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ACCELERATION OF THE ENZYMIC HYDROLYSIS OF BENZOYLCHOLINE

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During routine examination of new compounds in these laboratories, one rarely finds substances which enhance the action of cholinesterases. One such substance, however, is (+)-1-*cyclo*hexyl-1-phenyl-3-pyrrolidinopropan-1-ol methiodide ((+)-procyclidine methiodide, (+)-tricyclamol iodide). Activation of cholinesterases has been claimed for histidine, and some other amino acids, by Aron, Herschberg, and Frommel (1944), and Frommel (1946, 1947); for vitamin E by Bloch (1942); for folic acid by Davis (1946a, b, c); and for dihydrohydroxycodeinone hydrochloride (Eukodal) by Hazard, Cornec, and Pignard (1950). Furthermore, Todrick, Fellowes, and Rutland (1951) showed that certain alcohols activated the hydrolysis of acetylcholine by rat brain and human erythrocytes; the activation was less marked when the specific substrates, benzoylcholine and acetyl-β-methylcholine, were used with the cholinesterases of horse serum and rat brain, respectively.

This paper is concerned with analogous effects of a number of commonly used drugs on certain cholinesterase systems.

METHODS

Cholinesterase activity was determined by the Warburg manometric method as previously described (Fraser, 1954). The drug and cholinesterase preparation were kept in contact for 30 min. before starting the main experiment. The rates of substrate hydrolysis by cholinesterase in the presence of each compound over a wide range of concentrations were compared with that in its absence. The results, given as percentage acceleration of the normal rate of hydrolysis, are positive for acceleration and negative for deceleration, or inhibition. The possibility of liberation of gas from the interaction of the compounds under test and the esterase preparation was eliminated by suitable controls. Atropinase activity was similarly determined in the Warburg respirometer, with atropine sulphate as substrate, at a final concentration of 0.002 M.

Cholinesterase Preparations.—The tests were usually performed with the cholinesterases of human blood (Fraser, 1954). Plasma was the source of pseudo-cholinesterase, which had an activity of 1,500 μl. CO_2/ml./hr. with benzoylcholine (0.015 M) as substrate; lysed red cells were the source of the true cholinesterase, which had an activity of 2,000 μl. CO_2/ml./hr. with acetyl-β-methylcholine (0.03 M) as substrate. Sometimes rabbit plasma or horse serum was used as a source of cholinesterase. Unless otherwise stated, the substrates and their final concentrations were as follows: acetylcholine chloride, 0.015 M; acetyl-β-methylcholine chloride, 0.03 M; benzoylcholine chloride, 0.015 M; butyrylcholine iodide, 0.015 M; tributyrin, 0.03 M; and succinyldicholine dichloride, 0.015 M.

RESULTS

Effect on Pseudo-cholinesterase.

The Procyclidine Group.—The racemic and optically active forms of the hydrochloride, methiodide and ethiodide of procyclidine were studied. The nitrogen in the last two is quaternary, whereas in the hydrochloride it is tertiary. Duffin and Green (1955) have found that the *laevo* compounds possess a considerable degree of atropine-like activity. This is virtually absent from the *dextro* forms, and the racemic mixtures are intermediate in activity.

With acetylcholine, butyrylcholine, or tributyrin as substrate, all isomerides and the racemic mixtures inhibited the plasma pseudo-cholinesterase. With benzoylcholine as substrate (+)-, (−)- and (±)-procyclidine hydrochloride, and the atropine-like *laevo* isomerides of the methiodide and ethiodide, had an inhibitory effect, but the *dextro* forms of the methiodide and ethiodide accelerated the hydrolysis of benzoylcholine. As the concentration of these compounds was increased, the acceleration also increased, but was ultimately replaced by inhibition. Fig. 1 shows that with (+)-procyclidine methiodide the benzoylcholine curve had a peak acceleration of 34% at 5.0×10^{-4}M. Similarly, (+)-procyclidine ethiodide had a peak activity of 48% at 7.1×10^{-4}M. The racemic mix-

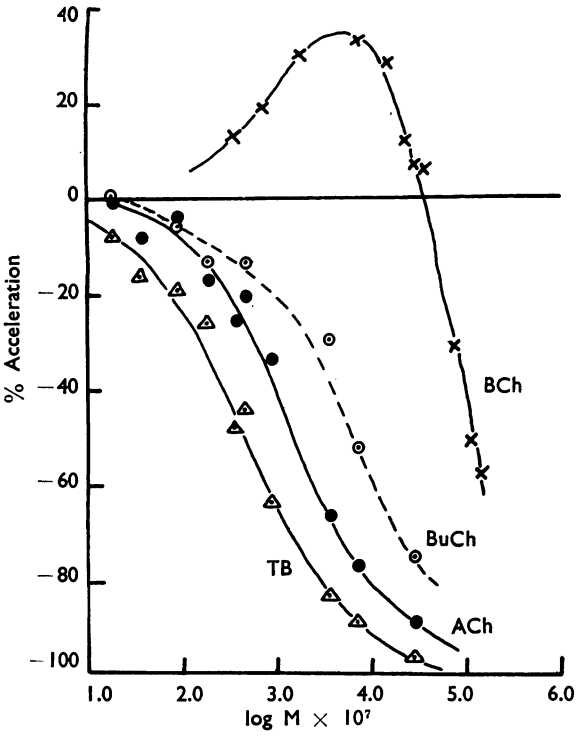

FIG. 1.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by (+)-procyclidine methiodide. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of (+)-procyclidine methiodide. Peak acceleration of benzoylcholine hydrolysis of 34% at 5.0×10^{-4}M. I 50's human plasma: with benzoylcholine 1.4×10^{-3}M; with butyrylcholine 6.3×10^{-4}M; with acetylcholine 1.6×10^{-4}M.

tures of the methiodide and of the ethiodide showed slight peak accelerations which were not statistically significant. (The peak acceleration figure is taken directly from the graph.)

From these results it appeared that the accelerating effect might be associated with one or more of the following: the quaternary nitrogen in the pyrrolidine ring; the alkyl group attached to this nitrogen; or the spatial configuration of the compounds. Further compounds containing nitrogen, or showing optical activity, were therefore tested.

The Hyoscyamines.—This group was compared with the procyclidine group for four reasons: it possesses optical isomerism; its members have a pyrrolidine ring with a substituent methyl group on the nitrogen in the molecule; the nitrogen is tertiary; and the atropine activity varies in intensity among the members of the group. Here again no acceleration of the hydrolysis of acetylcholine, butyrylcholine, or tributyrin was observed in the presence of either (+)-, (−)-, or (±)-hyoscyamine, but the rate of hydrolysis of benzoylcholine was markedly increased in the presence of any of the three forms. (+)-Hyoscyamine showed an approximate peak acceleration of 80% at 2.5×10^{-3}M, (−)-hyoscyamine 100% at 2.5×10^{-3}M, and (±)-hyoscyamine (atropine) 99% at 3.2×10^{-3}M. These figures are roughly comparable. Again, increase in the concentration beyond that giving peak acceleration led to inhibition. No atropinase activity was found in human plasma. Fig. 2 shows the results obtained with atropine. Atropine was further tested on a horse serum

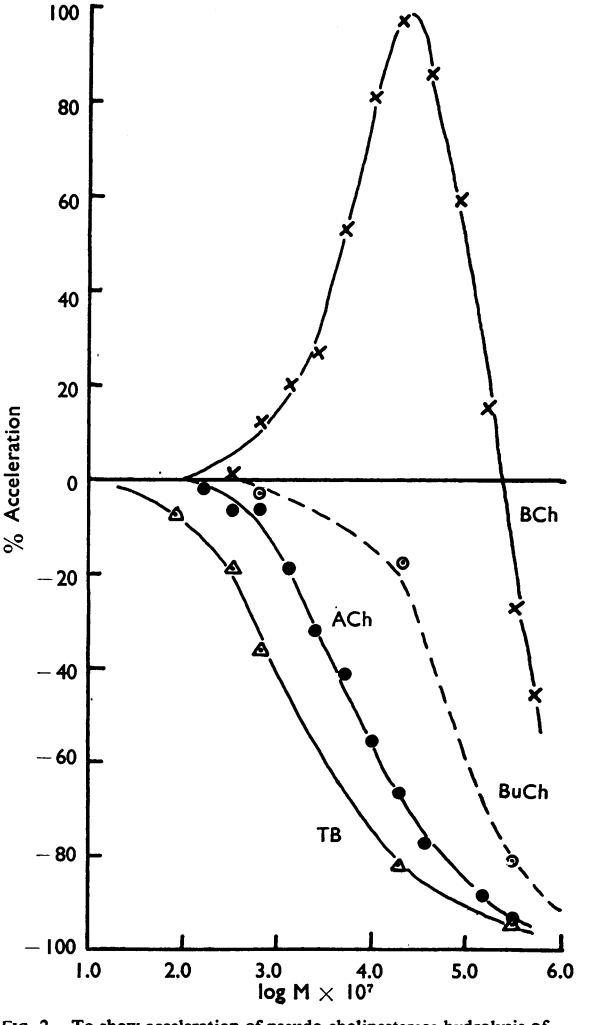

FIG. 2.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by atropine sulphate. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of atropine sulphate. Peak acceleration of benzoylcholine hydrolysis of 99% at 2.5×10^{-3}M. I 50's human plasma: with benzoylcholine 5.6×10^{-3}M; with butyrylcholine 7.1×10^{-3}M; with acetylcholine 7.1×10^{-4}M.

pseudo-cholinesterase preparation, also free from atropinase activity, and the results gave a curve for benzoylcholine hydrolysis identical with the benzoylcholine curve in Fig. 2.

Local Anaesthetics.—Cocaine was investigated because the structure of part of its molecule is closely similar to that of atropine. Only (−)-cocaine hydrochloride was examined and it produced a 30% peak increase in the rate of hydrolysis of benzoylcholine chloride at 1.3×10^{-3}M. With the other three substrates the rate of hydrolysis fell with increasing drug concentration ; there was no acceleration. On the other hand, procaine hydrochloride, although a substrate for an esterase in human plasma (Kisch, Koster, and Strauss, 1943), acted only as an inhibitor with all four substrates.

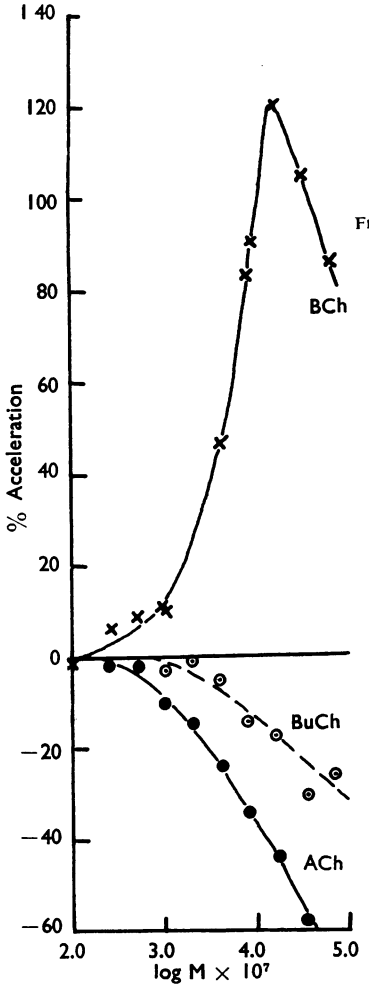

FIG. 3.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by morphine sulphate. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of morphine sulphate. Peak acceleration of benzoylcholine hydrolysis of 120% at 1.8×10^{-3}M. I 50's human plasma: with benzoyl choline $\geqslant 10^{-2}$M; with butyrylcholine $>10^{-2}$M; with acetylcholine 2.5×10^{-3}M.

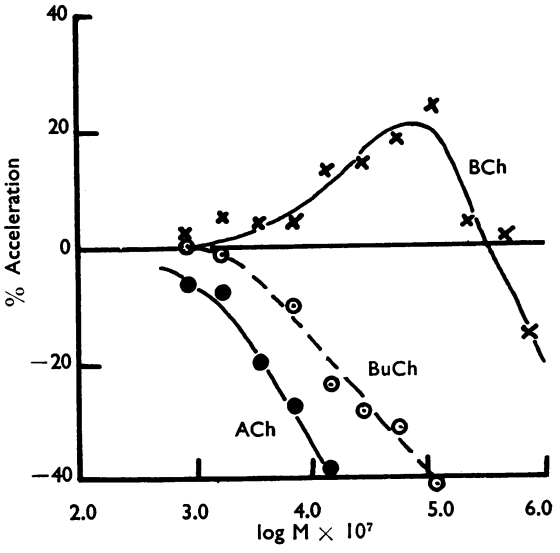

FIG. 4.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by hexamethonium iodide. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of hexamethonium iodide. Peak acceleration of benzoylcholine hydrolysis of 21% at 7.9×10^{-3}M. I 50's human plasma: with benzoylcholine $>10^{-1}$M; with butyrylcholine $\simeq 3.0 \times 10^{-2}$M; with acetylcholine $\simeq 3.0 \times 10^{-3}$M.

Analgesics.—Two analgesics were examined, morphine and thiambutene. Morphine, which contains a methyl substituted tertiary nitrogen in a piperidine ring, was a potent accelerator of the hydrolysis of benzoylcholine with a peak acceleration of 120% at 1.8×10^{-3}M. There was no acceleration of the hydrolysis of acetyl- or butyrylcholine ; tributyrin was not tested. Fig. 3 shows these effects. With nalorphine, in which the methyl group attached to the nitrogen is replaced by an allyl group, the peak acceleration of benzoylcholine hydrolysis was only 26% at 2.5×10^{-3}M.

Another analgesic with a morphine-like action, thiambutene (3-diethylamino-1 : 1-di(2'-thienyl)but-l-ene hydrochloride), which has a diethyl substituted tertiary nitrogen, inhibited cholinesterase with all four substrates.

Compounds with Two Quaternary Nitrogens.—Hexamethonium and decamethonium—both containing two trimethyl substituted quaternary nitrogens—were examined. Hexamethonium moderately accelerated the hydrolysis of benzoylcholine, with a maximum of 21% at 7.9×10^{-3}M, and inhibited the hydrolysis of acetylcholine and butyrylcholine (Fig. 4); decamethonium, on the other hand, inhibited with all three substrates.

Mephenesin.—This substance (3-(2-methylphenoxy) propane-1 : 2-diol) is unlike any of the

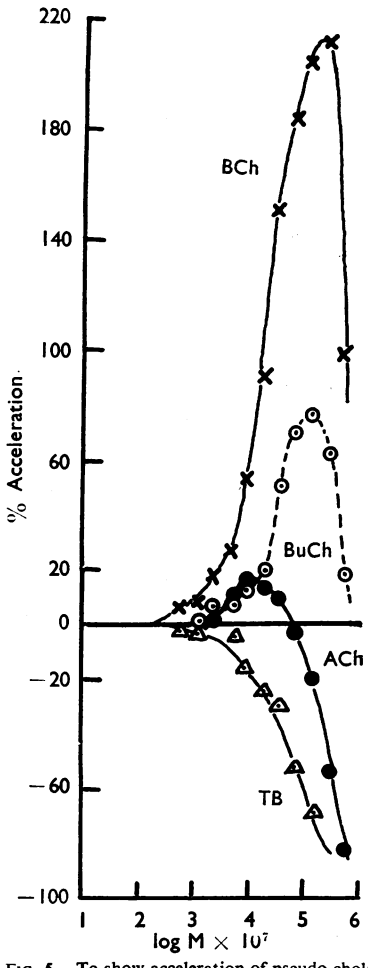

FIG. 5.—To show acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine, butyrylcholine and acetylcholine by mephenesin. Warburg respirometer. Ordinate: % acceleration of rate of hydrolysis; abscissa: log molar concentration ($\times 10^7$) of mephenesin. Peak acceleration of benzoylcholine hydrolysis of 214% at 2.5×10^{-2}M; of butyrylchcline 76% at 1.6×10^{-2}M, and of acetylcholine 16% at 10^{-3}M. I 50's human plasma: with benzoylcholine $>10^{-1}$M; with butyrylcholine $>10^{-1}$M; with acetylcholine 3.2×10^{-2}M.

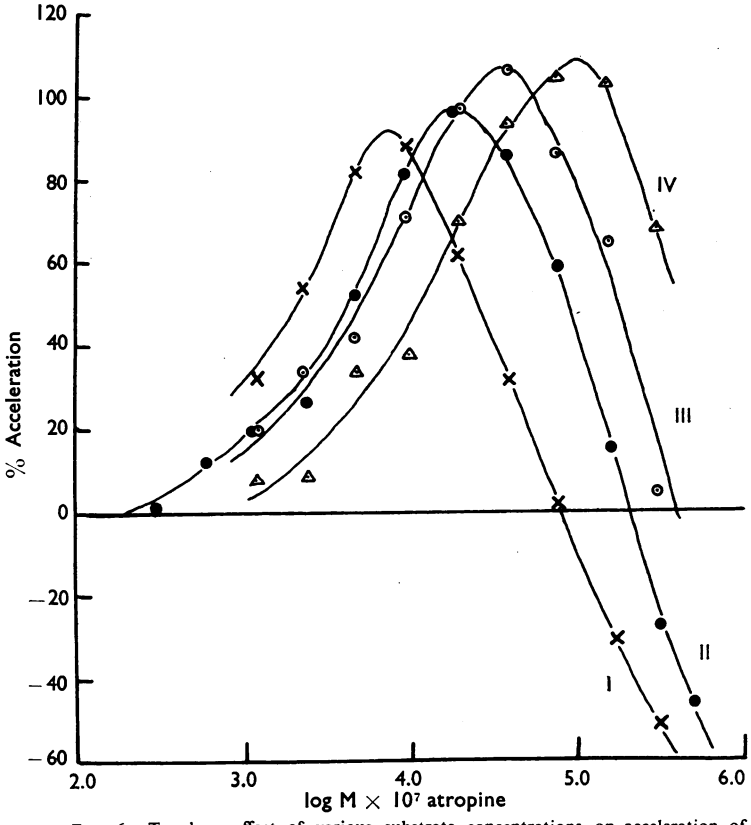

FIG. 6.—To show effect of various substrate concentrations on acceleration of pseudo-cholinesterase hydrolysis of benzoylcholine by atropine sulphate. Warburg respirometer. Ordinate: % acceleration; abscissa: log molar concentration ($\times 10^7$) of atropine sulphate. Concentrations of benzoylcholine: Curve I: 0.0075M; II, 0.015M; III, 0.03M; IV, 0.06M.

compounds so far discussed, since it contains no nitrogen, is not optically active and is not a salt. It was, however, a powerful accelerator of the hydrolysis of benzoylcholine by pseudo-cholinesterase, showing a peak acceleration of 214% at 2.5×10^{-2}M. Acceleration by mephenesin has been noted previously by Fraser (1951) and by Todrick (1954). It was, however, unique among the compounds discussed above in that it also accelerated the hydrolysis of acetylcholine (peak acceleration of 16% at 10^{-3}M) and butyrylcholine (peak acceleration of 76% at 1.6×10^{-2}M). The hydrolysis of tributyrin was inhibited (see Fig. 5).

Histidine and Folic Acid.—Aron *et al.* (1944), using a titration method for estimation, report an acceleration of the hydrolysis of acetylcholine by pseudo-cholinesterase when histidine is added to human, horse, or guinea-pig serum. This could not be confirmed using the manometric method described here. Whether this is due to a final *p*H difference, or to a difference in the duration of contact with the drug, is uncertain. No evidence of accelerated hydrolysis of the enzyme from human plasma in the presence of folic acid was obtained. The claim of Davis (1946a, b, c) limits the accelerating effect of folic acid to certain sera of dogs and human beings.

Effect on True Cholinesterase.—All the compounds examined inhibited the hydrolysis of acetyl-β-methylcholine by the true cholinesterase of human red cells.

Effect of Varying the Substrate Concentration.—To see if these acceleration effects were condi-

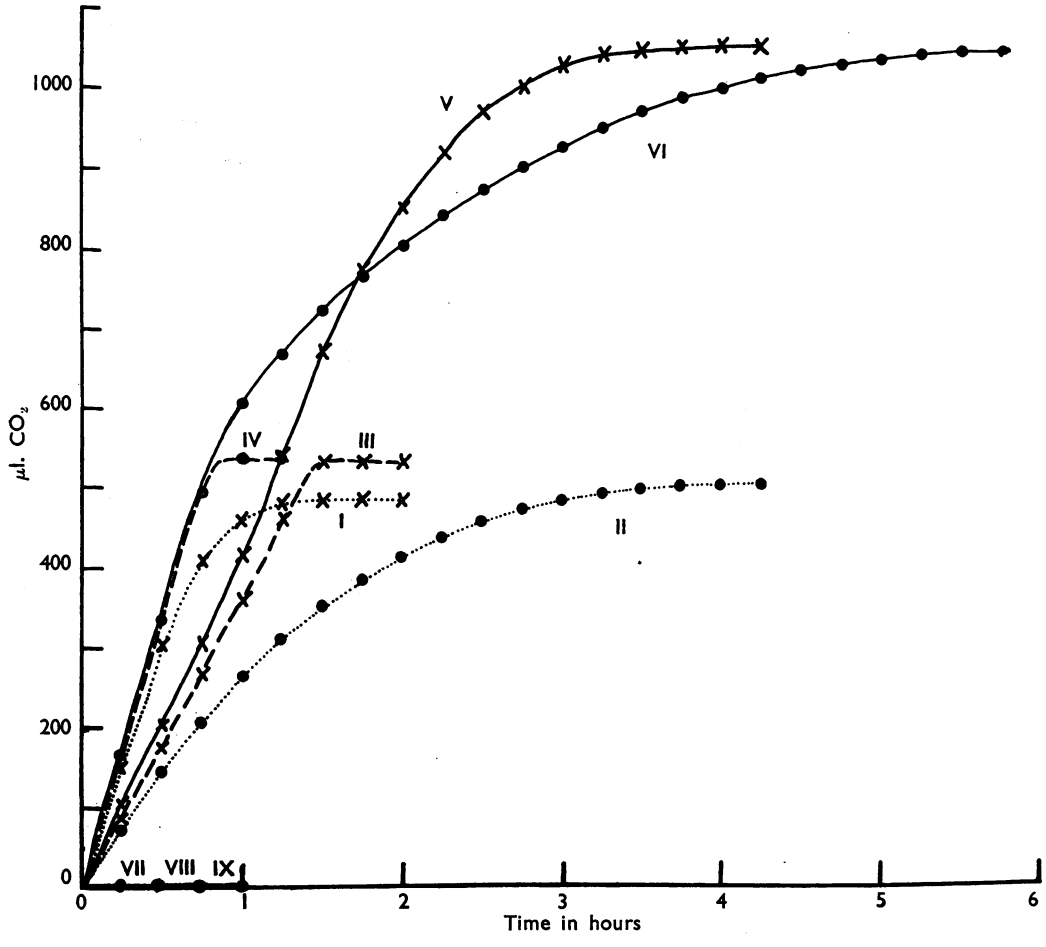

FIG. 7.—To show hydrolysis of benzoylcholine, acetylcholine and benzoylcholine+acetylcholine by human plasma in the presence and absence of atropine sulphate. Warburg respirometer. Ordinate: μl. CO_2 evolved; abscissa: time in hr. All concentrations of plasma are 1 in 15; of benzoycholine and acetylcholine 7.5×10^{-3}M, and of atropine sulphate 5×10^{-4}M. I, plasma+acetylcholine; II, plasma+acetylcholine+atropine; III, plasma+benzoylcholine; IV, plasma+benzoylcholine+atropine; V, plasma+acetylcholine+benzoylcholine; VI, plasma+acetylcholine+benzoylcholine+atropine; VII, benzoylcholine+atropine; VIII, acetylcholine+atropine; IX, plasma+atropine.

tioned by the substrate concentration, an experiment using atropine on human plasma cholinesterase was done over an eight-fold range of concentration of benzoylcholine. The results are shown in Fig. 6.

The amount of enzyme was kept constant throughout. Whatever concentration of benzoylcholine was used—from 0.0075M to 0.06M—atropine accelerated the hydrolysis. As the substrate concentration increased, the peak acceleration required higher concentrations of atropine. The nature of the effect remained the same throughout.

Effect of Substrate Combinations.—The effect of atropine on the pseudo-cholinesterase hydrolysis of benzoylcholine and acetylcholine, both singly and combined, is shown in Fig. 7; the reactions were allowed to go to completion. Control experiments with mixtures of benzoylcholine and atropine, acetylcholine and atropine, or plasma and atropine, showed no evolution of CO_2. Curves I and II show, respectively, the hydrolysis of acetylcholine in the absence and in the presence of atropine. Both reached the same maximum, but curve II took longer to reach it—that is, the hydrolysis of acetylcholine in the presence of atropine was slowed. A similar result could be obtained without atropine by reducing the amount of enzyme involved. With benzoylcholine as substrate (curves

III and IV) atropine produced the reverse effect, in that the time required to reach maximum CO_2 production was shortened. This effect could be obtained either by an increase in the rate of hydrolysis or an increase in the amount of enzyme present.

Curves V and VI show the rate of combined hydrolysis of benzoylcholine and acetylcholine in equimolar concentrations in the presence and absence of atropine. In the absence of atropine (curve V) the initial rate of hydrolysis was slightly greater than that of benzoylcholine alone, and less than that of acetylcholine. In the presence of atropine, the first part of the curve (curve VI) coincides with the benzoylcholine curve and later takes on a slope more closely approximating to the hydrolysis curve of acetylcholine in the presence of atropine. It would be reasonable to assume from these curves that, on the whole, benzoylcholine was hydrolysed preferentially to acetylcholine. Even in the mixed substrate the effect of atropine on the hydrolysis of acetylcholine is still seen, since the final attainment of complete hydrolysis was delayed by atropine.

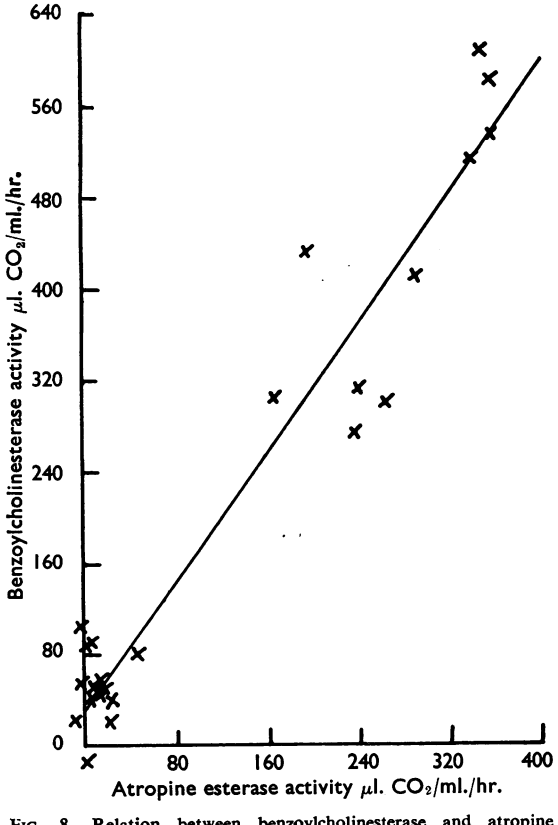

FIG. 8.—Relation between benzoylcholinesterase and atropine esterase in rabbit plasma. Warburg respirometer. Ordinate: benzoylcholinesterase activity expressed as μl. CO_2 evolved/ml. plasma/hr.; abscissa: atropine esterase activity expressed as μl. CO_2 evolved/ml. plasma/hr.

Action in the Absence of Enzyme.—In the absence of any enzyme and at high concentrations at elevated temperatures, acetylcholine and benzoylcholine may undergo spontaneous hydrolysis at a rate readily measurable in the Warburg respirometer. However, using molar concentrations of the substrate at 45° C. and a 256-fold range of atropine concentration, no acceleration of the spontaneous hydrolysis of benzoylcholine or of acetylcholine was obtained. In fact, both were slightly slowed—the hydrolysis of benzoylcholine more so than that of acetylcholine.

Rabbit Plasma.—It was of interest that, in rabbit plasma, atropine esterase activity was directly proportional to benzoylcholinesterase activity (Fig. 8). This figure gives the results obtained with the plasmas from twenty-four rabbits, and shows that in the rabbit the two enzymes were either one and the same, or occurred together in the same proportions. Ellis (1947), however, claims that they are two separable entities and has shown that, after destruction of the atropine esterase by heat, atropine inhibits the hydrolysis of benzoylcholine by benzoylcholinesterase. By incubation of rabbit liver globulin at 48° C. for 90 min. Ellis was able to destroy all the atropine esterase activity with only 20% loss of benzoylcholinesterase. Using rabbit plasma α and β globulins, as prepared by Glick, Glaubach, and Moore (1942), a temperature of 55° C. for 60 min. was necessary. Under these conditions 96% of the atropine esterase was destroyed, with the loss of only just over half of the benzoylcholinesterase activity. Using this preparation, atropine over the usual range of concentrations inhibited benzoylcholine hydrolysis by benzoylcholinesterase, similarly to Ellis's rabbit liver preparation, and at no concentration was acceleration obtained.

Purified Pseudo-cholinesterase.—Horse serum pseudo-cholinesterase was partly purified by the method of Glick *et al.* (1942). Atropine on this preparation gave an acceleration curve for benzoylcholine hydrolysis identical with that obtained from unpurified horse serum.

Atropine produced the same acceleration of benzoylcholine hydrolysis by horse serum pseudo-cholinesterase, purified by the method of Strelitz (1944), at all stages up to an estimated 5,000-fold purification.

Effects on Succinyldicholine Hydrolysis.—In the Warburg respirometer, the hydrolysis of succinyl-

dicholine by human plasma pseudo-cholinesterase was inhibited by atropine at concentrations above 10^{-4}M. Below this it had no action. At 3.2×10^{-3}M there was 50% inhibition.

Discussion

There are few reports of organic substances which activate cholinesterase. With the exception of the alcohols of Todrick *et al.* (1951), those that have appeared concern the hydrolysis of acetylcholine by pseudo-cholinesterase. In the experiments reported above, all cases of "activation" have involved benzoylcholine hydrolysis and one has further involved the hydrolysis of acetylcholine and of butyrylcholine. No activators of true cholinesterase, or of tributyrinase, have been found.

The typical action is an acceleration of the hydrolysis of benzoylcholine by pseudo-cholinesterase. As the concentration of "activator" was increased, the acceleration increased to a peak, then diminished, and was eventually replaced by inhibition.

Accelerating activity has been found in the presence of quaternary nitrogen, as in the procyclidine series; in the presence of tertiary nitrogen, in the hyoscyamine series; and in the absence of nitrogen, in mephenesin. Furthermore, activity is not necessarily associated with a particular stereochemical configuration, (+)- and (−)-hyoscyamine being active as well as (+)-procyclidine methiodide and ethiodide and (−)-cocaine. It therefore appears that this accelerating action is a function of each particular compound and is not dependent on pharmacological type or on any particular common chemical structure or configuration. The concentrations of compounds required to give these enzyme effects are, however, much higher than those required for their usual pharmacological actions.

Mechanism of Action.—In a closed system where temperature and amounts of enzyme and of benzoylcholine are kept constant there are several ways in which acceleration can be obtained. Firstly, the amount of enzyme could be increased by the activation of an inactive precursor present in the plasma. If this were so, it would be expected that the rate of hydrolysis of the other substrates would be similarly accelerated, but this has not been obtained, except with mephenesin. In addition, atropine produced acceleration curves of benzoylcholine hydrolysis which were identical with either purified or unpurified preparations of horse serum pseudo-cholinesterase. Secondly, there could be a direct action by these compounds on the substrate molecule, making benzoylcholine more susceptible to hydrolysis by pseudo-cholinesterase and the other substrates more resistant. Further, the formation of a complex between the compound and one or more of the substrates seems unlikely on chemical grounds. Additional information was provided by the experiments with rabbit plasma. This preparation contains benzoylcholinesterase instead of the pseudo-cholinesterase of human plasma, and yet no acceleration is obtained with atropine, as would be expected if a chemical complex had been formed between atropine and benzoylcholine which made it more susceptible to hydrolysis by enzyme. There does, however, appear to be some relationship between compound and substrate, since the substrate concentration experiment showed that the molar concentration of atropine at peak acceleration was, approximately, directly proportional to the molar concentration of the substrate.

Thus, by the elimination of other possibilities, it appears that these compounds probably modify the pseudo-cholinesterase molecule directly, making it more readily able to split benzoylcholine and, less readily, acetylcholine and butyrylcholine.

Another aspect of these results has been to show up the different *p*I 50 values that would be obtained with a compound of this type when tested as an *in vitro* inhibitor of pseudo-cholinesterase using benzoylcholine and butyrylcholine as specific substrates. As an example (Fig. 2), atropine sulphate has a *p*I 50 for human plasma pseudo-cholinesterase of 1.25, with benzoylcholine as substrate, but only 2.15 with butyrylcholine under identical conditions. Thus, atropine would be estimated to be eight times more powerful if butyrylcholine, rather than benzoylcholine, was the substrate. This again emphasizes the care which must be taken in the choice of substrate for anticholinesterase experiments and the necessity for the clear definition of the experimental conditions.

Summary

1. Acceleration of the hydrolysis of benzoylcholine by pseudo-cholinesterase *in vitro*, without acceleration of the hydrolysis of butyrylcholine or acetylcholine, is described.

2. Compounds shown to have this action were: (+)-procyclidine methiodide, (+)-procyclidine ethiodide, (+)-, (−)-, and (±)-hyoscyamine, (−)-cocaine, morphine, nalorphine, and hexamethonium. Allied compounds which did not possess this action were: (+)- and (−)-procyclidine hydrochloride, (−)-procyclidine methiodide and ethiodide, procaine, thiambutene, and decamethonium.

3. Mephenesin accelerated the hydrolysis of acetylcholine and butyrylcholine, as well as that of benzoylcholine.

4. None of these compounds accelerated the hydrolysis of tributyrin by tributyrinase, or that of acetyl-β-methylcholine by true cholinesterase.

5. It is postulated that this action is produced by a modification of the pseudo-cholinesterase molecule, making it more able to hydrolyse benzoylcholine and less able to hydrolyse acetylcholine and butyrylcholine.

6. The concentrations required are well above pharmacological levels.

7. It is emphasized that care must be taken in interpreting the results of *in vitro* tests for pseudocholinesterase inhibitors when employing either benzoylcholine or butyrylcholine as substrates.

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