cats. It might be of interest in this connection to determine whether physostigmine, the central actions of which are opposed to those of prostigmine, also potentiates the analgesic action of morphine.

Summary. 1. The D- and L-isomers of amidone and isoamidone inhibit the cholinesterases of rat brain and dog serum. 2. These drugs exert a greater inhibitory action on serum cholinesterase than on brain cholinesterase. 3. The L-isomers are more effective than the D-isomers in inhibiting these enzymes. 4. The greatest difference in effect between the D- and L-isomers was observed in experiments on the effect of D- and L-isoamidone on brain cholinesterase.

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Histochemical Demonstration of Sites of Choline Esterase Activity.*

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Glick[1] has shown that choline esters of higher fatty acids are hydrolyzed by choline esterase at an appreciable rate. Since fatty acids from C_{10} up form highly insoluble Ca, Ba and Co salts, the histochemical demonstration of choline esterase activity appeared feasible, provided the enzyme could survive the rigors of fixation and embedding.

Experimental. Previously, lauroyl-, myristoyl-, palmitoyl-, and stearoyl choline chloride had been synthesized by the addition of trimethylamine onto the corresponding β-chloroethyl esters.[2] For the present experiments the compounds were prepared by direct esterification of choline chloride with the corresponding acyl chlorides. An equimolar mixture of choline chloride and acyl chloride was heated to about 130 to 140°C under constant stirring. Evolution of HCl ceased after about 1 hour, and the mixture solidified into a thick paste. This mass was washed with petroleum ether and subsequently extracted by refluxing it with acetone. On cooling (sometimes in the ice box) the ester chloride precipitated in the form of a white crystalline solid. It was purified by 2 more recrystallizations from boiling acetone. It was impossible to determine the exact melting points because all esters became semifluid just above 58°C. Their purity was checked by analysis for N, Cl, and, after hydrolysis by 0.5 N alkali at 56°C, for fatty acid.

The rates of hydrolysis of the various esters by choline esterase were determined by the following methods:

Acetylcholine. A 0.01 M solution of acetylcholine bromide in 0.1 M phosphate buffer of pH 8 was prepared. 25 ml samples of the buffered substrate were placed in 3 flasks. To one of the flasks 4 drops of a 0.01 M solution of prostigmine bromide was added; this flask served as a control. One-half to 1 ml of tissue homogenate (usual ratio, 1:10 to 1:20) or serum was added to all flasks which were subsequently incubated for 1 hour at 37°C. At the end of this period, hydrolysis was stopped by the addition of 4 drops of prostigmine solution. The flasks were cooled to room temperature and titrated with 0.025 N NaOH to the same blue shade (pH $\pm$ 9.2), thymol blue being used as an indicator.

Lauroyl-, etc. choline. 0.002 M solutions of the substrates in 0.05 M tris(hydroxymethyl)-aminomethane-HCl buffer[3] of pH 8 were prepared. Ten ml samples of the buffered substrates were pipetted into 3 centrifuge tubes.

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1 Glick, D., *J. Biol. Chem.*, 1941, **137**, 357.

2 Fourneau, E., and Page, H. J., *Bull. soc. chim. de France*, 1914, **15**, 544.

3 Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1946, **62**, 33.

TABLE I.
Data of Analysis of Choline Ester Chlorides.

Ester	N%		Cl%		Fatty acid mEq/g	
	Theor.	Found	Theor.	Found	Theor.	Found
Lauroyl	4.35	4.16	11.03	10.33	3.11	3.35
Myristoyl	4.00	3.85	10.15	9.99	2.86	2.85
Palmitoyl	3.71	3.63	9.4	9.3	2.65	2.78
Stearoyl	3.46	3.53	8.75	8.64	2.47	2.35

TABLE II.
Rates of Enzymatic Hydrolysis of Choline Esters.
(Micromoles of ester hydrolyzed in 1 hr by 1 g of tissue or 1 ml of serum or enzyme preparation.)

Enzyme	Acetyl	Lauroyl	Myristoyl	Palmitoyl	Stearoyl
Dog serum	100	6.7	8.3	0.5	0
Dog adrenal	225	186	62	12	not done
Mouse brain	675	36	11.4	2.5	12.5
Rat brain	375	7.7	5	3.6	0
Pigeon brain	450	40	31.6	28	21.5
Purified bovine choline esterase (Winthrop); 1 mg	475	1.6	0	0	0

To one of the tubes 2 drops of prostigmine solution were added. One-half to 1 ml of tissue homogenate or serum (dialyzed against normal saline for 4 hours to remove the bulk of Ca) was added to each of the tubes which were subsequently incubated for 1 hour at 37°C. At the end of this period, hydrolysis was stopped by the addition of 2 drops of prostigmine solution. The tubes were spun for 10 minutes at 2400 rpm. Five ml samples were transferred to colorimetric tubes, and 5 ml of a 0.2% solution of $CaCl_2$ in 1.5% gelatin was added to each of them. The turbidity resulting from Ca soaps was determined with a photoelectric colorimeter, the sample incubated in the presence of prostigmine being used as a blank. The amount of fatty acid liberated was read from a curve previously calibrated with solutions of Na laureate, containing 1 to 12 μM of salt in 5 ml.

It is clearly realized that this latter method is not suitable for exact studies since part of the fatty acid liberated is precipitated by Ca and Mg in the tissues and another part may be adsorbed to the tissue fragments. However, it served as a useful guide for the approximate estimation of rates of hydrolysis, and its results were in good agreement with histological findings.

As shown, most of the crude enzymes used hydrolyze higher choline esters at an appreciable rate, although the pattern of relative rates varies considerably according to the source of the enzyme. In fact, a highly purified enzyme preparation of the electric organ of *Torpedo* (generously contributed by Dr. D. Nachmansohn of the Department of Neurology, Columbia University), although extremely active towards acetylcholine (4700 μM/ml/hr), failed to hydrolyze any of the higher esters.

By the use of Coujard's technic,[4] it could be proven that choline esterase survives, at least to some extent, the procedures of fixation and embedding. With a clean steel pen marks were made on microscopic slides, dog serum and homogenates tested in previous experiments being used as ink. The slides were dried rapidly and immersed in chilled acetone for 12 hours, subsequently treated with cold alcohol-ether mixture for 2 hours and finally with xylene for 1 hour at 56°C. The slides were then coated with a thin film of collodion and incubated for 2 to 12 hours at 37°C in a 0.002 M solution of myristoylcholine, buffered to pH 8 with tris(hydroxymethyl)-aminomethane and containing 0.01 M $CaCl_2$. When

[4] Coujard, R., *Bull. d'hist. appl.*, 1943, **20**, 161.

the slides were removed from the mixture and examined under the microscope, a thick deposit of typical crystals of calcium myristate was seen at the sites of the marks. No deposition of crystals could be observed at the marks made with brain homogenates if eserine or prostigmine was added to the incubating mixture in a concentration of 10^{-5} M, while in the marks made with serum, enzymatic activity was greatly reduced but not completely abolished.

On the basis of these findings a histochemical technic for the visualization of sites of choline esterase activity could be worked out. At first it was attempted to duplicate the technic for lipase[5] in all details except for the different substrate but after many trials it became apparent that conditions must be modified in several respects. The following technic, which is not to be considered final, gave good results in the overwhelming majority of cases. Rapid fixation seems to be critical since often only the more superficial layers of the block were entirely satisfactory while in the depth the preservation of the enzyme was spotty.

1. Fix and embed tissues the same way as in the lipase technic, except for a minor modification in embedding which improves the cutting qualities of the block. After acetone dehydration, run tissues through a/, a mixture of equal parts of absolute alcohol and ether (1 to 3 hours); b/, 4% collodion in alcohol-ether (12 hours); c/, 2 changes of chloroform, 1 hour each. All steps up to c/ should be performed at ice box temperature. From chloroform, embed in paraffin. Bring sections, as in the technic for lipase, to distilled water.

2. Incubate slides in the following mixture for from 2 to 16 hours at 37°C:

Stock solution I. 0.1 M maleate-tris(hydroxymethyl)-aminomethane buffer pH 7.5-7.7, 50 to 100 ml; 0.1 M cobaltous acetate solution, 30 to 50 ml; distilled water to make 300 ml; add about 1 mg each of $CaCl_2$, $MgCl_2$ and $MnCl_2$.

Preparation of the buffer. Dissolve 29 g of maleic acid and 30.4 g of tris(hydroxymethyl)-aminomethane in 500 ml of water, decolorize with a few g of charcoal and filter. Ten ml of this stock solution + the indicated number of ml of 0.5 N NaOH, diluted to a total of 50 ml, will give 0.1 M buffers of pH values from 5.08 to 8.45 at 23°C.

TABLE III.
Maleate-tris(hydroxymethyl)-aminomethane buffer.

ml 0.5N NaOH	pH	ml 0.5N NaOH	pH
1	5.08	9	6.86
2	5.30	10	7.20
3	5.52	11	7.50
4	5.70	12	7.75
5	5.88	13	7.97
6	6.05	14	8.15
7	6.27	15	8.30
8	6.50	16	8.45

Stock solution II. A 0.02 M solution of choline ester in distilled water. For most purposes the myristoyl ester is the most satisfactory; it requires an incubation time of about 6 hours. Both stock solutions, with a crystal of camphor added, will keep in the ice box indefinitely. For use, add 1 ml of stock solution II to 50 ml of stock solution I, previously heated to 37 to 40°C. At this temperature the mixtures should be perfectly clear, except in the case of the stearoyl ester which is not readily soluble in the presence of salts.

3. Wash slides under the tap for about 2 minutes.

4. Immerse them in a dilute solution of light yellow ammonium sulfide (a few drops to 50 ml of distilled water) for about 15 minutes. Darkening of sites of enzyme activity is very gradual, especially if palmitoyl choline was used as a substrate.

5. Wash slides under the tap. Counterstain as desired; dehydrate, clear and mount. Sites of choline esterase activity are black.

At first, in analogy to the technic for lipase, Ca, later Sr, Ba and Mg were tried instead of Co. However, all of them were found to give very coarse crystalline precipitates while Co gives a fine granular precipitate. The localization of the enzyme and the intensity of the reaction were the same, regardless of the metal ion used. When used in the technic for lipase, Co causes a moderate but noticeable

[5] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1945, 58, 362.

inhibition of the enzyme in certain locations. $CaCl_2$, $MgCl_2$ and $MnCl_2$ were added to the incubating mixture because their metal ions activate choline esterase *in vitro*[6] and seem to intensify the picture also in the histochemical experiment. The buffer used was chosen because, in comparison with other buffers (tris-(hydroxymethyl)-aminomethane-HCl, collidine), it seemed to inhibit the enzyme very little and to prevent the nonspecific impregnation of the tissues by Co which at the relatively high pH level of optimal enzymatic activity may be quite troublesome. The low substrate concentration was adopted because it was found that higher concentrations of substrate (over 0.001 M) inhibit the enzyme. Similar inhibition in even lower ranges of substrate concentration was observed by Mendel and Rudney,[7] and by Alles and Hawes.[8]

So far only a limited number of tissues were examined by the new technic. The presence of choline esterase could be ascertained in many tissues. Only some of the most interesting locations will be mentioned here: sympathetic ganglia in all species examined; brain of the pigeon, the dog (especially in the basal ganglia), the mouse (especially certain nuclei of the oblongata; Fig. 1); smooth muscle of the gastrointestinal tract, urinary bladder and uterus in several species; the conducting system in the dog's heart (Fig. 2); structures in the striated muscle of the mouse and the pigeon, tentatively identified as muscle spindles and not as motor end plates since they lie in the septa, separated by connective tissue from the muscle fibers proper (Fig. 3); adrenal medulla in some species. The reaction is completely inhibited in most tissues by the addition of prostigmine in a concentration of 10^{-5} M to the incubating mixture.

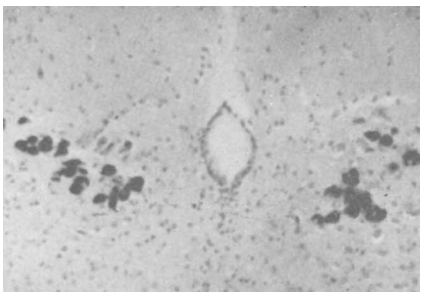

FIG. 1.
Brain of the mouse. A group of ganglion cells (nucleus of XIIth nerve?) stained selectively. Substrate: myristoyl-choline.

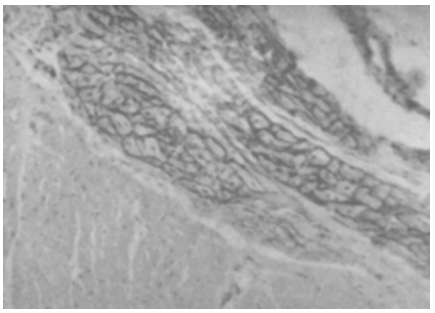

FIG. 2.
Conducting system of the dog's heart. Substrate: myristoyl-choline.

FIG. 3.
Typical structure in muscle of the mouse. Substrate: myristoyl-choline.

Besides the demonstration of enzymatic activity at the specific locations mentioned, the following observations of interest were made:

1. There is a considerable difference in the pattern of distribution of the enzyme in various species although the sympathetic nervous system or parts of it are stained in most species.

2. The finer distribution of the enzyme differs according to the substrate used. With lauroylcholine the enzyme presents a granular appearance and is often found in nuclei while

[6] Nachmansohn, D., *Nature*, 1940, **145**, 513.

[7] Mendel, B., and Rudney, H., *Bioch. J.*, 1943, **37**, 59.

[8] Alles, G. A., and Hawes, R. C., *J. Biol. Chem.*, 1940, **133**, 375.

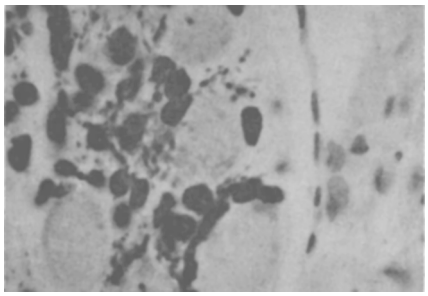

FIG. 4.
Sympathetic ganglion of the rabbit. Substrate: lauroyl-choline. Nuclei of satellite cells and granular material stained.

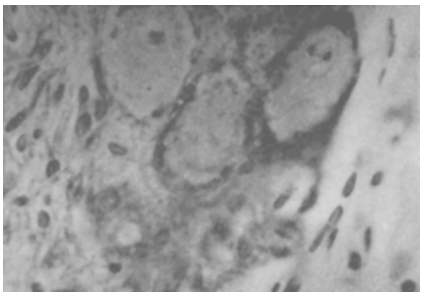

FIG. 5.
Another section of the same tissue block. Substrate: palmitoyl-choline. Nerve fibers around ganglion cells stained.

with palmitoylcholine it appears to be distributed in a uniform way mainly in nerve fibers. The most striking example is the sympathetic ganglia in which lauroylcholine produces an intense staining of the nuclei of the satellite cells (Fig. 4) while palmitoylcholine outlines the nerve fibers, the satellite cells being left entirely unstained (Fig. 5). This difference may indicate the existence of 2 (or more) different enzymes.

3. There is a species preference for certain substrates. Dog and mouse tissues give much more intense pictures with lauroyl- and myristoylcholine than with palmitoylcholine while for human and pigeon tissues the reverse holds true. Rat and guinea pig tissues fail to hydrolyze any of the substrates at a rate sufficiently high to give clearcut results.

Summary. Some choline esterases hydrolyze higher choline esters readily and they also survive to a demonstrable extent the procedures of histological fixation and embedding. It is possible to demonstrate them histochemically by incubating slides with the substrate in the presence of a cobalt salt; at the sites of choline esterase activity insoluble cobalt-soap will precipitate which can be transformed into black CoS. The histochemical distribution and properties of choline esterase are described.

16486

Characteristics of the Protein Groups Associated with the Hypotensive Activity of Trypsin.*

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In view of the action of trypsin and parenteral trypsin-like proteases and their inhibitors in certain types of shock,[1-5] blood coagulation[6-12] and other fields it is desirable to char-

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3 Burdon, K. L., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 24.

4 Rocha e Silva, M., and Teixeira, R. M., PROC. SOC. EXP. BIOL. AND MED., 1946, **61**, 376.