DL-α-Lipoic Acid from (+)- and (−)-α-Lipoic Acid.—Samples of 12.9 mg. of both (+)-α-lipoic acid and (−)-α-lipoic acid were mixed and ground together in a mortar. The mixture (m.p. 55–57°) was recrystallized from cyclohexane to give DL-α-lipoic acid, m.p. 60–61°.

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Hydrolysis of Ester-type Local Anesthetics and their Halogenated Analogs by Purified Plasma Cholinesterase[1]

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The reaction rate constants (K) and the Michaelis constants (K_m) of three *p*-aminobenzoic acid esters and their 2-chloro substituted derivatives[2] were determined in fresh human plasma and a human plasma cholinesterase concentrate (Cholase[3]). The K and K_m values of the 2-fluoro[4] and 2-bromo[2] substituted procaine HCl were also determined. Halogen substitution in the 2-position caused a 4- to 6-fold increase in the K values of the compounds investigated. The influence of halogen substitution on the K_m values was less marked.

The influence of halogen substitution on the enzymatic hydrolysis rate of *p*-aminobenzoic acid esters in human plasma[5] and on the local anesthetic activity and systemic toxicity of these compounds[6,7] have been previously reported. In these studies the hydrolysis rates of the various compounds were observed in plasma samples obtained on different days from different individuals and were expressed as the time necessary for 50% hydrolysis. Despite precautions taken to obtain valid comparative values for the hydrolysis rates it was felt that more characteristic data could be obtained by using a uniform source of enzyme (Cholase), and by determining, instead of the 50% hydrolysis time, the K and K_m values of the different compounds.

Material and Methods

The K and K_m of procaine·HCl (I), tetracaine·HCl (V) and 2-*sec*-butylaminoethyl-4-aminobenzoate·HCl (VII) as well as their 2-chloro substituted analogs (III, VI and VIII, respectively) were measured using a purified human plasma cholinesterase concentrate (Cholase). The K and K_m of the 2-bromo (IV) and 2-fluoro (II) analogs of procaine·HCl were also determined.[8] The K and K_m of all compounds were also measured in freshly obtained, heparinized, pooled human plasma samples and occasionally also in individual plasmas.

To avoid extreme variations in the times necessary for the completion of the experiments and in order to obtain more accurate K_m values the quantity of the enzymes used was varied according to the hydrolysis rate of the compound investigated. Since too high plasma concentrations interfered with spectrophotometric readings, this principle could be followed more closely with Cholase than with plasma.

Cholase dilutions of 1 to 125, 1 to 375, 1 to 750 and 1 to 1500 were made with a phosphate buffer (containing 6.07 g. of Na_2HPO_4 and 2.00 g. of $NaH_2PO_4 \cdot H_2O$ in 1 liter of distilled water). 10^{-3} M solutions of all substrates were prepared in distilled water. The systems used for the hydrolysis studies were prepared by adding at zero time, 0.2 ml. of the substrate solution, to a mixture of 1.0 ml. of buffer, 0.8 ml. of distilled water and 2.0 ml. of the appropriate dilution of Cholase or plasma used. All solutions were warmed to 37° before mixing. The 0.8 ml. of distilled water was included in the systems to allow for the use of cholinesterase inhibitors in subsequent studies.

TABLE I

CHOLASE AND PLASMA DILUTIONS AND RELATED SUBSTRATE CONCENTRATIONS

Substrate	Cholase: Dilution used	Cholase: Substrate concn. in cholase, moles/ml.	Plasma: Dilution used	Plasma: Substrate concn. in plasma, moles/ml.
Procaine·HCl (I)	1–375	3.75×10^{-5}	1–5.0	5.0×10^{-7}
2-Fluoroprocaine·HCl (II)	1–1500	1.50×10^{-4}	1–10.0	1.0×10^{-6}
2-Chloroprocaine·HCl (III)	1–1500	1.50×10^{-4}	1–10.0	1.0×10^{-6}
2-Bromoprocaine·HCl (IV)	1–750	7.50×10^{-5}	1–10.0	1.0×10^{-6}
Tetracaine·HCl (V)	1–125	1.25×10^{-5}	1–2.5	2.5×10^{-7}
2-Chlorotetracaine·HCl (VI)	1–750	7.50×10^{-5}	1–5.0	5.0×10^{-7}
2-*sec*-Butylaminoethyl-3-aminobenzoate·HCl (VII)	1–125	1.25×10^{-5}	1–2.5	2.5×10^{-7}
2-*sec*-Butylaminoethyl-2-chloro-4-aminobenzoate·HCl (VIII)	1–750	7.50×10^{-6}	1–5.0	5.0×10^{-7}

The *p*H (7.4) and the Na^+ concentration (0.025 M) were identical with those present in the systems generally used for the measurement of the activities of the various enzyme sources with acetylcholine substrate in Warburg experiments. The volume of all systems was 4 ml. and their final substrate concentration was 5×10^{-5} M.

The only variables in the composition of the systems were the dilution of enzymes used and, consequently, the enzymes substrate ratio. These variables are summarized in Table I.

The hydrolysis rates of the various substrates were determined with a modification of the ultraviolet spectrophotometric method of Kalow.[9] The changes of the optical den-

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(2) These compounds were kindly supplied by Dr. Robert H. Hall of Wallace & Tiernan, Inc., Belleville, New Jersey.

(3) Cholase was kindly supplied by Dr. Edwin B. McLean, Cutter Laboratories, Berkeley, California.

(4) The 2-Fluoroprocaine HCl was placed at our disposal by Dr. A. C. Bratton, Jr., of Parke, Davis & Co., Detroit, Michigan.

(5) D. L. Davis and F. F. Foldes, *Federation Proc.*, **13**, 346 (1954).

(6) F. F. Foldes and D. H. Rhodes, *Anesth. & Analg.*, **32**, 305 (1953).

(7) F. F. Foldes, D. L. Davis and O. J. Plekss, "Anesthesiology," in press.

(8) From here on, for the sake of brevity, the various compounds will be referred to by their serial number in Table II.

(9) W. Kalow, *J. Pharmacol. & Exper. Therap.*, **104**, 122 (1952).

sities, observed at a wave length of 313 mμ, were recorded at 30 or 60 second intervals until completion of the hydrolysis, in a model DU Beckman spectrophotometer. The absorption chamber was kept at a temperature of 37 ± 0.2° by circulating water with an electric pump, from a thermostatically controlled source, through two thermospacers mounted on the sides of the sample compartment. Readings were made against a blank in which the substrate solution was replaced by an equal amount of distilled water. This blank, which does not correct for alkaline hydrolysis, was selected because preliminary observations indicated that the alkaline hydrolysis of the substrates, with the experimental conditions used, was negligible. The K_m values were determined, as suggested by Lineweaver and Burk,[10] by plotting S/V against S (where S is the substrate concentration and V the reaction rate with the corresponding S).

All determinations were done in duplicate. The data represent the averages of several experiments.

Results

The K_m and K values for the eight compounds investigated are presented in Table II.

TABLE II

MICHAELIS (K_m) AND REACTION RATE (K) CONSTANTS

Compd.	K_m (mole/l.) Cholase	K_m (mole/l.) Plasma	K (mole/l. min.) Cholase	K (mole/l. min.) Plasma
I	2.6×10^{-6}	3.0×10^{-6}	3.6×10^{-3}	2.2×10^{-3}
II	1.3×10^{-5}	1.2×10^{-5}	1.3×10^{-2}	6.8×10^{-5}
III	3.1×10^{-6}	1.8×10^{-6}	2.0×10^{-2}	1.0×10^{-4}
IV	2.7×10^{-6}	1.2×10^{-6}	1.0×10^{-2}	5.8×10^{-5}
V	2.8×10^{-6}	5.0×10^{-7}	9.6×10^{-4}	7.9×10^{-6}
VI	8.6×10^{-7}	1.1×10^{-6}	4.2×10^{-3}	3.1×10^{-6}
VII	1.3×10^{-6}	1.3×10^{-5}	1.5×10^{-3}	7.3×10^{-6}
VIII	3.0×10^{-6}	2.3×10^{-6}	8.8×10^{-3}	4.3×10^{-5}

Independent of the source of the enzyme the K_m values of most of the compounds ranged from 1.2 to 3.1 $\times 10^{-6}$; the K_m of II and VII, however, were 1.2 to 1.3 $\times 10^{-5}$. Whereas the K_m of V in Cholase and that of its 2-chloro analog in plasma were in line with the other K_m values, the K_m of V in plasma was 5×10^{-7} and the K_m of VI in Cholase was 8.6×10^{-7}.

It is evident from Table II that halogen substitution markedly increased the hydrolysis rate both in Cholase and in plasma, of all compounds investigated.

Discussion

The data presented indicate that the introduction of a chlorine atom in the 2-position markedly increased the enzymatic hydrolysis rate of *p*-aminobenzoic acid esters. The factor by which the K of the three compounds investigated was increased by the chloro substitution was the greatest for VII, less for I, and the least for V. The rate of increase in the K values was about the same with heparinized plasma and with Cholase (see Table III).

TABLE III

THE INFLUENCE OF CHLORINE SUBSTITUTION ON THE REACTION RATE CONSTANTS (K) OF *p*-AMINOBENZOIC ACID ESTERS

Compd. compared	K chlorine substituted / K non-substituted Cholase	Plasma
I and III	5.6	5.0
V and VI	4.4	4.0
VII and VIII	5.9	6.0

(10) H. Lineweaver and D. Burk, THIS JOURNAL, **56**, 658 (1934).

The introduction of a fluoride or bromide radical in the 2-position into I also increased the enzymatic hydrolysis rate both in human plasma and in Cholase (see Table IV).

TABLE IV

THE INFLUENCE OF THE SUBSTITUTION OF VARIOUS HALOGEN RADICALS IN THE PROCAINE MOLECULE ON ENZYMATIC HYDROLYSIS RATE

Halogen substituted	K substituted / K non-substituted Cholase	Plasma
Fluroide	3.6	3.1
Chloride	5.6	5.0
Bromide	2.8	2.6

The influence of the various halogens was chlorine > fluorine > bromine; and the effect was more marked when the enzyme source was Cholase than when it was plasma.

The K_m values of four of the eight compounds investigated (see Table II) were of the order of 10^{-6} both with plasma and Cholase. The order of magnitude of the K_m of II and VII, however, was 10^{-5}. The difference of the K_m values of V and VI with plasma and Cholase cannot be readily explained. It is possible that because of considerable self inhibition observed with the enzymatic hydrolysis of V and VI the K and the K_m values could not be determined as accurately as those of the other compounds.

It was previously reported by Kalow[9] that the affinity of I to human plasma cholinesterase is 220 times greater than the affinity of ACh to this enzyme. Since the K_m of ACh in Cholase and fresh human plasma determined by Warburg's manometric technique was found to be 1.8×10^{-3} M and 2.0×10^{-3} M, respectively,[11] our data indicate that the affinity of I to plasma cholinesterase, as calculated from their K_m values, is about 700 times greater than that of ACh. The affinities of III, IV, V, VI and VIII are of the same order of magnitude, whereas these II and VII are about one-tenth as great.

Measuring the hydrolysis rate of ACh with Michel's electrometric method[12] the activity of 1 ml. of Cholase was found to be equivalent to the activity of 300 ml. of fresh pooled human plasma.[13] With Warburg's manometric technique, also using ACh substrate, this ratio was found to be 220 in our laboratory.[11] In contrast to this the relative ability of Cholase to hydrolyze various *p*-amino-

TABLE V

VARIATION IN THE COMPARATIVE ABILITY OF CHOLASE AND FRESH HUMAN PLASMA TO HYDROLYZE ACh AND *p*-AMINOBENZOIC ACID ESTERS

Compd.	Cholase / Plasma, activity ratio	Compd.	Cholase / Plasma, activity ratio
ACh	220	V	120
I	164	VI	135
II	192	VII	203
III	200	VIII	202
IV	173		

(11) F. F. Foldes, G. R. Van Hees and S. P. Shanor, to be published
(12) H. O. Michel, *J. Lab. Clin. Med.*, **34**, 1564 (1949).
(13) E. B. McLean, personal communication.

benzoic acid derivatives varied from a 120- to 200-fold as compared to plasma (see Table V).

The discrepancy in the relative potency of plasma and Cholase to hydrolyze ACh could be explained partly by loss of potency in storage and partly by the different methods used. It is more difficult, however, to explain the relatively lower activity of Cholase against *p*-aminobenzoic acid esters in general, and the considerable variation of its activity against various members of this group of compounds (see Table V).

The possibilities to be considered include: (1) That more than one enzyme capable of hydrolyzing ACh is present in human plasma and that the activity of these different enzymes toward other esters hydrolyzed by human plasma is variable. It is furthermore conceivable that with the method of purification used in the preparation of Cholase relatively less of the fraction primarily responsible for the hydrolysis of *p*-aminobenzoic acid esters is extracted. (2) That there is only one plasma cholinesterase but the activity of this one enzyme toward various substrates is affected to a variable degree by the extraction process and/or storage.

An answer to these questions would have considerable theoretical and practical importance. It might clarify whether the variation in activity toward ACh and I observed in certain mammalian plasmas as compared to human plasma[14] is due to marked species variation in the properties of a single enzyme or the uneven distribution of several enzymes in different mammalian plasmas.

Of the halogen substituted compounds included in this study, III, IV and VIII have proved to be excellent as local anesthetic agents under clinical circumstances. III[15] was found to be about twice and IV and VIII[16] about four times as potent as I. Their activity was characterized by rapid onset, great intensity, and excellent penetrating capacity. Because of their relatively fast enzymatic hydrolysis rate in human plasma, the possibility for the accumulation of toxic concentrations in the organism is limited. Indeed, no systemic absorption reactions have been encountered with the clinical use of these halogen substituted local anesthetic agents.

(14) M. H. Aven, A. Light and F. F. Foldes, *Federation Proc.*, **12**, 299 (1953).

(15) F. F. Foldes and P. G. McNall, *Anesthesiology*, **13**, 287 (1952).

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[CONTRIBUTION FROM THE ROLLIN H. STEVENS MEMORIAL LABORATORY OF THE DETROIT INSTITUTE OF CANCER RESEARCH]

The Preparation of Four 3,17β-Dihydroxyestrenes[1]

BY JOHN A. HARTMAN

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Preparations of the unsaturated alcohols derived from $\Delta^{5(10)}$-estrene-3-one, 19-nortestoster-one and the enoldiacetate of the latter are described.

The catalytic hydrogenation of estrone, estradiol and estriol has yielded mixtures of mono-, di- and triols, along with some incompletely saturated material.[2-5] The stereochemistry of the newly formed asymmetric centers is unknown with the exception that both estrone and estradiol give the same dihydroxyestrane, reported to be identical with a naturally occurring steroid found in human pregnancy urine.[6]

The partial reduction of estradiol by the Birch[7] method provides a convenient synthesis of two 17β-hydroxyestrene-3-ones, *i.e.*, the $\Delta^{5(10)}$-(I) and Δ^{4}-(II). The latter has been assigned the "normal" or anti configuration at C_{10} by Wilds on the basis of molecular rotation.[7] Since it may be converted into a $\Delta^{3,5}$-enolacetate (III)[8] the formation of three estrene diol epimer pairs (3α- and 3β-hydroxy) is possible with unsaturation at carbon five common to all, and the stereochemistry at C_{17} and C_{10} known.

Since we were primarily interested in obtaining the 3β-epimers for biological testing, conditions were selected on the assumption that any hydrogen attached to C_{10} would have qualitatively, and perhaps quantitatively, the same effect as an angular methyl group on reduction with complex hydrides. Thus the ketones I and II were reduced with lithium aluminum hydride[9] and the enol diacetate III was reduced with sodium borohydride.[10]

None of the crude reduction mixtures formed an insoluble fraction when treated with a warm saturated 95% ethanol solution of digitonin. Negative results with this test are not indicative of the absence of the 3β-alcohols and previous work has indicated the lack of digiton in precipitates in the completely saturated compounds.[3,6] However, Dirscherl reported that two different "hexahydro" derivatives of estrone formed insoluble digitonides.[3]

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