Cholinesterase Hydrolysis and Substrate Inhibition of Lactoylcholines[1]

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The reaction kinetics of the hydrolysis of lactoylcholine (LCh) by cholinesterase (ChE)[2] was studied by manometric techniques. DL-LCh was hydrolyzed by ChE three to four times faster than ACh. The stereospecificity of ChE was not very significant for the enantiomers of LCh (L/D = 1.5–1.7). The strong ChE inhibitors of ACh hydrolysis were also strong inhibitors of DL-LCh hydrolysis. ChE was not inhibited by ACh, while it was inhibited by LCh at high substrate concentrations. Therefore, ACh and DL-LCh were used as substrates to study the mechanisms of substrate inhibition of ChE by LCh. These studies gave the following results. (1) The rate of evolution of CO_2 with DL-LCh as substrate was linear during the first 10 min of interaction. The rate of evolution of CO_2 with ACh as substrate was linear during the whole reaction period of the first 40 min. (2) The rate of hydrolysis of DL-LCh during reaction period 0–10 min (475 μl/hr) was higher than that of 10–20 min (275 μl/hr). There was no significant difference in the rates of hydrolysis of ACh during these reaction periods (132 μl/hr). (3) Choline or lactate alone did not inhibit the enzyme; together they depressed the enzymic hydrolysis of ACh. (4) Prior incubation of the enzyme with DL-LCh depressed the rate of hydrolysis of ACh. (5) Physostigmine (12×10^{-9} *M*) exhibited competitive kinetics with ACh as substrate. The kinetic characteristics of the inhibition caused by physostigmine with DL-LCh as substrate were not clear. The Lineweaver–Burk plots of DL-LCh hydrolysis without inhibitor and with 12×10^{-9} *M* physostigmine were parallel suggesting that the inhibition might be "uncompetitive." However, at 60×10^{-9} *M* physostigmine, the inhibition of DL-LCh hydrolysis did seem to approach to that of the "partially competitive type."

Although acetylcholine (ACh) has been shown beyond reasonable doubt to be the transmitter at certain cholinergic nerve endings, there are several investigations which suggest that choline esters other than ACh may occur in animal tissues.[3] Acetylcholine-like activity occurs in nonnervous tissue and is so widely distributed in nature so as to suggest a nonnervous or hormonal function to choline esters. It is known that choline esters, other than ACh, contribute to the ACh-like activity of nervous as well as nonnervous tissues of mammals.[4] There are many possibilities for the occurrence of lactoylcholine[3a] (LCh) in animal tissues and it bears close structural similarities to propionylcholine (PCh) and acrylylcholine (ArCh) both of which were isolated from animal tissues[4a,5] (Figure 1).

The potential identification of LCh as one of the naturally occurring choline esters warranted a detailed kinetic study of its hydrolysis by AChE and ChE from different sources. The kinetics of the hydrolysis of LCh and its isomers by acetylcholinesterase (AChE) were described elsewhere.[6] It was shown that DL-LCh was hydrolyzed approximately one and one-half times faster than ACh by thawed human erythrocytes.[6c] The hydrolytic rate of L-(+) isomer was about twice that of ACh and was approximately four times greater than that observed for D-(−)-LCh. All isomers of LCh, as well as ACh, inhibited AChE at high substrate concentrations. Our studies have indicated that the isomers of LCh were hydrolyzed faster than ACh by ChE. Further, the isomers of LCh inhibited ChE at high substrate concentrations, while ACh did not exhibit the characteristics of ChE inhibitor. Therefore, we have used DL-LCh and ACh as substrates to investigate the details of the substrate inhibition of ChE by LCh.

Experimental Section

Substrates and Inhibitors.—The DL-, D-(−)-, L-(+)-LCh iodides[7] were prepared according to the methods described by Sastry, *et al.*[8] The synthesis of DL-lactoyl-DL-β-methylcholine (LMCh) iodide and DL-glyceroylcholine (GCh) iodide were described elsewhere.[3a] The ACh iodide,[9] PCh iodide,[10] butylcholine (BCh) iodide,[9] physostigmine sulfate,[9] neostigmine methyl sulfate,[9] 2-diethoxyphosphinylthioethyldimethylamine acid oxalate,[11] and 2-diethoxyphosphinylthioethyltrimethylammonium iodide (phospholine)[11] were obtained from commercial sources.

Cholinesterase was prepared commercially[12] by a modified Strelitz's procedure[13] from horse serum and supplied as a stable lyophilized powder containing about 5 units/mg. One unit is equal to 1 μmol of ACh hydrolyzed/min. The enzyme solutions were 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 2.3×10^{-2} *M* $NaHCO_3$, 7.5×10^{-3} *M* KCl, 7.5×10^{-3} *M* NaCl, and 4×10^{-3} *M* $MgCl_2 \cdot 6H_2O$, according to the methods described by Umbreit, *et al.*[14] The pH of this

(1) (a) Preliminary reports of this investigation were orally presented at the Meetings of the Federation of American Societies for Experimental Biology and Medicine in Chicago, Ill., March–April 1964, and IIIrd International Pharmacological Congress in Sao Paulo, Brazil, July 1966. (b) This investigation was supported by U. S. Public Health Service Research Grant No. NB-04699.

(2) The name "cholinesterase" was recommended for pseudocholinesterase or serum cholinesterase by the International Union of Biochemistry. Systematic name: acylcholine acylhydrolase.

(3) (a) B. V. R. Sastry, C. C. Pfeiffer, and A. Lasslo, *J. Pharmacol. Exp. Ther.*, **130**, 346 (1960); (b) V. P. Whittaker, *Handbuch Exp. Pharmakol.*, **15**, 1 (1963).

(4) (a) J. Banister, V. P. Whittaker, and S. Wijesundera, *J. Physiol.* (London), **121**, 55 (1953); (b) K. Kuriaki, T. Yakushiji, T. Noro, T. Chimizu, and Sh. Saji, *Nature*, **181**, 1336 (1958); (c) H. Kewitz, *Arch. Exp. Pathol. Pharmakol.*, **237**, 308 (1959).

(5) V. P. Whittaker, *Biochem. Pharmacol.*, **1**, 342 (1958).

(6) (a) B. V. R. Sastry and J. V. Auditore, *Fed. Proc.*, **19**, 283 (1960); (b) B. V. R. Sastry and J. V. Auditore, *Proc. Intern. Pharmacol. Meeting, 1st, Stockholm, 1961*, **7**, 323 (1963); (c) J. V. Auditore and B. V. R. Sastry, *Arch. Biochem. Biophys.*, **105**, 506 (1964); (d) B. V. R. Sastry and E. C. White, *Pharmacologist*, **6**, 198 (1964).

(7) 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.

(8) B. V. R. Sastry, A. Lasslo, and C. C. Pfeiffer, *J. Org. Chem.*, **23**, 2005 (1958).

(9) Mann Research Laboratories, Inc., New York, N. Y.

(10) Dajax Laboratories, Leominster, Mass.

(11) These compounds were kindly provided by Dr. Robert A. Lehmann, Campbell Pharmaceutical Co., New York, N. Y.

(12) Worthington Biochemical Corp., Freehold, N. J.

(13) F. Strelitz, *Biochem. J.*, **38**, 86 (1944).

(14) W. W. Umbreit, R. H. Burris, and J. F. Stauffer, "Manometric Techniques," Burgess Publishing Co., Minneapolis, Minn., 1959, pp 25, 149.

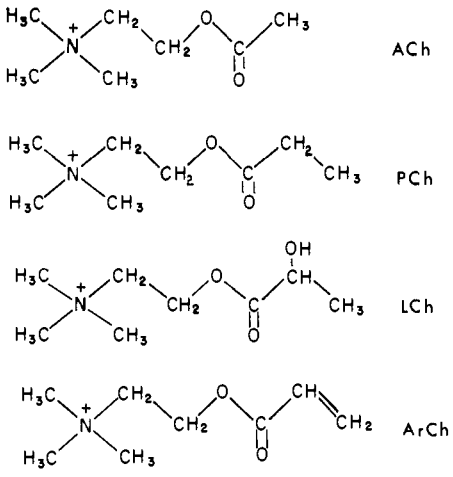

Figure 1.—Structural relationships of lactoylcholine (LCh) to other choline esters occurring in nature. Propionylcholine (PCh), acrylylcholine (ArCh), and LCh form a closely allied series containing a three-carbon unit in the acyl portion.

buffer was found to be 7.5 at 37° when measured according to the methods described by Siggaard-Andersen[15] using thermostated capillary glass electrode and thermostated calomel electrode. The buffer should have a pH of 7.6 in a gas phase of 5% CO_2 and 95% N_2 at 37° (740 mm), when theoretically calculated with the indicated bicarbonate concentration.

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 and 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 the contents was preincubated for 15 min. The manometers were read at 1-min intervals during the first 10 min after the addition of the substrate. During the reaction period 10–50 min, the manometers were read at every 5 or 10 min. The activity–pS curves were constructed from the initial linear velocities.

The concentrations of inhibitors for 50% inhibition (I_{50}) were determined graphically by plotting V/V_1 (V = velocity without inhibitor, V_1 = 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 carbon dioxide was linear.

Results

Rates of ChE Hydrolysis of LChs and ACh at Various Reaction Times.—The rates of evolution of CO_2 were linear during the initial 0–10 min with LChs as substrates and were depressed significantly after 10 min of interaction. With ACh, PCh, and BCh as substrates the rate of evolution of CO_2 was linear for more than 30 min. To illustrate these observations more clearly, the activity–pS curves were constructed separately for (1) initial linear reaction velocities (0–10 min) (Figure 2) and (2) mean reaction velocities during 10–20 min (Figure 3).

The activity–pS curve of ACh for the reaction period 0–10 min is almost identical with its activity–pS curve for the reaction period 10–20 min (Figures 2 and 3). For example, at pS 2.0, the rate of hydrolysis of ACh in both periods is about 132 μl of CO_2/hr. Similarly, there are no significant differences between the activity–pS curves of BCh for the reaction periods 0–10 and 10–20 min. For example, the rate of hydrolysis of BCh at pS 2.0 is about 300 μl of CO_2/hr in both periods.

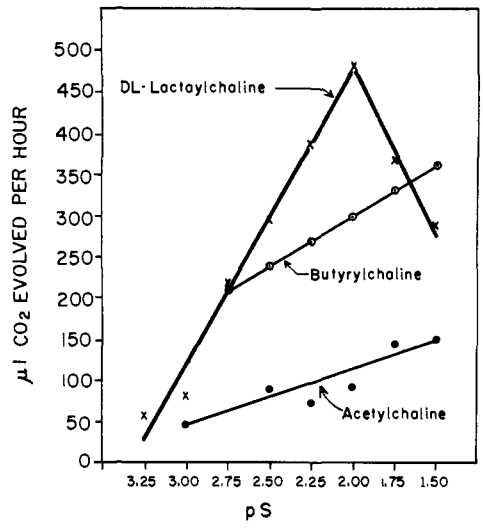

Figure 2.—Activity–pS curves for the ChE hydrolysis of LCh and related compounds during the period of initial linear velocities (0–10 min). Similar curves were obtained in five different experiments.

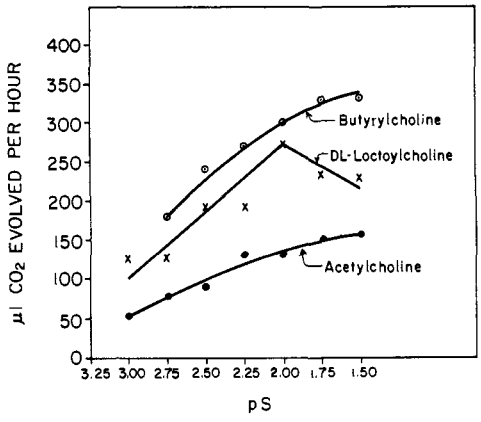

Figure 3.—Activity–pS curves for the ChE hydrolysis of LCh and related compounds during the reaction period 10–20 min. Similar curves were obtained in five different experiments.

In the case of DL-LCh, the activity–pS curve for the reaction period 10–20 min fell at a lower level than that for 0–10 min. The rate of hydrolysis of DL-LCh was higher than that of BCh during the reaction period 0–10 min between the pS values 2.75–1.75, but its rate of hydrolysis was lower than that of BCh during the reaction period 10–20 min at all substrate concentrations used in this study. At pS 2.0, the rate of hydrolysis of DL-LCh during the reaction period 0–10 min is about 475 μl of CO_2/hr, while it is 275 μl during 10–20 min, *i.e.*, the rate of hydrolysis of DL-LCh was depressed to about half in 10 min.

The lactoylcholines exhibited higher rates of autohydrolysis than those of ACh at all substrate concentrations. Corrections were made for autohydrolysis of various cholinesters in drawing the activity–pS curves.

The DL-lactoyl-DL-β-methylcholine (DL-LMCh) and DL-glyceroylcholine (DL-GCh) were not hydrolyzed significantly by the enzyme. The significance of this observation was discussed elsewhere.[16]

Activity–pS Curves of DL-LCh and ACh for the Initial Linear Velocities.—The activity–pS curve of DL-LCh (Figure 2) was bell shaped, indicating that

(15) O. Siggaard-Andersen, "The Acid-Base Status of the Blood," Williams & Wilkins Co., Baltimore, Md., 1964, pp 93–95.

(16) A. Lasslo, A. L. Meyer, and B. V. R. Sastry, *J. Med. Pharm. Chem.*, **2**, 91 (1960).

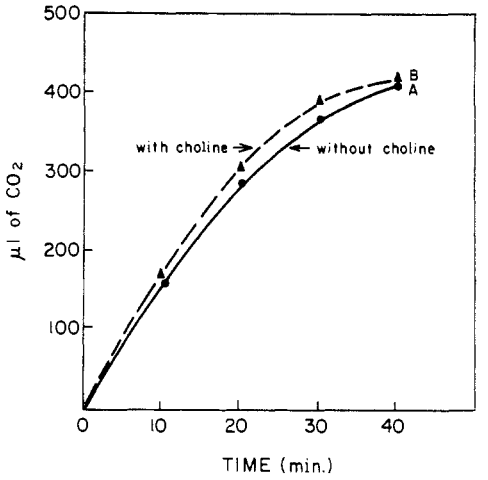

Figure 4.—Influence of choline on the hydrolysis of DL-LCh by ChE. pS of DL-LCh, 2.0; concentration of choline chloride, 40.62 μmol/3.0 ml of reaction mixture. Each point is a mean of three values. The difference between A and B was not significant ($P > 0.1$).

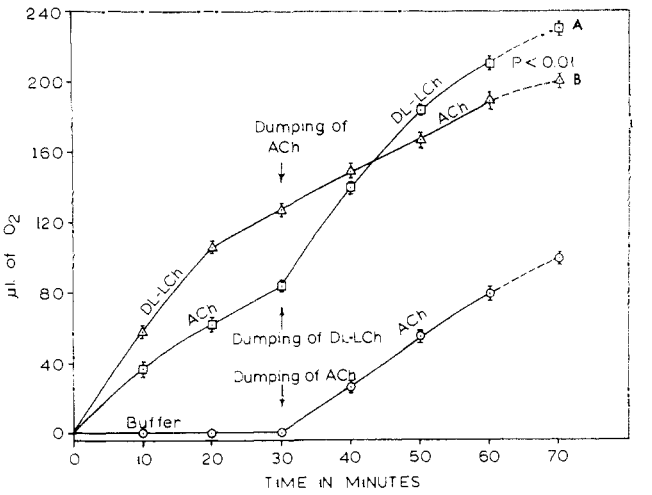

Figure 5.—Hydrolysis of ACh by ChE incubated with DL-LCh and *vice versa*. pS of substrates, 2.5. Each point is a mean of five values. The vertical lines indicate two standard errors. The same batch of ChE was used in all experiments. A, ACh was dumped at 0 time followed by DL-LCh at 30 min; B, DL-LCh was dumped at 0 time followed by ACh at 30 min.

DL-LCh would inhibit the enzyme at high substrate concentrations. The pS optimum was 2.0. The D-(−)- and L-(+)-LCh gave similar activity–pS curves. The activity–pS curves of BCh and ACh did not exhibit a pS optimum between the pS values 3.0 and 1.5 studied in the present investigation. The relative molar activities were calculated from the initial linear velocities at the pS optimum (2.0) of LChs.

At pS 2.0, DL-LCh was hydrolyzed at a rate three to four times faster than that of ACh and 1.5 times faster than that of BCh (Table I). The enzymic rate of L-(+) isomer was only slightly higher than that of D-(−) isomer (isomeric ratio, 1.1–1.5). Their affinities and V_{max} have the following order: L > DL > D.

Influence of Choline on the Hydrolysis of DL-LCh by ChE.—The enzymic hydrolysis of DL-LCh (pS, 2.0) was studied simultaneously in two reaction media: (1) the Krebs–Ringer bicarbonate buffer to which no choline chloride was added, and (2) the Krebs–Ringer bicarbonate buffer to which 40.6 μmol of choline chloride/3.0 ml of reaction mixture was added. Choline chloride that was added to the second medium was about five times higher than the amount of choline that would have formed from the enzymic and the nonenzymic hydrolysis of DL-LCh in the 40 min under the conditions described in methods. There was no significant difference between the enzymic rates of hydrolysis in both media (Figure 4). Therefore, choline which was formed during the reaction did not depress the enzymic hydrolysis of DL-LCh.

Hydrolysis of ACh by ChE Prior Incubated with DL-LCh.—In series A, ACh was dumped at 0 time, DL-LCh was dumped at 30 min, and the readings were taken for 60 min (Figure 5). Therefore, the reading at 60 min would indicate the combined value for the hydrolysis of ACh in 60 min and DL-LCh in 30 min. In series B, DL-LCh was dumped at 0 time and ACh was dumped at 30 min. Therefore, the reading at 60 min would indicate the combined rate of hydrolysis of DL-LCh in 60 min and ACh in 30 min.

At pS 2.5, the rate of hydrolysis of DL-LCh was at least twice greater than that of ACh. In series B, the substrate (DL-LCh) that was hydrolyzed faster was in contact with the enzyme for a longer period than the substrate (ACh) which was hydrolyzed relatively slowly. Therefore, one would expect that the CO_2

TABLE I

RELATIVE RATES OF CHOLINESTERASE HYDROLYSIS OF LACTOYLCHOLINE AND RELATED COMPOUNDS

Substrate	pS	Serum ChE	Rel rate of hydrol (ACh = 1)
L-(+)-LCh	2.00	Horse[a]	4.09
	2.09	Man[b]	2.67
	1.90	Dog[c]	2.22
	1.30	Dog[c]	2.73
D-(−)-LCh	2.00	Horse[a]	3.64
	2.09	Man[b]	1.70
	1.90	Dog[c]	1.22
	1.30	Dog[c]	1.29
DL-LCh (obsd)	2.00	Horse[a]	3.92
	2.09	Man[b]	2.15
DL-LCh (calcd)	2.00	Horse[a]	3.86[f]
	2.09	Man[b]	2.41[f]
	1.90	Dog[c]	1.72[f]
	1.30	Dog[c]	2.01[f]
DL-LMCh	2.09	Man[b]	0.20[e]
DL-GCh	2.09	Man[b]	0.23[e]
PCh	2.00	Horse[d]	1.82
	2.00	Man[e]	1.86
ArCh	2.00	Man[e]	1.01
BCh	2.00	Horse[d]	2.50
	2.00	Man[e]	2.39

[a] Purified enzyme. The rates of hydrolysis were measured at pS optimum (Figure 2). Calculated from initial linear velocities (0–10 min). $K_m \times 10^{-3}$ *M*: ACh, 3.80; L-(+)-LCh, 2.10; DL-LCh, 2.11; D-(−)-LCh, 2.2. V_{max} (μl of CO_2/hr): ACh, 337; L-(+)-LCh, 782; DL-LCh, 742; D-(−)-LCh, 718. [b] Purified enzyme. The values were quoted from the work of Lasslo, *et al.*[16] No pS optima were determined. Values were reported for only one substrate concentration and initial linear velocities were not measured. [c] The enzyme was an impure preparation from dog plasma with a pS optimum of 1.3. [d] No pS optima were found in the range of pS values 3.25–1.5 studied. [e] Purified enzyme. The values were quoted from the work of A. A. Sekul, W. C. Holland, and A. E. Breland, Jr., *Biochem. Pharmacol.*, **11**, 487 (1962). No pS optima were reported. Initial linear velocities were calculated. [f] The values were calculated from the rates of hydrolysis of D-(−) and L-(+) isomers.

evolved in 60 min in series B (DL-LCh and ACh) should be greater than that in series A (ACh and DL-LCh). But the total CO_2 evolved during 60 min in series B was significantly lower than that evolved in series A. Therefore, prior incubation of the enzyme with DL-LCh did seem to depress the rate of hydrolysis of ACh.

Influence of Sodium Lactate or a Mixture of Sodium Lactate and Choline Chloride on ACh Hydrolysis by ChE.—About 6.2 μmol of DL-LCh was split at pS 2.5 in 45 min by both enzymic and nonenzymic hydrolysis under the conditions described in the method. This would result in the formation of about 6.2 μmol of sodium lactate and 6.2 μmol of choline chloride in 3.0 ml of reaction medium. In order to verify as to whether the reaction products would change the enzyme activity, ChE was incubated in Krebs bicarbonate buffer containing sodium lactate (6.2 μmol/3.0 ml) or a mixture of sodium lactate and choline (each, 6.2 μmol/3.0 ml) for 45 min before ACh was dumped and CO_2 evolution was determined.

The results (Figure 6) have indicated that preincubation with sodium lactate alone did not depress the enzyme activity significantly. However, incubation with a mixture of sodium lactate and choline chloride depressed the enzyme activity (18%) significantly. Therefore, the depression of the rate of evolution of CO_2 as a function of time could be explained partially by product inhibition. However, this does not explain the descending limb in the activity–pS curve (Figure 2).

The conditions of this experiment were similar to those in series B of a preceding experiment (Figure 5). Instead of dumping DL-LCh an equimolar mixture of choline and sodium lactate were added initially to the reaction medium and incubated for 45 min (equivalent to 15 min of preincubation period *plus* 30 min of reaction time).

Enzymatic Hydrolysis of ACh and DL-LCh with Physostigmine as Inhibitor.—The enzyme was incubated for 15 min with 12×10^{-9} *M* physostigmine prior to the addition of the substrate. The activity–pS curves with ACh and DL-LCh as substrates were plotted for consecutive reaction periods 0–10, 10–20, 20–30, and 30–40 min (Figure 7). In the case of ACh the activity–pS curves for different reaction periods coincided with one another. This would suggest that a steady state was attained between the substrate and the inhibitor in their competition for the esteratic site within the first 10 min and was maintained in the consecutive 30 min.

With DL-LCh as substrate, and the same concentration of physostigmine as the inhibitor, a typical bell-shaped activity–pS curve for the reaction period 0–10 min was obtained. An "apparent steady state" did seem to be established between the substrate and inhibitor to compete for the active site. The pS–optimum without the inhibitor was 2.0 and with the inhibitor 1.75. Therefore, the pS–optimum moved to high substrate concentrations, which was generally observed under steady-state conditions in the presence of a competitive inhibitor.[17] However, the activity–pS

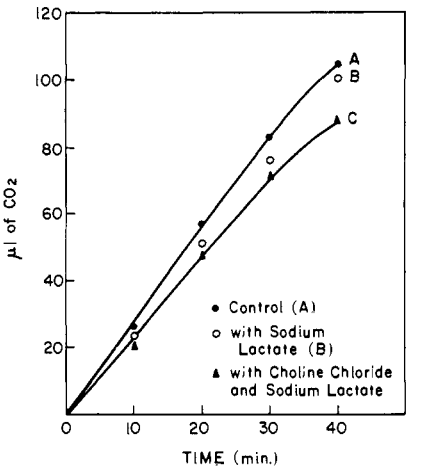

Figure 6.—Influence of sodium lactate or a mixture of sodium lactate and choline chloride on the hydrolysis of ACh by ChE. pS of ACh, 2.5. Each point is a mean of five values. A (solid circles), evolution of CO_2 with ACh only; B (hollow circles), evolution of CO_2 with ACh and sodium lactate (6.2 μmol/3.0 ml); C (solid triangles), evolution of CO_2 with ACh, sodium lactate (6.2 μmol/3.0 ml), and choline chloride (6.2 μmol/3.0 ml). The difference between A and B was not significant ($P > 0.1$). The difference between A and C was significant ($P < 0.01$).

curve for the reaction period 10–20 min would suggest that the "steady state" was disturbed in about 20 min and a part of the inhibition due to physostigmine was reversed. The whole activity–pS curve has shifted to higher rates of hydrolysis and the descending limb at high substrate concentrations disappeared. This was more apparent at high substrate concentrations in the activity–pS curve for the reaction period 20–30 min. The highest rate of hydrolysis of DL-LCh was observed during 20–30 min at pS 1.5. In about 30–40 min, a new set of "steady-state" conditions did seem to be established. The activity–pS curve was again bell shaped with pS optimum at 1.75. The descending limb was at least partially due to the reappearance of substrate inhibition. The rate of evolution of CO_2 (280 μl/hr) at pS optimum during 30–40 min in the presence of the inhibitor was almost equal to that (275 μl/hr) at 10–20 min without the inhibitor. These observations have suggested that an actual steady state was not attained between DL-LCh and physostigmine in competing for the active site. Although the enzyme was incubated with physostigmine prior to the addition of the substrate, DL-LCh was able to reverse the inhibition, at least partially, in about 20 min.

Lineweaver–Burk plots of the kinetics of the hydrolysis of ACh with and without physostigmine suggested that the mechanism of inhibition was competitive in the conditions adapted in the present investigation (Figure 8). Lineweaver–Burk plots of DL-LCh hydrolysis without inhibitor and with 12×10^{-9} *M* physostigmine were parallel. It has been shown that such parallel lines will be obtained during "uncompetitive" (anticompetitive) inhibition in which the inhibitor combines with the enzyme–substrate complex, but not with the enzyme.[18] This would suggest that physostigmine did interact with ChE–DL-LCh complex.

(17) K. B. Augustinsson, *Acta Chem. Scand.*, **5**, 699 (1951); K. B. Augustinsson and D. Nachmansohn, *Science*, **110**, 98 (1949); F. Bergmann, I. B. Wilson, and D. Nachmansohn, *J. Biol. Chem.*, **186**, 693 (1950).

(18) K. S. Dodgson, B. Spencer, and K. Williams, *Nature*, **177**, 432 (1956); J. L. Webb, "Enzyme and Metabolic Inhibitors," Vol. 1, Academic Press Inc., New York, N. Y., 1963, pp 59–60, 160–165.

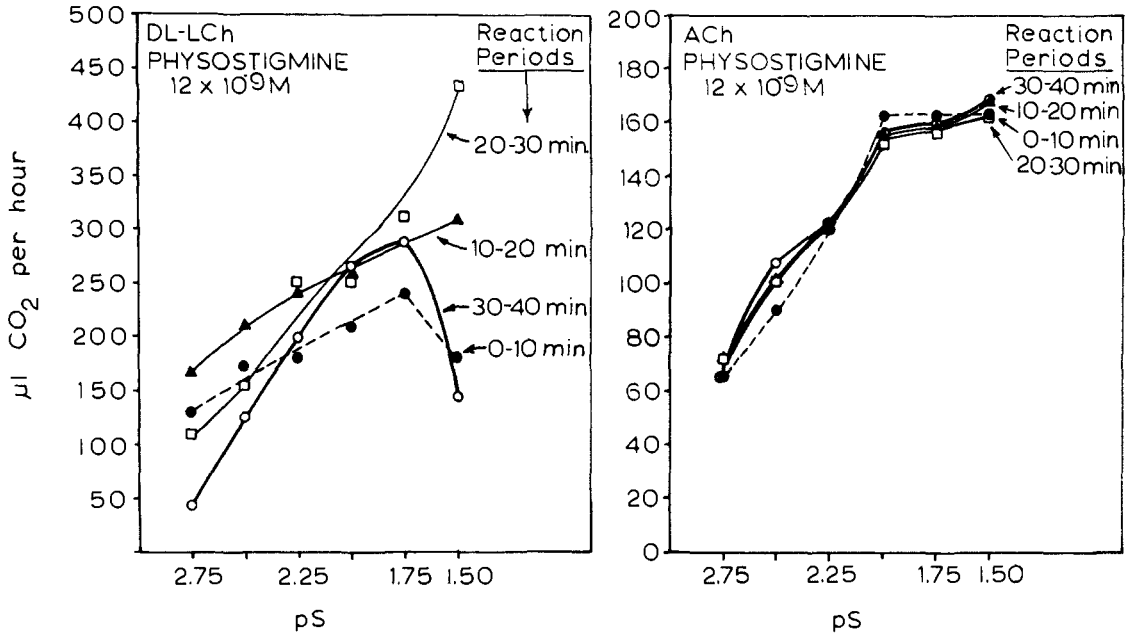

Figure 7.—Activity–pS curves for ChE hydrolysis of DL-LCh and ACh in the presence of physostigmine during various reaction periods. The enzyme was incubated with the inhibitor for 15 min before the substrate was added.

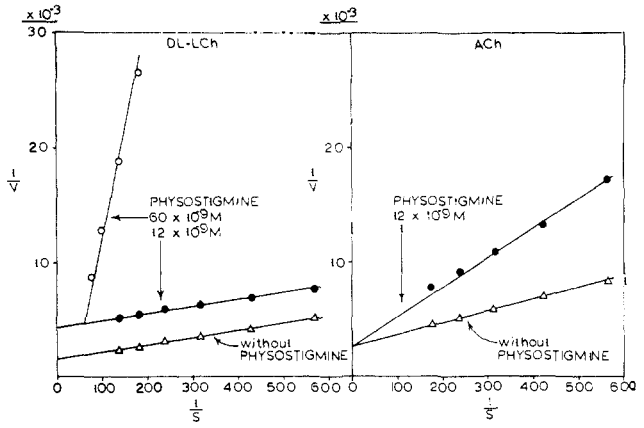

Figure 8.—Lineweaver–Burk plot of the kinetics of ChE hydrolysis of DL-LCh and ACh in the presence of physostigmine ACh, steady-state conditions; DL-LCh, apparent steady-state conditions.

When the inhibitor concentration was increased to 60 $\times$ 10^{-9} M, physostigmine did seem to show partially competitive kinetics. In other words, with DL-LCh as substrate, physostigmine interacts with the enzyme as well as enzyme–substrate complex and shows "mixed kinetics."

Activities of ChE Inhibitors with DL-LCh and ACh as Substrates.—The activity–pS curve of ACh was not parallel to that of DL-LCh. Consequently, a question might arise as to whether the same active sites on the enzyme were involved in the hydrolysis of both DL-LCh and ACh. Therefore, the I_{50} values of different types of ChE inhibitors were determined with ACh and DL-LCh as substrates (Table II). All types of strong ChE inhibitors (tertiary or quaternary, reversible or irreversible) with ACh as substrate were also strong inhibitors of DL-LCh hydrolysis.

DL-GCh and DL-LMCh were not only poor substrates but also poor inhibitors of ChE hydrolysis of both ACh and DL-LCh.

Table II
Activities of Cholinesterase Inhibitors with Acetylcholine and Lactoylcholine as Substrates

Substrate[a]	Inhibitor[b]	I_{50}, M[d]
Acetylcholine iodide	Physostigmine sulfate	3.2×10^{-8}
	Neostigmine methyl sulfate	8.0×10^{-8}
	Phospholine iodide	2.2×10^{-10}
	217-AO[c]	7.5×10^{-9}
	DL-Glyceroylcholine iodide	2.2×10^{-2}
	DL-Lactoyl-β-methylcholine iodide	9.5×10^{-3}
DL-Lactoylcholine iodide	Physostigmine sulfate	2.5×10^{-8}
	Neostigmine methyl sulfate	6.5×10^{-8}
	Phospholine iodide	2.8×10^{-11}
	217-AO[c]	1.1×10^{-10}
	DL-Glyceroylcholine iodide	1.4×10^{-2}
	DL-Lactoyl-β-methylcholine iodide	2.4×10^{-2}

[a] pS of substrates, 2.0. [b] A minimum of three inhibitor concentrations were used. [c] A tertiary analog of phospholine. [d] Calculated from initial linear velocities, when equilibrium conditions were established.

Discussion

A direct comparison of the rates of hydrolysis of the isomers of LCh with that of ACh is not possible because the activity–pS curve of ACh intersects those of LCh (Figure 2). Further, all isomers of LCh inhibited the enzyme at high substrate concentrations. However, the initial rates of hydrolysis suggest that LCh's were hydrolyzed faster than ACh at all substrate concentrations. The L-(+) isomer was hydrolyzed faster than DL- or D-(−) isomer (Table I). As the activity–pS curves of all isomers were nearly bell shaped and showed the same pS optimum (2.0), their values of K_m and V_{max} could be compared with one another. If K_m and V_{max} were to be taken as approximate measures of the two stages, formation and breakdown of ES complex,[19] both of these processes were faster with L-(+) isomer than D-(−) isomer.

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The strong ChE inhibitors of ACh hydrolysis were also strong inhibitors of DL-LCh hydrolysis (Table II). Therefore, the hydrolysis of both substrates did seem to be catalyzed by the same esteratic site on the enzyme.

There are some suggestions in the published literature that, while one enantiomer is hydrolyzed by the enzyme, the second enantiomer may behave like an inhibitor.[20] Therefore, a question may arise that, while L-(+) isomer was hydrolyzed, accumulation of D-(−) isomer would inhibit ChE, when DL-LCh was used as a substrate. The isomeric ratio between the rates of hydrolysis of the enantiomers of LCh by ChE was only 1.1–1.7. The arithmetic mean between the rates of hydrolysis of the enantiomers is not significantly different from that of the racemate. Further, the enantiomers and the racemate were hydrolyzed by ChE faster than ACh. The activity–pS curves of the enantiomers, as well as the racemate, were bell shaped. Therefore, the difference in the configuration of L-(+)- and D-(−)-LCh is not a factor by which the substrate inhibition of ChE by DL-LCh could be explained. Similarly, the substrate inhibition could not be explained completely by the products of hydrolysis, because either choline or lactate alone did not inhibit the enzymic hydrolysis of ACh.

The mechanism of hydrolysis of cholinesters by AChE is explained by assuming that a labile acyl enzyme is formed as an intermediate during hydrolysis.[21] Further, the recent investigations by Krupka and Laidler[22] and Wilson and Alexander[23] suggest that deacetylation of acetyl-AChE is prevented by binding of a molecule of ACh to the acyl enzyme at the anionic site at high substrate concentrations. A similar possibility exists for the accumulation of lactoyl-ChE because (1) the pS optimum moves to high substrate concentrations in the presence of physostigmine, (2) physostigmine shows "mixed kinetics" (uncompetitive and partially competitive) with DL-LCh as a substrate, while it exhibits competitive kinetics with ACh as a substrate, and (3) incubation of ChE with DL-LCh or a mixture of equimolar amounts of sodium lactate and choline depresses the rate of hydrolysis of ACh by the enzyme.

The existence of an anionic site in ChE has been the subject of much discussion, and two different opinions were expressed: (1) an anionic site of the same strength as in AChE does not exist in ChE,[24,25] and (2) one anionic site is present for each esteratic site on ChE.[25,26] Even if an anionic site was present on ChE, that site does not seem to be involved in the substrate inhibition caused by LCh because ACh and BCh do not inhibit ChE. However, it is possible that a second molecule of LCh can combine at a nonspecific site on the enzyme and prevent delactoylation of lactoyl-ChE.

A fair correlation was reported between the capacity to inhibit ChE and the electrophilic strength of the carbon of carboxamides derived from piperidine.[27] The inhibitory strength increases with the enhancement of the electrophilic character of the carbonyl carbon. Further, it has been reported that, in the course of the inhibition of ChE by carbamates, the inhibitor is slowly hydrolyzed[28] (possibly through the formation of a carbamyl-ChE) due to the strong binding between electrophilic carbonyl carbon and nucleophilic group at the esteratic site. The carbonyl carbon of LCh (pK of lactic acid = 3.86) is more electrophilic than that of ACh or BCh (pK of acetic and butyric acids = 4.76 and 4.82, respectively).

The effectiveness of LChs as substrate inhibitors may be related partly to the electrophilic strength of their carbonyl carbons. It is interesting to record that several other esters exhibit the characteristics of the substrate inhibitors of ChE. Haloacetic acid esters of aliphatic alcohols[24,29] and choline esters of fluoro-, chloro-, bromo-, and iodoacetic acids (pK = 2.66, 2.85, 2.85, 2.91, respectively),[30] benzoic acid (pK = 4.19),[31] acetylsalicylic acid (pK = 3.49),[32] and furoic acid (pK = 3.11) have been shown to inhibit ChE at high substrate concentrations. In all of these esters, the carbonyl carbons are more electrophilic than that of ACh or BCh. Therefore, the presence of strong electrophilic carbonyl carbons does seem to be one of the common features of carbamate inhibitors as well as substrate inhibitors. The electrophilic character of carbonyl carbon in BCh seems to be optimum for rapid hydrolysis without substrate inhibition.

The electrophilic carbons in LCh, ACh, and carbamates compete for the same nucleophilic site on the enzyme. The carbonyl carbon in LCh is more electrophilic than that of ACh and competes far better for the nucleophilic group at the esteratic site. With physostigmine (12×10^{-9} M) as inhibitor and ACh as substrate steady state was obtained within 10 min and reversal of inhibition was not found even after 30 min. With DL-LCh as substrate, true "steady-state conditions" were not established and partial reversal of the inhibition by physostigmine was observed within 20 min.

If L-(+)-LCh were to occur in nature as a potent neurohumor or local hormone, there are adequate biochemical mechanisms for its disposal or to prevent its accumulation in animal tissues because it was hydrolyzed rapidly both by AChE and ChE. It is hydrolyzed faster than two of its analogs, propionyl- and acrylylcholines, reported to occur in animal tissues. Studies on its synthesis from lactoyl-coenzyme A and choline by choline acyltransferase are in progress.

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