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MOLECULAR ASPECTS OF THE INTERACTION OF LACTOYL- AND GLYCEROYLCHOLINES WITH ACETYLCHOLINESTERASE*

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SUMMARY

The hydrolyses of lactoyl-, glyceroyl- and lactoyl-β-methylcholines by acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7)** were studied using manometric techniques. Although the activity–pS curves of acetylcholine were not parallel to those of lactoylcholines, the hydrolysis of all substrates was catalyzed by the same active sites. The strong acetylcholinesterase inhibitors of acetylcholine hydrolysis were also strong inhibitors of DL-lactoylcholine hydrolysis. L(+)-Lactoylcholine was hydrolyzed at a rate about 1.5 times faster than acetylcholine, while D(−)-lactoylcholine had a rate of hydrolysis of about 1/6 that of acetylcholine. Acetylcholinesterase was inhibited at high substrate concentrations of lactoylcholine (pS optimum: 2.0) or acetylcholine (pS optimum: 2.25). The hydrolyses of DL-glyceroylcholine and DL-lactoyl-β-methylcholine by acetylcholinesterase were not significant. The lack of hydrolysis of DL-lactoyl-β-methylcholine could be interpreted in terms of steric hindrance effected by the β-methyl group in the area of enzyme–substrate interactions. The lack of hydrolysis of DL-glyceroylcholine might be due to the formation of a six-membered chelate ring in which β-hydroxyl hydrogen atom would form a hydrogen bond with the carbonyl keto oxygen. The carbonyl frequencies in the infrared spectra of compounds related to DL-glyceroylcholine and atropine suggest the presence of the chelate ring structure in DL-glyceroylcholine.

INTRODUCTION

Propionylcholine and acrylylcholine are two of the naturally occurring and pharmacologically active analogs of acetylcholine[2,3]. Lactoyl- and glyceroylcholines are closely related to propionylcholine and acrylylcholine; furthermore, since lactic and glyceric acids occur in animal tissues, the actual existence of natural lactoylcholine

* Part of this investigation was orally presented at the meetings of the American Society for Pharmacology and Experimental Therapeutics[1], Lawrence, Kan., August, 1964.

** Recommended name for true cholinesterase by International Union of Biochemistry on the Nomenclature and Classification of Enzymes.

and glyceroylcholine cannot be entirely excluded. The possibilities for the occurrence of lactoylcholine and related compounds in the animal tissues were discussed in a previous publication by SASTRY, PFEIFFER AND LASSLO[4]. Therefore, we have studied the hydrolysis of lactoylcholine and glyceroylcholine by acetylcholinesterase in connection with our investigations to develop methods to isolate and characterize the cholinesters that may occur in animal tissues. Lactoyl-β-methylcholine and acetyl-β-methylcholine are included in the present study in view of their structural analogies to lactoylcholine and acetylcholine (Fig. 3).

The enzymatic hydrolysis of lactoylcholine and related compounds by serum cholinesterase were described elsewhere[5,8]. It was shown that the rates of hydrolysis of both isomers of lactoylcholine were faster than those of acetylcholine. The isomeric ratios between the rates of hydrolysis of the enantiomers of lactoylcholine by cholinesterase from various species indicate that asymmetry in the acyl component is not a very significant factor in their hydrolysis.

MATERIALS AND METHODS

Substrates and inhibitors

The DL-, D(−)-, L(+)-lactoylcholine iodides* were prepared according to the methods described by SASTRY, LASSLO AND PFEIFFER[6]. The synthesis of DL-lactoyl-DL-β-methylcholine iodide and DL-glyceroylcholine iodide were described elsewhere[4]. The acetylcholine iodide (Mann Research Laboratories, Inc., New York, N.Y.), propionylcholine iodide (Dajax Laboratories, Leominster, Mass.), physostigmine sulfate (Mann Research Laboratories, Inc., New York, N.Y.), neostigmine methylsulfate (Mann Research Laboratories, Inc., New York, N.Y.), 2-diethoxyphosphinylthioethyldimethylamine acid oxalate (217-AO)** and D-diethoxyphosphinylthioethyltrimethylammonium iodide (217-MI, phospholine)** were obtained from commercial sources.

Enzyme

Acetylcholinesterase (1000 units/mg protein) was prepared commercially (Nutritional Biochemicals Corp., Cleveland, Ohio) from bovine erythrocytes. 1 unit of acetylcholinesterase activity is equivalent to the disappearance of 1 μmole of acetylcholine ($2.7 \cdot 10^{-3}$ M) per min at 25° as determined by HESTRIN's[7] reaction. The solution of the enzyme was prepared in Krebs–bicarbonate buffer containing 1% albumin for kinetic studies.

Kinetics of the hydrolysis of the substrates

The volume of CO_2 liberated from a bicarbonate buffer by the acid formed during the hydrolysis of the ester was measured at 37° by Warburg manometric method. The Krebs–Ringer bicarbonate buffer was prepared consisting of $2.3 \cdot 10^{-2}$ M $NaHCO_3$, $7.5 \cdot 10^{-2}$ M KCl, $7.5 \cdot 10^{-2}$ M NaCl and $4 \cdot 10^{-2}$ M $MgCl_2 \cdot 6\,H_2O$, according to the methods

* The configuration and specific rotation designated by D(−)- and L(+)- refer to the original lactic acid molecules from which the enantiomers of lactoylcholine were synthesized. D-Lactoylcholine is levo and L-lactoylcholine is dextro in their specific rotations in methanol solutions[6].

** The compounds 217-AO and 217-MI were kindly provided by Dr. ROBERT A. LEHMANN, Campbell Pharmaceutical Co., New York, N.Y.

described previously[8]. The pH of this buffer was found to be 7.5 at 37° when measured according to the methods described by SIGGARD-ANDERSEN[9] using thermostated capillary glass electrode and thermostated Calomel electrode.

The total volume of the reactants was 3.0 ml in a 15-ml flask. The main compartment contained 2.5 ml of the buffer, 0.2 ml of the enzyme. The substrate (0.3 ml) was placed in the side arm. The air in the reaction vessels was displaced with 5% CO_2 and 95% N_2, and contents were preincubated for 15 min. The manometers were read every 2 min during the first 20 min and every 10 min during 20–60 min. The pS–activity curves were constructed from initial linear velocities. The relative rates of the hydrolysis of the substrates were calculated from their rates of hydrolysis at their pS optimum.

The concentrations of inhibitors for 50% inhibition (I_{50}) were determined graphically by plotting V/V_I (V = velocity without inhibitor, V_I = velocity with inhibitor) against the concentration of the inhibitor, $[I]$, at optimum substrate concentration. The enzyme was incubated with the inhibitor for 15 min before the substrate was added, and the velocity was calculated when the rate of evolution of CO_2 was linear.

Infrared spectra of the substrates

All spectra were recorded using a Perkin–Elmer Model 21 Recording Infrared Spectrophotometer. The KBr pressed pellet technique[10] for preparing solid samples was employed for all spectra bearing the "KBr disc" phase designation. The pellets were approx. 1 cm^2 in area. The sample thickness in mg/cm^2 is, therefore, equal to the mass of the sample pressed into the pellet. Spectra of liquid phase samples were obtained using a liquid cell of 0.05-mm path length.

RESULTS

Activity–pS curves for the enzymatic hydrolysis of acetylcholine and DL-*lactoylcholine*

The activity–pS curves were approximately bell-shaped for both acetylcholine and DL-lactoylcholine (Fig. 1). High substrate concentrations of acetylcholine, as

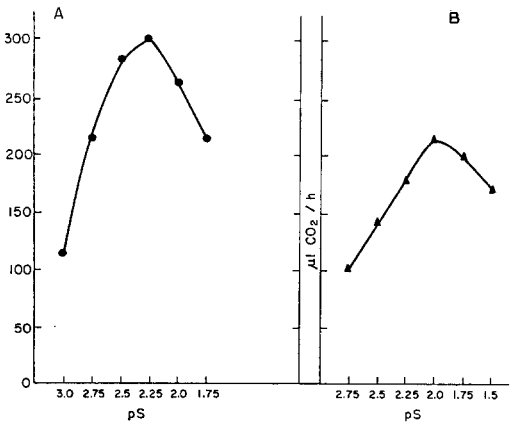

Fig. 1. Activity–pS curves for the enzymatic hydrolysis of acetylcholine (A) and DL-lactoylcholine (B) by acetylcholinesterase. The reaction rates were calculated from the initial linear velocities.

well as DL-lactoylcholine, inhibited the enzyme. Similarly, high substrate concentrations of L(+) and D(−) isomers inhibited the enzyme.

The pS optimum of acetylcholine was about 2.25 ($0.56 \cdot 10^{-2}$ M) and for the DL-, D(−)- or L(+)-lactoylcholine was 2.0 (10^{-2} M). The pS optimum of lactoylcholine was significantly higher with pure enzyme (2.0) than with thawed erythrocytes (1.6)[11]. The activity–pS curves of acetylcholine and DL-lactoylcholine were not parallel with purified acetylcholinesterase as well as with thawed erythrocytes.

Relative rates of hydrolysis

The relative rates of hydrolysis were calculated from their rates of hydrolysis at their pS optimum (Table I). Acetyl-β-methylcholine was hydrolyzed at a rate 5 times slower than acetylcholine. This indicates that introduction of the β-methyl function into the acetylcholine molecule decreases the rate of hydrolysis 5-fold. DL-Lactoyl-

TABLE I

SYNOPSIS OF THE RELATIVE RATES OF ACETYLCHOLINESTERASE HYDROLYSIS OF LACTOYLCHOLINE AND RELATED COMPOUNDS (ACETYLCHOLINE = 1)

Source of acetylcholinesterase	*Substrate*	*pS*	*Relative rate of hydrolysis (molar basis)*
Bovine red blood cells*	Acetylcholine	2.25***	1.00
	DL-Acetyl-β-methylcholine	2.00	0.24
	L(+)-Lactoylcholine	2.00***	1.47
	D(−)-Lactoylcholine	2.00***	0.21
	DL-Lactoylcholine (obs.)	2.00***	0.68
	DL-Lactoylcholine (calc.)	2.00	0.82
	DL-Lactoyl-β-methylcholine	2.00	Negligible
	DL-Glyceroylcholine	2.00	Negligible
Human red blood cells**	Acetylcholine	2.5	1.00
	L(+)-Lactoylcholine	1.6	1.71
	D(−)-Lactoylcholine	1.6	0.41
Bovine red blood cells*	Acetylcholine	2.30	1.00§
	Propionylcholine	2.30	0.62§
	Acrylylcholine	2.30	0.12§
	Butylcholine	2.30	0.02§

* Purified enzyme preparation (see MATERIALS AND METHODS).
** Thawed erythrocytes. The values were quoted from AUDITORE AND SASTRY[11].
*** pS optimum was established in the present experiments.
§ The values were quoted from SEKUL, HOLLAND AND BRELAND[12]. Only initial reaction rates were reported.

choline was about 2/3, and L(+)-lactoylcholine was about 1.5 times as active as acetylcholine. The enzymic rate of hydrolysis of lactoyl-β-methylcholine was not significant. Therefore, the introduction of the β-methyl function into lactoylcholine molecule abolished its activity completely. Similarly, the introduction of the β-hydroxyl group forming glyceroylcholine abolished the activity of lactoylcholine.

L(+)-Lactoylcholine was hydrolyzed at a rate approx. 1.5 times faster than acetylcholine. D(−)-Lactoylcholine was hydrolyzed at a rate about 7 times slower

than L(+)-lactoylcholine. On the basis of the rates of hydrolysis of the enantiomers of lactoylcholine, a 1:1 mixture of the optical isomers should be about 8/10 as active as acetylcholine. However, DL-lactoylcholine was about 7/10 as active as acetylcholine. Therefore, D(−)-lactoylcholine was a very weak inhibitor of L(+)-lactoylcholine hydrolysis by acetylcholinesterase.

The rate of L(+)-lactoylcholine was higher than those of propionylcholine or acrylylcholine. DL-Lactoylcholine and propionylcholine were hydrolyzed approximately at about the same rate.

Activities of acetylcholinesterase inhibitors with DL-*lactoylcholine and acetylcholine as substrates*

The I_{50} values for various acetylcholinesterase inhibitors were listed in Table II. The p*S* optimum of DL-lactoylcholine (2.00) was different from that of acetylcholine

TABLE II

ANTI-ACETYLCHOLINESTERASE ACTIVITIES OF VARIOUS INHIBITORS

*Substrate**	*Inhibitor***	I_{50} *(M)*
Acetylcholine iodide	DL-Glyceroylcholine iodide	$9 \cdot 10^{-3}$
	Atropine methylsulfate	$4.5 \cdot 10^{-2}$
	DL-Lactoyl-β-methylcholine iodide	$4 \cdot 10^{-2}$
	Physostigmine sulfate	$2.2 \cdot 10^{-7}$
	Neostigmine methylsulfate	$1.7 \cdot 10^{-7}$
	Phospholine iodide	$3 \cdot 10^{-8}$
	217-AO	$4 \cdot 10^{-8}$
DL-Lactoylcholine iodide	DL-Glyceroylcholine iodide	$8.0 \cdot 10^{-3}$
	Atropine methylsulfate	$3.0 \cdot 10^{-3}$
	DL-Lactoyl-β-methylcholine iodide	$1.3 \cdot 10^{-2}$
	Physostigmine sulfate	$5.1 \cdot 10^{-7}$
	Neostigmine methylsulfate	$1.0 \cdot 10^{-7}$
	217-AO	$8.0 \cdot 10^{-8}$

* Optimum substrate concentrations were used. p*S* values: acetylcholine, 2.25; DL-lactoylcholine, 2.0.

** The enzyme was incubated with the inhibitor for 15 min before the substrate was added. The velocity was measured when the rate of evolution CO_2 was linear.

(2.25). However, the strong carbamate and phosphate inhibitors of acetylcholine hydrolysis were also strong inhibitors of DL-lactoylcholine hydrolysis suggesting that the same active site was involved in the hydrolysis of acetylcholine and DL-lactoylcholine by acetylcholinesterase.

Anti-acetylcholinesterase activities of DL-*glyceroylcholine and related compounds*

DL-Glyceroylcholine and DL-lactoyl-β-methylcholine were not only weak substrates but also weak inhibitors. They were about 10^4–10^5 times less active than the well known reversible and irreversible inhibitors of acetylcholinesterase (Table II), *e.g.*, neostigmine methylsulfate and phospholine iodide. Similar to DL-glyceroylcholine, atropine methylsulfate was found to be a weak inhibitor of acetylcholinesterase with

acetylcholine and DL-lactoylcholine as substrates. These observations suggest that these compounds are not effective to form enzyme–substrate or enzyme–inhibitor complexes.

Infrared spectra of substances

The carbonyl carbon in glyceroylcholine (α,β-dihydroxy-propionylcholine) is less electrophilic than that of lactoylcholine (α-hydroxy-propionylcholine), but is more electrophilic than that of acetylcholine (K_{25} for acetic, glyceric and lactic acids: $1.76 \cdot 10^{-5}$, $28 \cdot 10^{-5}$, $84 \cdot 10^{-5}$, respectively). Therefore, based on the electronic characteristics of the carbonyl keto function, one would expect that the rate of hydrolysis of DL-glyceroylcholine by acetylcholinesterase should be very close to those of acetylcholine and DL-lactoylcholine. However, the rate of hydrolysis of DL-glyceroylcholine was negligible which should be explained by other molecular factors. The β-hydroxyl function in DL-glyceroylcholine could lend itself readily to the formation of a six-membered chelate ring, in which the β-hydroxyl hydrogen would form a hydrogen bond with the carbonyl keto oxygen (Fig. 2). Since the classical work of WILSON, NACHMANSOHN, BERGMANN and many others[13–15] has indicated that the variations in the electronic characteristics of the carbonyl keto function have considerable effect upon enzyme–substrate interactions at the esteratic site, one could reasonably predict that the influence of hydrogen bonding upon the carbonyl keto group may reduce the affinity of glyceroylcholine to the active sites. Further, the six-membered ring would

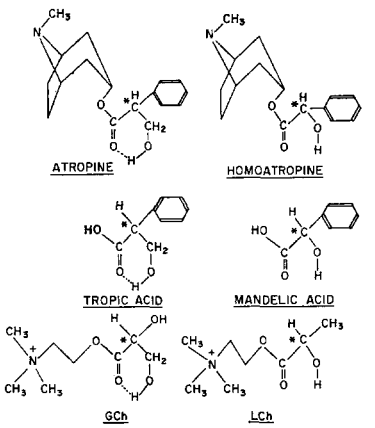

Fig. 2. Structural resemblances in the configurations of glyceroylcholine (GCh) and lactoylcholine (LCh) with those of atropine and related compounds. Asterisk indicates asymmetric carbon.

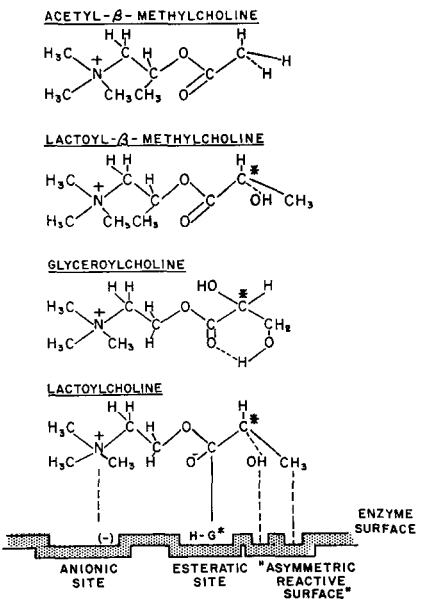

Fig. 3. Hypothetical interaction between the active sites of acetylcholinesterase and lactoylcholine and related compounds. Asterisk indicates the asymmetric carbon.

cause steric hindrance for the formation of proper enzyme–substrate complex (Fig. 3). Therefore, we studied the infrared spectra of glyceroylcholine and related compounds to obtain evidence for the presence of six-membered chelate ring in glyceroylcholine.

The chelation would shift the carbonyl band to longer wavelengths in the infrared spectrum[16–18]. However, this technique could not be used directly to glyceroylcholine and other quaternary ammonium compounds because the inductive effect of quaternary ammonium group would shift the carbonyl band to shorter wavelengths (Table III). The tertiary analog of glyceroylcholine (2-dimethylaminoethyl glycerate) was found to be unstable to obtain a pure sample for spectroscopic studies. Therefore, we have selected atropine and related compounds for this study because (a) glyceroylcholine is structurally related to atropine (Fig. 2), (b) atropine contains a tertiary nitrogen and its carbonyl frequency could be determined accurately, and (c) if the chelate ring could be demonstrated to be present in a complex molecule like atropine, it should be present in a simple molecule like glyceroylcholine.

There are no significant differences in the carbonyl frequencies of ethylacetate and 2-dimethylaminoethylacetate (Table III). The carbonyl absorption band moved to higher frequencies in acetylcholine, propionylcholine, DL-lactoylcholine as well as in DL-glyceroylcholine. This shift of the carbonyl peak towards the higher energy of the infrared absorption was due to the inductive influence of quaternary nitrogen[19–21].

TABLE III

C=O STRETCHING VIBRATIONS IN THE INFRARED SPECTRA OF ACETYLCHOLINE, ATROPINE AND RELATED COMPOUNDS

Comparison	*Phase or solvent*	*Compounds*	*Wave number* (cm^{-1})*
1	Abs. ethanol**	Ethylacetate	1727, 1743
		2-Dimethylaminoethylacetate	1729, 1743
2	Abs. ethanol**	Acetylcholine iodide	1763
		Propionylcholine iodide	1761
		DL-Lactoylcholine iodide	1761
		DL-Glyceroylcholine iodide	1765
3	Chloroform	Methyllactate	1736
		Methylacetate	1735
4	KBr	DL-Mandelic acid	1720
		DL-Tropic acid	1711
5	KBr	Homatropine	1757
		Atropine	1731
		Scopolamine	1727
6	Chloroform	Homatropine***	1730
		Scopolamine***	1723
		Atropine***	1719

* The absorption bands are within the limits of $\pm$ 2 cm^{-1}. Differences of 4 cm^{-1} are significant.

** The absorption of abs. alcohol in this region is minimal. However, the bands were corrected for solvent absorption.

*** The bases were freshly prepared from their salts.

The acid carbonyl band occurs in the spectrum of tropic acid at a lower frequency than that of mandelic acid. Similarly, the absorption band of the ester carbonyl group in atropine and scopolamine has shifted to lower frequency than that of homatropine both in KBr and chloroform solution. In dilute chloroform solutions, the intermolecular associations will become negligible and intramolecular bonds will predominate. Further, chelation is known to shift the ester carbonyl band to a lower frequency[16–18]. These observations indicate the formation of a six-membered chelate ring in atropine and scopolamine. Similarly, such possibility exists in DL-glyceroylcholine.

In most of the substances, hydrogen bond formation involves closing a six-membered ring (*e.g.*, DL-glyceroylcholine, atropine, scopolamine), the values of interatomic distances and bond angles being such to favor the formation of a strong hydrogen bond. On the other hand, strong hydrogen bond is not formed with completion of a five-membered ring[22], the conditions being unfavorable (*e.g.*, DL-lactoylcholine, homatropine). The carbonyl frequency occurs at the same position both in methyllactate and methylacetate.

DISCUSSION

Kinetic data alone is not sufficient to compare the enzymic hydrolysis of the isomers of lactoylcholine with acetylcholine because their activity–pS curves cross one another. Acetylcholine has lower K_m and higher affinity to acetylcholinesterase than L(+)-lactoylcholine. However, the v_{max} of L(+)-lactoylcholine is higher than that of acetylcholine.

A direct comparison of the isomers of lactoylcholine is possible because they have the same pS optimum. Their affinities for acetylcholinesterase fall in the following order: L(+)-lactoylcholine > DL-lactoylcholine > D(−)-lactoylcholine. The L(+) isomer was hydrolyzed at a rate 7 times faster than the D(−) isomer (Table I). The observed rate of the hydrolysis of the DL isomer was significantly lower than the calculated rate for 1:1 mixture from the rates of hydrolysis of L(+) and D(−) isomers. Therefore, the D(−) isomer is a very weak inhibitor of L(+)-lactoylcholine hydrolysis by acetylcholinesterase.

Although the activity–pS curves of acetylcholine cross those of DL-lactoylcholine, the same catalytic site was involved in the hydrolysis of both substrates. The compounds which were strong inhibitors of acetylcholine hydrolysis by acetylcholinesterase were very active to inhibit DL-lactoylcholine hydrolysis (Table II).

The forces of binding between acetylcholinesterase and acetylcholine could be analyzed in terms of two enzyme subunits, anionic and esteratic sites (Fig. 3). The anionic site binds and orients substituted ammonium structures by coulombic and van der Waals forces[15]. The esteratic site, G–H, contains an acidic (H) and a basic (..) group, both of which are required for catalytic activity. Lactoylcholine contains an asymmetric carbon in the α-position to the carbonyl group. The difference in the rates of hydrolysis of the enantiomers of lactoylcholine was explained by assuming the existence of an asymmetric reactive surface adjacent to the esteratic site[23]. The anionic site, as well as the methyl and hydroxyl groups on the asymmetric carbon of L(+)-lactoylcholine would contribute for the binding and would align the carbonyl group into proper orientation with the G–H group of the esteratic site. In the case of D(−)-lactoylcholine, while the anionic site binds with the quaternary nitrogen and the

hydroxyl group binds with its site, the methyl group would be placed in an improper position. Further, this would disturb orientation of ester carbonyl with the G–H group and would prevent its hydrolysis.

The fact that the rate of enzymatic hydrolysis of DL-glyceroylcholine* is negligible as compared with that of DL-lactoylcholine could be explained by their molecular constitutions. Glyceroylcholine could form a six-membered chelate ring (counting the hydrogen atom) due to the formation of a strong hydrogen bonding between the hydrogen of the hydroxyl group and the carbonyl oxygen. The carbonyl group of glyceroylcholine as a part of the chelate ring could not be aligned properly with the G–H group of the esteratic site. The infrared spectra of tropic acid and atropine and related compounds would indicate the presence of six-membered ring in these compounds. Further, the six-membered chelate ring would induce steric hindrance for the binding of glyceroylcholine at the esteratic site.

The formation of a strong hydrogen bond was not insured in ethyllactate (or in lactoylcholine) because (a) the hydrogen–oxygen distance is long, and (b) the hydrogen atom is not well directed towards the outer part of the oxygen atom, where the unshared electron pairs are located[22].

Acetyl-β-methylcholine is a selective substrate for acetylcholinesterase because it is not hydrolyzed by plasma cholinesterase[24]. However, this substrate was hydrolyzed at about 1/4 the rate of acetylcholine hydrolysis. This should represent the hydrolysis of the L(+) isomer, because the D(−) isomer was not hydrolyzed by the homogenate of bovine erythrocytes and was only a weak inhibitor[25,26]. Similarly, the hydrolysis of DL-lactoyl-β-methylcholine** by acetylcholinesterase (Table I) or cholinesterase[8] was not significant. In these compounds, the methyl group is located in the area of enzyme–substrate interaction and the volume occupied by a methyl group is considerably larger than a hydrogen atom. Therefore, one would expect that the methyl group would exert steric hindrance and the substrate was not properly bound to the enzyme for activation. Due to the similar reasons, DL-glyceroylcholine and DL-lactoyl-β-methylcholine are very weak inhibitors of acetylcholine and DL-lactoylcholine hydrolysis by acetylcholinesterase.

The investigations of KRUPKA AND LAIDLER[27] and WILSON AND ALEXANDER[28] suggest that deacetylation of the acetyl enzyme was prevented by binding of a second molecule of acetylcholine to the acetyl enzyme at high substrate concentrations. A similar possibility exists in the case of the isomers of lactoylcholine. It has been shown that only one molecule of lactoylcholine could combine at the esteratic and asymmetric site[11]. Both of the enantiomers of lactoylcholine could bind to the enzyme and exhibit characteristics of substrate inhibition. Therefore, the substrate inhibition observed with DL-lactoylcholine might be due to accumulation of lactoylenzyme.

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* No attempts were made to prepare the optical isomers of glyceroylcholine, because DL-glyceroylcholine was both a poor substrate and a poor inhibitor of cholinesterase[5] and acetylcholinesterase. Further, its muscarinic and nicotinic activities were very low compared to DL-lactoylcholine[4].

** Lactoyl-β-methylcholine contains 2 asymmetric carbons; and, therefore, 4 isomers are possible. Due to the low activities of DL-lactoyl-β-methylcholine as a substrate and inhibitor of cholinesterase and acetylcholinesterase, and as a stimulant of cholinergic receptors[4,5] no attempts were made to prepare all isomers.

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REFERENCES

1 E. C. WHITE AND B. V. R. SASTRY, *Pharmacologist*, 6, No. 2 (1964) 198.
2 R. BANISTER, V. P. WHITTAKER AND S. WIJESUNDERA, *J. Physiol. London*, 121 (1953) 55.
3 V. P. WHITTAKER, *Biochem. Pharmacol.*, 1 (1959) 342.
4 B. V. R. SASTRY, C. C. PFEIFFER AND A. LASSLO, *J. Pharmacol. Exptl. Therap.*, 130 (1960) 346.
5 B. V. R. SASTRY AND E. C. WHITE, *Federation Proc.*, 23 (1964) 177.
6 B. V. R. SASTRY, A. LASSLO AND C. C. PFEIFFER, *J. Org. Chem.*, 23 (1958) 2005.
7 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
8 A. LASSLO, A. L. MEYER AND B. V. R. SASTRY, *J. Med. Pharm. Chem.*, 2 (1960) 91.
9 O. SIGGARD-ANDERSEN, *The Acid–Base Status of Blood*, Williams Wilkins, Baltimore, Md., 1964, p. 93.
10 D. N. INGEBRIGTSON AND A. L. SMITH, *Anal. Chem.*, 26 (1954) 1765.
11 J. V. AUDITORE AND B. V. R. SASTRY, *Arch. Biochem. Biophys.*, 105 (1964) 506.
12 A. A. SEKUL, W. C. HOLLAND AND A. E. BRELAND, JR., *Biochem. Pharmacol.*, 11 (1962) 487.
13 I. B. WILSON AND F. BERGMANN, *J. Biol. Chem.*, 186 (1950) 683.
14 F. BERGMANN, I. B. WILSON AND D. NACHMANSOHN, *J. Biol. Chem.*, 186 (1950) 693.
15 R. A. OOSTERBANN AND H. S. JANSZ, in M. FLORKIN AND E. H. STOTZ, *Comprehensive Biochemistry*, Vol. 16, Elsevier, Amsterdam, 1965, p. 1.
16 R. S. RASMUSSEN AND R. B. BRATTAIN, *J. Am. Chem. Soc.*, 71 (1949) 1073.
17 R. R. HAMPTON AND J. E. NEWELL, *Analyt. Chem.*, 21 (1949) 914.
18 L. J. BELLAMY, *The Infrared Spectra of Complex Molecules*, John Wiley, New York, 1958, p. 184.
19 J. H. FELLMAN AND T. S. FUJITA, *Biochim. Biophys. Acta*, 56 (1962) 227
20 J. H. FELLMAN AND T. S. FUJITA, *Biochim. Biophys. Acta*, 71 (1963) 701.
21 B. V. R. SASTRY AND E. C. WHITE, *Federation Proc.*, 24 (1965) 610.
22 L. PAULING, *The Nature of Chemical Bond and the Structure of Molecules and Crystals*, Cornell University Press, Ithaca, N.Y., 1962, p. 495.
23 B. V. R. SASTRY AND J. V. AUDITORE, *Proc. 1st Intern. Pharmacol. Meeting*, 7 (1963) 323.
24 E. C. WEBB, *Biochem. Soc. Symp.*, 19 (1960) 90.
25 A. H. BECKETT, N. J. HARPER AND J. W. CLITHEROW, *J. Pharm. Pharmacol.*, 15 (1963) 362.
26 F. C. G. HOSKIN, *Proc. Soc. Exptl. Biol. Med.*, 113 (1963) 320.
27 R. M. KRUPKA AND K. J. LAIDLER, *J. Am. Chem. Soc.*, 83 (1961) 1448.
28 I. B. WILSON AND J. ALEXANDER, *J. Biol. Chem.*, 237 (1962) 1323.