (K_I) FOR AChE–INHIBITOR COMPLEXES

Temp., 25.12°; pH 7.4; [S] = $3.33 \times 10^{-3} M$

Compound	$K_I \times 10^3$	Compound	$K_I \times 10^3$
IV	2.3 ± 0.1	VII	6.4 ± 0.2
V	1.6 ± .1	Eserine (I)[a]	6.1
VI	4.9 ± .3	Prostigmine[a]	16

[a] See reference 2.

For the dissociation process 2, in which

$$\text{Enz·I} \underset{}{\overset{K_I}{\rightleftharpoons}} \text{Enz} + \text{I} \qquad (2)$$

smaller K_I values imply a greater degree of inhibition of the reaction with substrate, it is seen from Table I that all of the compounds tested are at least as potent as eserine and far more potent than prostigmine. Compounds IV and V in particular are, respectively, 2.7 and 3.8 times more effective in covering catalytic sites than the most powerful natural inhibitor, eserine.

In terms of structure of the inhibitor *vs.* the geometry of the enzyme surface, with the lowering of K_I values being taken as indicative of a progressively

(6) I. B. Wilson and F. Bergmann, *J. Biol. Chem.*, **186**, 683 (1950).

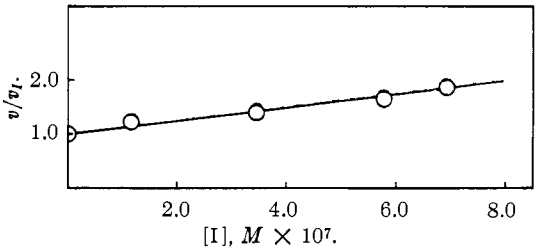
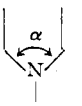


Fig. 2.—Plot of v_I/v *vs.* [I] for compound VII.

closer approach to the complement of the surface structure,[7] compounds IV and V offer some interesting support to the theory discussed in the Introduction. With the quaternary methylated nitrogen function serving as the point of attachment to the "anionic" enzymatic site,[2] as for the natural substrate III, and two methylene groups serving to bridge the requisite distance on the surface to the second site of binding, it would appear that a simple functional source of localized high electron density such as the ring N atom satisfies the electrostatic and steric requirements of the second site, to complete the binding process and produce a really effective inhibitor. This high degree of attraction on the part of AChE for a molecule having *both* the quaternary and high electron density functions (resulting in a very small K_I value) is in marked contrast to the enzymatic response to entities such as $(CH_3)_4N^+$ ion,[8] which possess only the positive quaternary function and are many orders of magnitude less efficient in their function as inhibitors of the enzyme.

The relative efficiency of IV and V, differing in essential structure chiefly at the angular disposition of groups about the ring nitrogen, seems to be in line with the relative order of basicities of the secondary ring bases themselves, *i.e.*, K_B values in water at 25° for pyrrolidine[9] and piperidine are, respectively, 1.3 and 1.6×10^{-3}. Hence, the larger ring size leads to greater basicity of the N function, and inferentially to greater basicity and electron localization on the ring N of V as compared to IV with the resulting increase in inhibitory power of V over IV as given by the K_I values in Table I. It might also be noted in this connection that the ring N atom in the six-membered cycle of V has somewhat smaller steric requirements than those for compound IV, so that the relative order of reactivity for these two might reflect a cavitation in the enzyme's surface best fitted by a structure with a small angle α, less than that found for pyrrolidine and approaching in value that found in piperidine.



It is also evident from the data of Table I that a

(7) This treatment neglects the contribution to K_I arising from any differences in degree of solvation among the individual members of the series IV–VII, but such differences might be expected to be small, in at least the pairs of compounds IV–V, and VI–VII. Here, the pairwise variation in structure is not a deep-seated one, and solvation should not vary significantly.

(8) See, for example, the compilation of data in the paper by I. B. Wilson, *J. Biol. Chem.*, **197**, 215 (1952).

(9) L. C. Craig and R. M. Hixon, THIS JOURNAL, **53**, 4367 (1931).

quaternized nitrogen atom is not essential for achieving relatively high anticholinesterase activity. Compounds VI and VII are as good as eserine and far better than prostigmine in inhibitory power, and only possess two tertiary amine centers. Here, the order of activity with respect to ring size is reversed, as compared to compounds IV and V, but an unequivocal comparison between the two groups is not possible since either end of the ditertiary base might be serving as the high electron density function for adsorption at the esteratic site. Interestingly, though, the loss of quaternization in VI and VII leads to a drop in activity (as compared to IV and V) by a factor of 2 to 4.

Effect of Ionic Strength on AChE Activity.—A series of kinetic runs was made in which, at constant initial concentrations of enzyme, substrate and buffering constituents, the total ionic strength μ was varied by the addition of successive increments of sodium chloride. The rate results are shown in Table II. Relative rates were taken as ratios from the slopes of the rate plots.

TABLE II

VARIATION IN RELATIVE RATE WITH IONIC STRENGTH μ

pH 7.40; temp., 25.12°

μ of reaction mixture	Relative rate
0.176[a]	1.00
.205	1.02
.238	0.99
.265	1.02
.292	0.97
.563	0.95
Av.	0.99 ± 0.02

[a] Standard phosphate buffer.

From Table II it is seen that over a factor of threefold variation in μ of the medium, the catalyzed hydrolysis rates are quite insensitive to change in ionic strength, and indeed are essentially constant within the limits of experimental error. This of course holds true for the conditions used here in which the enzyme is operating at or near saturation with respect to the substrate, and hence the rates probably reflect only the inertness toward a medium change of the final reaction of the enzyme–substrate complex leading directly to products. At lower substrate concentrations, the association process might well demonstrate sensitivity to change in the ionic strength of the medium.

Effect of Buffering Constituents on AChE Activity.—The use[2] of a 0.01 M concentration of the cation Mg^{++} to achieve high catalytic activity from the enzyme made it a matter of interest to investigate the effect of buffering agents offering a variation in complexing power for the bivalent metal. Also, the possibility of a specific requirement for phosphates in AChE activity was checked in the same set of experiments.

Without regard for the efficiency of buffering attainable at pH 7.4, four separate solutions were made which contained 0.1 M NaCl and 0.01 M $MgCl_2$ in common, and individual 0.015 M concentrations of the following constituents: phosphate, tris-(hydroxymethyl)-aminomethane (THAM), citrate and borate. Dilute acid or base were used as needed to adjust initial pH values to 7.40, and the resulting solutions were used as buffer base for enzymatic hydrolyses run under identical conditions. The results of these runs are shown in Table III.

TABLE III

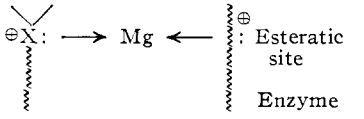
EFFECT OF BUFFERING CONSTITUENT ON ENZYMATIC AC HYDROLYSIS

$[Ac]_0 = 3.33 \times 10^{-3} M$; temp., 25.12°; pH 7.40

Buffer constituent	Slope of rate plot, (ml. base/min.) × 10^3	Relative rate
Phosphate	6.20	1.00
THAM	5.16	0.83
Borate	1.07	.17
Citrate	0.27	.04

Comparison of relative rates in Table III for phosphate *vs.* THAM buffers indicates that monophosphate species are not essential for the enzymatic catalysis of AC hydrolysis. However, the ions of boric and citric acids are observed to lower the activity by large factors, presumably by their sequestering effect on the essential Mg^{++} ion.

This vital role of free Mg^{++} as an essential trace metal for AChE enzymatic catalysis has been somewhat neglected in published discussions[2] of the rôle of the AChE surface in catalysis. Conceivably, the binding of a locus of high electron density (in substrate or inhibitor) to the esteratic site on the enzyme might be mediated by the Mg^{++} ion through some such coördinate bonding as



in which the site is assumed to possess nucleophilic character exclusively.

Experimental[10,11]

Enzyme Preparation.—In the preparation of the stock enzyme solution used for the major portion of this work, 1 kg. of raw electric eel tissue was subjected to the toluene treatment, extraction procedure and fractionation with ammonium sulfate solutions of varying concentrations, according to the procedures of Rothenberg and Nachmansohn.[3] The final enzyme fraction was dissolved in the standard buffer employed by Nachmansohn and co-workers (pH 7.40, 0.1 M in sodium chloride, 0.01 M in magnesium chloride, 0.015 M in phosphate) and possessed a total hydrolytic activity of 0.63 kg. of acetylcholine per hour, and a specific activity of 10.2 g. of AC hydrolyzed per hour per mg. of protein. Very high dilutions of this stock material (stored at about 4°) were made with buffer and used for kinetic work within a few hours after preparation. However, kinetic tests indicated that there was little or no drop in hydrolytic activity when diluted AChE solutions (1:12,500) were allowed to stand for 12 hours at room temperature.

Preparation of Inhibitors.—In general, the amine salts were prepared by condensation of the primary chloride (β-dimethylaminoethyl chloride or its methochloride derivative) with the appropriate cyclic amine. After refluxing the chloride with excess amine for periods of 24 hours or more, the product free bases were extracted and distilled through a 70-plate column. The quaternary salts IV and V were isolated as the dipicrates, which were crystallized to purity and then converted to the chloride derivatives by passage through columns of the anion-exchanger Dowex-1. The

(10) Melting points are uncorrected.

(11) Analyses by courtesy of: (a) Dr. W. C. Alford, Microanalytical Laboratory, National Institute for Arthritis and Metabolic Diseases, N.I.H.; and (b) Micro-Tech Laboratories.

esulting solutions were evaporated to dryness and the salts recrystallized repeatedly from absolute methanol.

The physical and analytical properties of these materials are summarized in Table IV. The quaternary salts were analyzed as the dipicrates, because of the extreme ease of decomposition of the chlorides at elevated temperatures.

TABLE IV

PHYSICAL AND ANALYTICAL CONSTANTS

Compound		IV	V	VI[a]	VII
B.p.	°C.			83–84[b]	84–85
	Mm.			40	25
$n^{25}D$				1.4551	1.4617
M.p., °C.		202–203 dec.	209–210 dec.		
Dipicrate: %					
Carbon	Calcd.	41.04	42.04	67.59[a]	41.04
	Found	41.07	42.16	67.68[a]	41.54
Hydrogen	Calcd.	4.27	4.49	12.70	4.27
	Found	4.22	4.34	12.65	3.92
Nitrogen	Calcd.	18.24		19.71	
	Found	18.24		19.75	

[a] Analyzed as the free base. [b] Lit. value, 92–94° (48 mm.); L. M. Rice, C. H. Grogan and E. E. Reid, THIS JOURNAL, **75**, 2261 (1953).

Enzymatic Rate Determinations.—The hydrolysis reaction of acetylcholine catalyzed by AChE was followed by titration of the liberated acetic acid, at a constant *p*H of 7.4. Triply recrystallized AC was used in all the rate determinations.

For the reaction vessel, a 4-ml. titration cell was fitted with a water jacket through which water held at constant temperature (25.12 ± 0.02°) was circulated, with a small glass capillary rod for mechanical stirring, and with a small pair of standard electrodes for the Beckman *p*H Meter.

In a representative rate run, 2.50 ml. of buffer was pipetted into the cell and brought to temperature. Then in succession were added 0.50 ml. of the diluted enzyme solution, 0.10 ml. of an inhibitor solution whenever used, and finally 0.10 ml. of an acetylcholine chloride solution of sufficient strength to make its initial concentration in the reaction mixture $3.33 \times 10^{-3} M$. Enzyme concentrations were of the order of 2.0×10^{-5} mg. per ml. After approximately 1 to 2 minutes of stirring to come to bath temperature, readings of the volume of 0.01274 *N* base required to keep the *p*H constant at its initial value were begun. Base was added manually from an ultramicroburet dipping into the reaction mixture. Plots of volume of base added *vs.* time were in general quite linear for about the first five or six minutes of reaction, during which time eight to ten experimental points were obtained.

Runs to determine the normal activity of any given dilution of enzyme were made just prior to the use of that dilution for any determination employing an inhibitor. All inhibitor solutions were freshly prepared.

All points in an enzymatic hydrolysis run were corrected for the spontaneous, non-enzymatically-catalyzed hydrolysis of AC. Under the conditions of these experiments, this spontaneous reaction was found to have a first-order rate constant of 1.1×10^{-6} sec.$^{-1}$, which led to rates amounting to only 1 or 2% of the observed total rate of hydrolysis in any given run. Results on duplicate rate determinations indicated that they were reproducible in general to well within 5%. In any given run, the slope of the reaction curve could be determined within about this same degree of precision (5% or better).

Acknowledgment.—We are indebted to Mr. C. W. Coates and Drs. D. Nachmansohn and H. S. Polin for their most generous aid and advice in the procurement of electric eel tissue and its initial extracts.

BETHESDA, MARYLAND

[CONTRIBUTION FROM THE UNIVERSITY OF TEXAS, M. D. ANDERSON HOSPITAL FOR CANCER RESEARCH]

The Optical Rotation of Human Serum Albumin and γ-Globulin[1]

BY B. JIRGENSONS AND S. SIROTZKY

RECEIVED SEPTEMBER 24, 1953

Serum albumin and γ-globulin were isolated by Cohn's method (#10) from normal sera and from sera of patients with confirmed cancer. The dependence of the optical rotation of these proteins on *p*H was studied, and their resistance to the denaturing influence of guanidine hydrochloride was investigated. It was found that the proteins isolated from sera of cancer patients had somewhat lower rotation values than the normal ones. The rotation values of albumin were not constant between the *p*H limits of 4–10, but a flat maximum of levorotation was found at *p*H 5–7.

Introduction

Blood proteins have been the objects of many investigations but in most instances the studies have been confined to distribution patterns and changes in concentration. Only a few authors have tried to compare the properties of the same kind of proteins isolated from various sources.[2–6] The present study was undertaken in order to ascertain whether the proteins isolated from the blood of cancer patients differ from those isolated from the blood of normal (healthy) individuals. If there were some slight differences in the molecular configuration of proteins from normal serum and from "cancer" serum, the differences could be detected most readily by optical rotation measurements. This assumption is based on previous experience in work on denaturation of proteins. The measurement of optical rotation has been found to be a sensitive means to detect denaturation and also a convenient method for protein characterization.[7]

Experience with the optical activity studies on amino acids showed long ago that single data are of little value. The dependence of the specific rotation of amino acids and proteins on *p*H is, however, very important. Almquist and Greenberg[8] initiated the study of optical rotation of proteins as a function of *p*H, and recently data for more proteins was reported.[7] This study has now

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