The Effect of Alcohols on Cholinesterase

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Interest in the mode of action of narcotics has induced a number of workers to study the effect of this group of compounds on cholinesterase, on account of the significance of the latter in the transmission of nervous impulses.

The effect of alcohols has been investigated in some detail by Plattner & Galehr (1928), Bernheim & Bernheim (1936) and Mik'helson (1941, 1943, 1946), who have all found that they cause inhibition of the enzyme. Ettinger, Brown & Megill (1941) have also brought forward evidence in support of this. Heim & Fahr (1940), however, have reported that ethyl and amyl alcohols in low concentrations activate cholinesterase, though inhibition is observed at higher concentrations. Genuit & Labenz (1941) completely failed to substantiate these findings as far as the activation was concerned.

In the course of other work we have repeatedly observed that *n*-propanol and *iso*propanol activate cholinesterase.

The object of the present investigation has been to study the effect of alcohols on cholinesterase in the hope of resolving these apparent contradictions.

EXPERIMENTAL

Cholinesterase activity was determined by a modification of the method of Ammon (1933), i.e. by the Warburg manometric technique. The saline introduced by Krebs & Henseleit (1932) was employed. It was equilibrated with 5% CO_2, 95% N_2 before use, in addition to the final equilibration of the filled flasks which was carried out in the thermostat at 38°. In the earlier part of the work, the enzyme and substrate were mixed, and 15 min. was then allowed for temperature equilibration before reading. In the later experiments, in which the activity at low substrate concentrations was studied, the linear period of CO_2 output was inevitably reduced (see Figs. 8 and 11); to obtain satisfactory results, it was necessary to allow only the minimum effective equilibration period after mixing and to read more frequently. In practice readings were taken every 5 min.

An attempt was made to measure the output during the first 5 min. by reading the manometer before mixing. This led to the observation that the addition of the alcohol caused in the first 5 min. an immediate evolution of gas which might be several times greater than the subsequent 5 min. output. This initial evolution has been shown to occur in the absence of both enzyme and substrate.

The output rises steeply with butanol concentration, but is independent of the presence of substrate (Fig. 1). The latter point is of considerable practical significance, since it means that the substrate concentration present during the period when useful measurements can be taken has not been affected by the initial output. The gas evolution may be due to the influence of the alcohol on either the pK of $NaHCO_3$ or the solubility of CO_2, for if the alcohol and $NaHCO_3$ are mixed in the well of the flask before equilibrating with the gas mixture and the enzyme is placed in the side arm, there is no abnormal gas evolution after mixing.

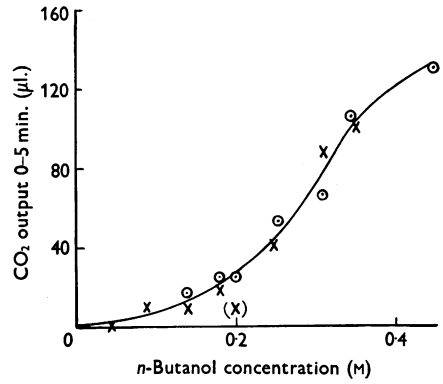

Fig. 1. Effect of *n*-butanol concentration on the CO_2 output during the first 5 min. following the mixing of butanol and buffer (enzyme absent). ×——×, substrate (0·018M-acetylcholine) present; ⊙——⊙, substrate absent.

Enzyme preparations. The greater part of the work has been carried out on a homogenate of rat brain. Male white rats were killed, exsanguinated and the brain removed rapidly, washed in Krebs-Henseleit saline, blotted and homogenized in the saline (18·0 ml. of saline to 1 g. of tissue). The homogenizer used was similar to that of Folley & Watson (1948). The brain was homogenized for 10–15 min. at 12,000 rev./min.

The human erythrocyte preparation used consisted of stroma from cells which had been washed several times to remove serum. They were haemolysed in 6 vol. of water, washed with several changes of water until the supernatant was colourless and then freeze dried. The preparation consisted of a 0·2% suspension in Krebs-Henseleit saline. Horse serum was diluted 1 in 5 with this saline before use. In each case 0·5 ml. of enzyme was employed in a total volume of 3·0 ml.

Substrates. Acetylcholine, acetyl β-methylcholine (Amechol: Savory and Moore) and benzoylcholine have all been used. The substrate solutions were made up freshly for each experiment to minimize falling-off in concentration prior to the experiment due to non-enzymic hydrolysis. For the same reason, they were dissolved in distilled water and not in buffer solution.

Alcohols. The alcohols used were the purest grade available commercially and the *n*-butanol, in particular, was of A.R. quality. We are indebted to British Drug Houses Ltd. for providing the latter before it actually came on the market.

Controls. The possibility that the alcohol would affect the rate of non-enzymic hydrolysis has been checked. The effect of *n*-butanol was negligible in the case of all three substrates.

A control experiment was also made in which the substrate only was omitted. This was to investigate the possibility that the apparent activation might be merely an oxidation of the alcohol by other enzymes in the tissue. The output for the 20 min. period between 5 and 25 min. after mixing was found to be:

(i) In the absence of butanol, $3{\cdot}9 \pm 2{\cdot}6$ (S.D.) mm.^{3} CO_2.

(ii) In the presence of butanol, $4{\cdot}7 \pm 1{\cdot}1$ (S.D.) mm.^{3} CO_2.

There is obviously no significant difference between the two values (Student's $t = 0{\cdot}77$), the mean of which is 4·3. The output falls to less than half this value for the ensuing 30 min.

In the experiments at low substrate concentrations, in which the 5 min. output has been the basis for calculation, a correction of 1·1 has been deducted, but in the earlier long-period experiments this correction, which would have been less, has been neglected. This slight output is assumed to be due to residual metabolism.

RESULTS

The effect of the nature and concentration of aliphatic alcohols on the activity of rat-brain cholinesterase

Straight-chain alcohols. The effect of primary alcohols on the activity of the enzyme is shown in Fig. 2*a*. The activity was determined after the enzyme had been incubated with the alcohol for 60 min. at 38°, the substrate being 0·018M acetylcholine. The main conclusions to be drawn are: (i) Alcohols containing 3–6 carbon atoms activate the enzyme. The effect is maximal with *n*-butanol, the maximum activation being 75%. (ii) At concentrations above the optimum required for the production of activation the alcohol brings about an inactivation of the enzyme. This is practically complete at a concentration three times the optimum. (iii) The optimum activating concentration decreases rapidly as the length of the carbon chain increases, being approximately halved for each additional carbon atom.

It should be pointed out that the falling-off in activation observed after the four-carbon alcohol cannot be explained in terms of decreasing solubility, since the six-carbon alcohol, which hardly activates at all, will inhibit completely when its concentration is further increased. The optimum activating concentrations are actually about one-third to one-half of the saturation concentrations (Seidell, 1941).

Branched-chain alcohols. Only two alcohols containing branched chains have been studied, namely *iso*propanol and *iso*butanol. They were tested under the same conditions as the straight-chain alcohols. The general form of the curves relating alcohol concentration and activity has been found to be the same as for the latter (Fig. 2*b*). *iso*Propanol is, however, considerably more active than its primary isomer, being only just less active than *n*-butanol, whereas *iso*butanol is less active than its isomer. In both cases the optimum activating concentration is some 50% higher than that for the corresponding straight-chain alcohols.

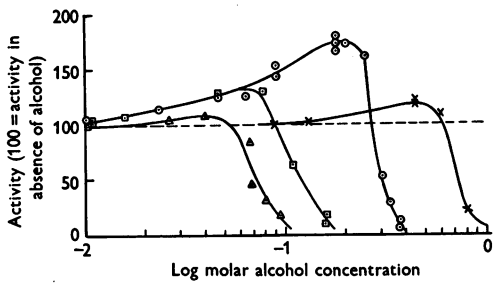

Fig. 2*a*. Effect of different concentrations of primary alcohols on the cholinesterase activity of rat-brain homogenate. *n*-Propanol, ×——×; *n*-butanol, ⊙——⊙; *n*-pentanol, ⊡——⊡; *n*-hexanol, △——△. Enzyme and alcohol incubated for 60 min. at 38° (pH 7·6) prior to measurement of activity. Substrate 0·018M-acetylcholine.

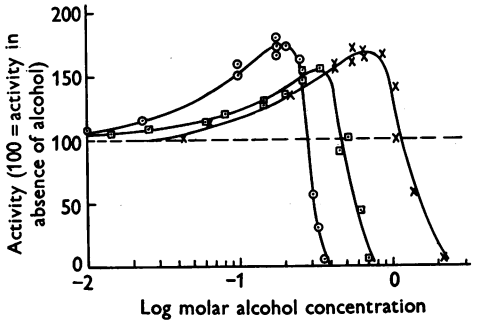

Fig. 2*b*. Effect of different concentrations of secondary alcohols on the cholinesterase activity of rat-brain homogenate. *iso*Propanol, ×——×; *iso*butanol, ⊡——⊡; *n*-butanol, ⊙——⊙ (reference standard taken from Fig. 2*a*). Enzyme and alcohol incubated for 60 min. at 38° (pH 7·6) prior to measurement of activity. Substrate 0·018M-acetylcholine.

The consistent pattern of the phenomenon, namely activation rising to a maximum followed by actual inhibition as the alcohol concentration is further increased, suggests that two effects are involved. The inhibition phase is not unexpected, since the concentration required to produce it might easily bring about denaturation of the enzyme. However, there appears to be no obvious explanation of the activation. Since *n*-butanol exhibits the effect to the greatest extent and moreover gives more reproducible results than are obtained with

n-propanol, work designed to throw light on the mechanism of the process has been carried out with the former compound only.

The effect of n-*butanol on brain cholinesterase*

The influence of time and temperature. The period of incubation used in the work described above had been arbitrarily set at 60 min., but as equilibrium seemed to be reached in a much shorter time the technique was altered to eliminate the initial incubation period.

This actually leads to a greater degree of activation at all alcohol concentrations, a maximum of over 100% activation being achieved (Fig. 3). The

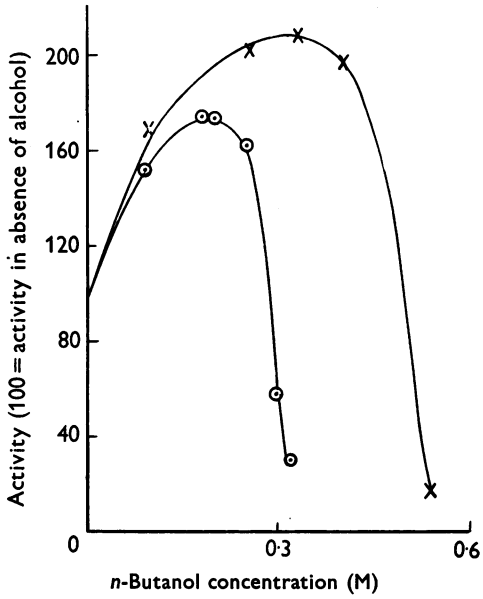

Fig. 3. Influence of incubation time on the effect of *n*-butanol on the activity of cholinesterase (rat-brain homogenate). Substrate 0·018M-acetylcholine. ⊙——⊙, 60 min. incubation at 38° prior to measurement of activity (curve taken from Fig. 2*a*); × —— ×, no incubation prior to measurement of activity.

optimum activation concentration is also higher. These findings fit in well with the previous suggestion that two processes are occurring, provided it is assumed that the activation is rapid and the inhibition slow. The upper curve is then a measure of the activation process, and the difference between the two curves a measure of the rate of inactivation.

Both processes are dependent on the alcohol concentration. However, the rate of inactivation appears to increase very rapidly in the neighbourhood of 0·4M so that almost instantaneous inactivation occurs.

The effects of time and temperature on activation and inactivation, shown in Fig. 4, suggest (*a*) that the activation is instantaneous and (*b*), that as the inactivation is very rapid at 38° but practically negligible at 23°, the mechanism may be analogous to denaturation, a process frequently found to have a high temperature coefficient (cf. Stearn, 1949).

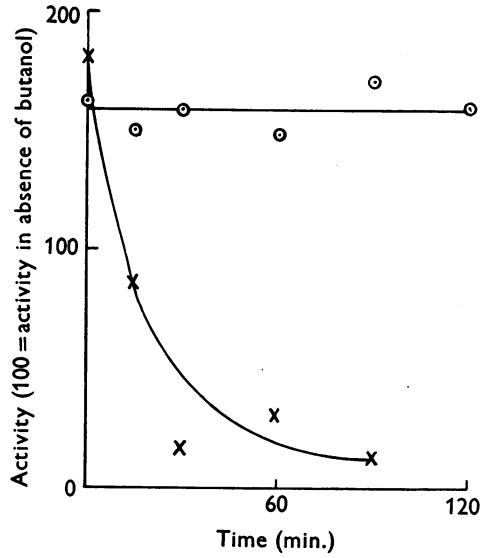

Fig. 4. The influence of temperature on the effect of *n*-butanol on the activity of cholinesterase (rat-brain homogenate). Abscissae: length of incubation prior to measurement of activity. ⊙——⊙, incubated at 23°; × —— ×, incubated at 38°.

The reversibility of the action of butanol on brain cholinesterase

The enzyme, activated by the addition of 0·19M-butanol, was dialysed against several changes of Krebs-Henseleit saline. The activation was found to be completely reversible by dialysis (Table 1*a*).

Table 1. *The effect of dialysis on butanol-activated and butanol-inactivated cholinesterase (rat-brain homogenate)*

(Substrate, 0·018M-acetylcholine.)

	Relative cholinesterase activity	
	In absence of butanol	In presence of butanol
(*a*) Activated		
Before dialysis	100	178
After dialysis	103	93
(*b*) Inactivated		
Before dialysis	100	10
After dialysis	104	5

If, however, the enzyme is dialysed after inactivation by prolonged contact with a higher concentration of butanol, there is no sign of reversal of the

inactivation, even after 44 hr. dialysis in which the smell of the alcohol disappeared with the third of five changes of saline.

These results provide additional evidence for the suggestion that two separate mechanisms are involved: a reversible activation process and an irreversible inactivation. Denaturation is to be expected at the concentration at which the latter occurs, hence the inactivation phenomenon has not been examined further.

The effect of altering the enzyme and substrate

The mechanism of activation was examined by studying the influence of the enzyme and the substrate. The following combinations of enzyme and substrate were employed: (*a*) rat-brain homogenate and acetyl β-methylcholine, 0·028M; (*b*) human stroma and acetylcholine, 0·018M; (*c*) horse serum and acetylcholine, 0·018M; (*d*) horse serum and benzoylcholine, 0·006M.

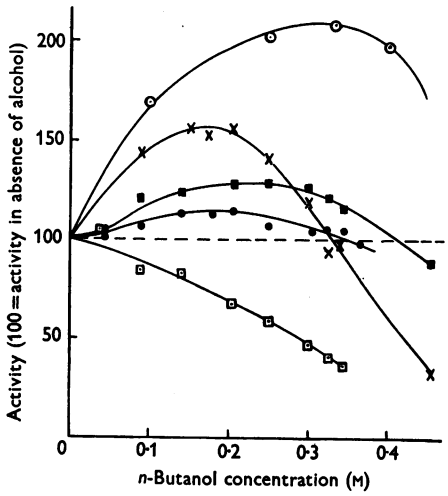

Fig. 5. Effect of varying concentrations of *n*-butanol, on the activity of cholinesterase from different sources towards different substrates. ⊙——⊙, rat-brain homogenate, 0·018M-acetylcholine; ×——×, human erythrocyte, 0·018M-acetylcholine; ■——■, horse serum, 0·006M-benzoylcholine; ●——●, rat brain, 0·028M-acetyl β-methylcholine; ⊡——⊡, horse serum, 0·018M-acetylcholine. No preliminary incubation.

The effects of varying the butanol concentration have been investigated in each case by mixing the butanol and substrate in the side arm of the Warburg flask. The enzyme activities shown in Fig. 5 have been plotted as percentages of the control activity in the absence of butanol.

With one exception, all the systems react in a qualitatively similar manner to butanol. They are all activated, the effect becoming noticeable at a butanol concentration of about 0·05M, reaching a maximum at 0·18–0·30M and changing to an inhibition at 0·35–0·45M. The maximum activation varies considerably with the nature of the enzyme and substrate, being higher where the 'true' enzyme and the physiological substrate are involved and falling off when either is replaced. The exception is the system comprising horse serum and acetylcholine. There is here no significant activation at any butanol concentration and inhibition begins at the same concentration level as activation does in the other cases and increases progressively with butanol concentration.

The marked variation with change of substrate suggests that activation is in some way competitive. The abnormal behaviour of the serum enzyme-acetylcholine system supports this, since this system is the only one of the five investigated in which inhibition of the enzyme by excess substrate does not occur (Augustinsson, 1948).

The influence of substrate concentration

The hypothesis that the activation observed might be the prevention of inhibition by excess substrate accounts for the known facts. It can also be tested in a simple manner. If the alcohol is acting as a competitive inhibitor of the inhibition by excess substrate the activation should disappear at substrate concentrations below the optimum. It is also highly probable that the alcohol will inhibit the formation of the enzyme-substrate complex which leads to hydrolysis since the molecules involved are the same; this should cause actual inhibition of the enzyme at substrate concentrations below the optimum.

The influence of substrate concentration on the effect of butanol on cholinesterase has therefore been studied in some detail. In these experiments a butanol concentration of 0·19M has been used throughout. Following Goldstein (1944) and Augustinsson (1948), the concentrations of substrate have been quoted in terms of pS (the negative logarithm of the molar substrate concentration).

The influence of acetylcholine concentration on the inhibition of the horse serum enzyme. This case is dealt with first since it involves a simpler system. According to the hypothesis put forward, no activation should be observed, since there is no inhibition by excess substrate. Competitive inhibition of the enzyme by the alcohol might, however, be expected. The experimental results indicate that inhibition occurs throughout the range of acetylcholine concentrations tested (pS, 0·74–2·74) and, furthermore, that the inhibition increases markedly as the substrate concentration is lowered. Such behaviour is typical of a competitive reversible inhibitor, and the data have therefore been subjected to Lineweaver & Burk's (1934) method of testing for competitive reversible inhibition by plotting the reciprocals of the activity and the substrate concentrations against

one another. It can be seen (Fig. 6) that the curves for the inhibited and uninhibited enzyme tend to meet at infinite substrate concentration which confirms the idea that this is basically a case of competitive inhibition.

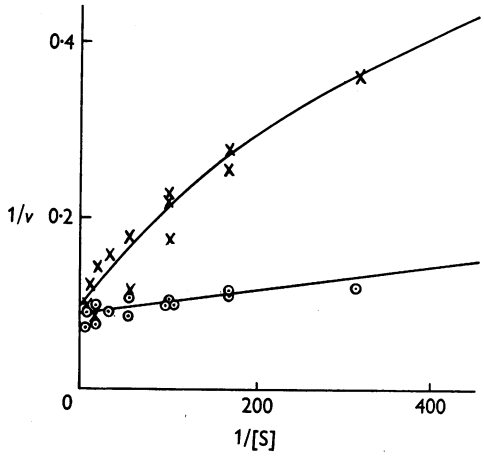

Fig. 6. Test of competitive nature of reversible inhibition in accordance with the procedure of Lineweaver & Burk (1934). Ordinates: reciprocals of activity of serum enzyme towards acetylcholine; activities in each of three experiments calculated as percentages of activity against 0·01 M-substrate. Abscissae: reciprocals of acetylcholine concentration. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of butanol.

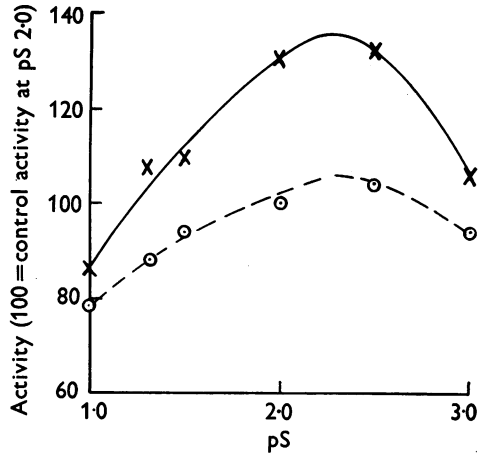

Fig. 7. Influence of benzoylcholine concentration on the effect of *n*-butanol on serum cholinesterase. *pS* = negative log of substrate concentration. ×——×, in presence of 0·19 M-*n*-butanol; ⊙——⊙, in absence of butanol.

The activation of the horse serum-benzoylcholine system. The activation already noted at pS 2·2 has now been observed over the pS range 1–3 (Fig. 7). It is maximal (30 %) at pS 2·3, i.e. about the optimum pS and falls to around 10 % at the extreme ends of the range. There is no definite shift in the optimum pS.

At pS > 2·7 a different effect is observed (Fig. 8). A rapid falling-off in enzyme activity occurs with time in contrast to the control. The significance of this will be considered later.

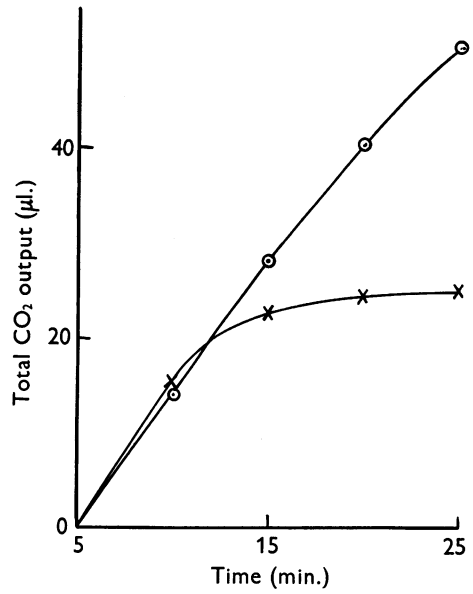

Fig. 8. The effect of *n*-butanol on the course of the hydrolysis of 0·001 M-benzoylcholine by horse serum cholinesterase. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of *n*-butanol.

The activation of the rat brain-acetylcholine system. Because the effect of butanol is most marked in the case of the true enzyme, acetylcholine being the substrate, and as this is the system of primary physiological interest, a considerable effort has been made to obtain a clear picture of the effect of substrate concentration. Unfortunately the optimum substrate concentration is very low (pS 2·6), and the experimental difficulties are therefore great.

The results of three experiments in the higher concentration range are shown in Fig. 9. The activities have been calculated in terms of the activity at pS 2·0 in the absence of butanol, in order to eliminate variations due to the differing activities of individual enzyme preparations. At high concentrations the degree of activation is almost independent of concentration (approx. 100 %), but it starts to decrease at pS 2·0.

A separate series of experiments was carried out to study the effect of butanol in the neighbourhood of the optimum substrate concentration, i.e. between pS 2 and 3. The mean results show satisfactory uniformity when calculated in terms of the control activity at pS 2·5 (Fig. 10). It seems clear that there

is a fall of 0·2–0·3 unit in the optimum pS value due to the presence of butanol, an effect which, if it were linked to an inhibition, would according to Lineweaver & Burk (1934) be indicative of a competitive

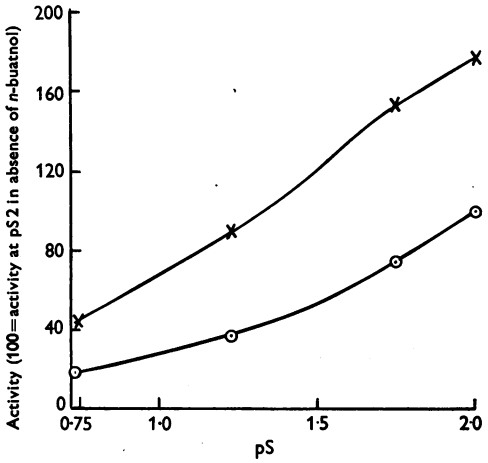

Fig. 9. Influence of acetylcholine concentration on the effect of *n*-butanol on cholinesterase (rat-brain homogenate). ×——×, in presence of 0·19 M-*n*-butanol; ⊙——⊙, in absence of *n*-butanol.

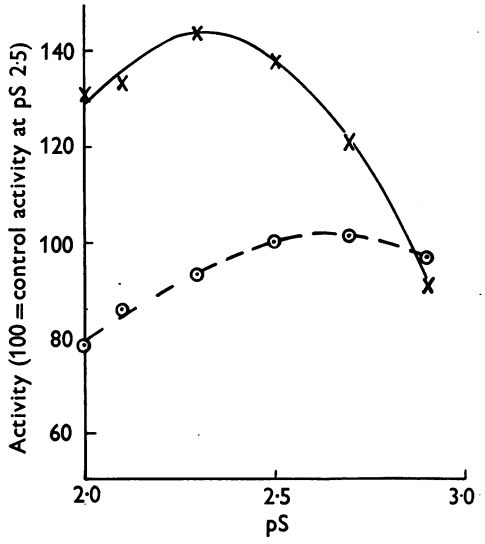

Fig. 10. The influence of acetylcholine concentration on the effect of *n*-butanol on the activity of cholinesterase (rat-brain homogenate) between *pS* 2 and 3. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of butanol.

reversible inhibition. These results also indicate that the activation decreases as the concentration falls, disappearing altogether at pS 2·9, which is in conformity with the hypothesis.

While it is not possible to measure the effect of decreasing the concentration still further, it is worth while to look closely at the results obtained at the lowest concentration (Fig. 11). The rapid decay in activity in contrast to that of the control, previously noted with the serum enzyme and benzoylcholine, can again be observed, though not to the same extent.

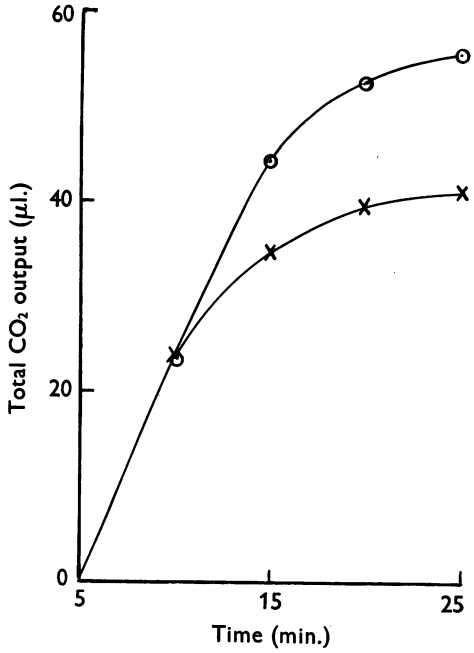

Fig. 11. The effect of *n*-butanol on the course of the hydrolysis of 0·001 M-acetylcholine by rat-brain homogenate. ×——×, in the presence of 0·19 M-*n*-butanol; ⊙——⊙, in the absence of butanol.

This effect could be interpreted as an inhibition due to butanol which becomes evident only at a pS < 3, and which is observed only after the hydrolysis has proceeded for some time and so brought about the necessary lowering of the substrate concentration. This is not advanced as evidence for the hypothesis under consideration, but merely as an explanation of observed results should it be otherwise proved correct.

The activation of the rat-brain acetyl β-methylcholine system. It has become increasingly evident that, in order to test whether activation is in reality a suppression of the inhibition by excess substrate, it is necessary to work at substrate concentrations well below the optimum. In the two cases examined, the evidence has not been conclusive owing to the limitations of the experimental technique. One way out of the difficulty is to find a substrate with a higher optimum concentration. Acetyl β-methylcholine is such a substrate, since it has an optimum pS of about

2 compared with 2·4 for benzoylcholine and 2·6 for acetylcholine with the brain enzyme (Augustinsson, 1948). This substrate had been less used on account of the slight maximum activation observed (cf. Fig. 5), but, despite this, the system has been re-studied (Fig. 12).

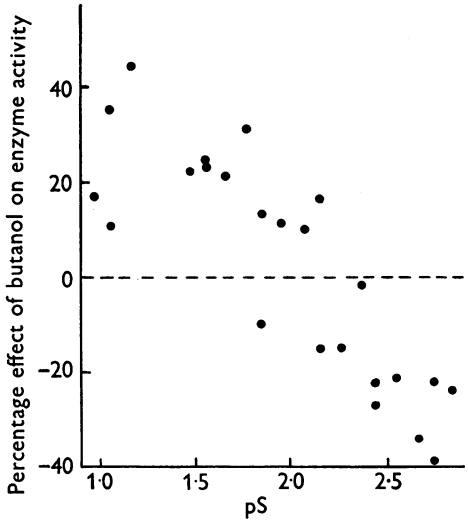

Fig. 12. Influence of concentration of acetyl β-methylcholine on the effect of *n*-butanol on cholinesterase (rat-brain homogenate).

The results show that butanol activates the enzyme between pS 1·0 and 1·8, but that at pS 2·4–2·7 there is a clear-cut inhibition. Neither the activation nor the inhibition exceeds 40 %, but the abrupt change in the sign of the phenomenon affords strong evidence for the hypothesis that the activating effect of butanol is due to depression of the inhibition by excess substrate and should change to an inhibitory effect as the substrate concentration falls below the optimum.

The effect of surface active agents

An alternative hypothesis which has been considered is that the enzyme protein is associated or aggregated in the natural state, and that alcohols by virtue of their surface-active properties cause dissociation, with consequent exposure of additional active centres.

If this were the case compounds with highly specific surface-active properties such as the anionic and kationic soaps should bring about activation at remarkably low concentrations. It has been found, however, that dodecylpyridinium bromide and sodium dodecylsulphonate show no signs of activating cholinesterases. They do inhibit the enzymes at concentrations of the order of 10^{-4} M (Table 2), the kationic soap being the stronger inhibitor of the two by a factor of about 10.

Table 2. *The effect of surface active agents on cholinesterases*

Enzyme	Rat brain	Horse serum	
Substrate	0·018M-acetylcholine	0·018M-acetylcholine	0·006M-benzoylcholine
Surface active agent	50% inhibition concn. (no pre-incubation)		
Dodecylpyridinium bromide	$4·3 \times 10^{-4}$	$4·4 \times 10^{-5}$	$3·5 \times 10^{-4}$
Sodium dodecylsulphonate	$1·7 \times 10^{-3}$	$8·2 \times 10^{-4}$	$1·6 \times 10^{-3}$

The absence of activation, though not necessarily conclusive, is evidence against the dissociation hypothesis. The behaviour of the serum enzyme towards the two substrates affords additional strong evidence against this hypothesis. A theory based on simple dissociation could hardly account for the fact that the hydrolysis of acetylcholine is inhibited while that of benzoylcholine is activated.

DISCUSSION

The picture which seems to emerge from the foregoing is that the activation of cholinesterase by alcohols is not a positive but a negative effect, in which the alcohols act as inhibitors of the enzyme at all times, but can, in certain circumstances, interfere with another inhibitory process to such an extent as to cause an apparent activation. This type of activation appears to be quite distinct from the two types of activation previously described (Baldwin, 1947).

Objections may be raised on the grounds that the enzyme preparations used were not purified and may have contained both types of cholinesterase; it might then be suggested that differential inhibition coupled with the different optimum substrate concentrations might give the appearance of activation by alcohols.

Mendel, Mundell & Rudney (1943) have, however, shown that horse serum hydrolyses acetyl β-methylcholine at one-tenth of the rate at which it hydrolyses benzoylcholine. From Table 2 of their paper it can be calculated that this is equivalent to an activity of the 'true' enzyme in terms of acetylcholine splitting activity of 2 % of that of the 'pseudo' enzyme. The human erythrocyte enzyme used in this work was purified in such a manner as to free the stroma from other components. Like Mendel & Rudney's (1943) preparation it should be free from serum esterase. Ord & Thompson (1950) have put forward evidence, based on the activity against the specific substrates, that rat brain contains less than 10 % of the 'pseudo' cholinesterase.

Since, in the experiments reported, the specific substrates have been used with enzyme preparations

which are known to act specifically on them, the criticism that the activity of a mixture of enzymes is being measured does not appear to us to be valid. The question of aliesterase activity (Adams & Whittaker, 1949; Richter & Croft, 1942) does not appear to be involved.

The inhibition of cholinesterase by alcohols observed by the majority of the earlier workers is to be expected since, without exception, they employed biological assay techniques to measure the rate of destruction of acetylcholine; these rendered desirable the use of very low initial acetylcholine concentrations. Those employed were: Plattner & Galehr (1928), $1{\cdot}4 \times 10^{-4}$M; Bernheim & Bernheim (1936), $3{\cdot}7 \times 10^{-4}$M; Heim & Fahr (1940), $1{\cdot}9$–$5{\cdot}7 \times 10^{-5}$M; Genuit & Labenz (1941), $3{\cdot}7$–$9{\cdot}3 \times 10^{-7}$M.

It is difficult to understand the activation observed by Heim & Fahr, since the acetylcholine concentration they employed is well below the optimum.

It would probably be correct to say that the activation of cholinesterase by alcohols is of no physiological significance since, although there is no convincing evidence available regarding the actual acetylcholine concentration which develops at the cholinergic nerve endings, it must be relatively low judged by the total acetylcholine liberated.

Our results are perhaps of more significance from the point of view of enzyme technique, particularly in connexion with the study of inhibition. Augustinsson (1949) has recently stressed the point that the degree of inhibition brought about by a reversible competitive inhibitor is markedly dependent on the substrate concentration employed, though he did not go on to suggest that a negative inhibition could be obtained by a mere variation of the substrate concentration.

Our findings confirm his view that the choice of a single substrate concentration for the measurement of enzyme activity is extremely difficult in the case of those enzymes which are inhibited by excess substrate. It is not suggested that all the inhibitors of enzyme activity will also interfere with the inhibition by excess substrate, but, since it appears to have occurred in one set of cases, it is not permissible to assume that it does not occur in others. Richter & Croft (1942) have, for instance, reported the activation of cholinesterases by quinine and atoxyl. It does not seem impossible that interference could occur to an extent that would not show up obviously as an activation.

On these grounds, it is suggested that, in studies on the reversible inhibition of those enzymes which are also inhibited by excess substrate, a substrate concentration or concentrations fairly well below the optimum should be chosen. This would be unnecessary, however, if it could be proved beforehand that the inhibitor did not interfere with the inhibition by excess substrate.

SUMMARY

1. The effect of alcohols on cholinesterases has been investigated. They have been found to bring about activation of the enzyme up to an optimum concentration which decreases as the chain length of the alcohol increases. At higher concentrations inactivation occurs.

2. The activation is practically instantaneous and reversible, the inactivation slower and irreversible.

3. No activation of the hydrolysis of the acetylcholine by the serum enzyme is observed, only a competitive reversible inhibition over the same alcohol concentration range.

4. The hypothesis that the activation is primarily an interference with the inhibition by excess substrate, has been examined by studying the effect of substrate concentration. It has been shown that, in the hydrolysis of acetyl β-methylcholine by the brain enzyme, the activation observed at high substrate concentrations changes at low substrate concentrations to an inhibition.

5. Specific surface-active agents do not cause activation of the enzyme.

6. The importance of the substrate concentration in the study of cholinesterase activity is underlined by this work.

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REFERENCES

Adams, D. H. & Whittaker, V. P. (1949). *Biochem. J.* **44**, 62.
Ammon, R. (1933). *Pflüg. Arch. ges. Physiol.* **233**, 486.
Augustinsson, K. B. (1948). *Acta physiol. scand.* **15**, Suppl. no. 52.
Augustinsson, K. B. (1949). *1st Int. Congr. Biochem. Abstr.*, p. 586.
Baldwin, E. (1947). *Dynamic Aspects of Biochemistry*, 1st ed. Cambridge University Press.
Bernheim, F. & Bernheim, M. L. C. (1936). *J. Pharmacol.* **57**, 427.
Ettinger, G. H., Brown, A. B. & Megill, A. H. (1941). *J. Pharmacol.* **73**, 119.
Folley, S. J. & Watson, S. C. (1948). *Biochem. J.* **42**, 204.
Genuit, H. & Labenz, K. (1941). *Arch. exp. Path. Pharmak.* **198**, 369.
Goldstein, A. (1944). *J. gen. Physiol.* **27**, 529.

Heim, F. & Fahr, A. (1940). *Arch. exp. Path. Pharmak.* **195**, 59.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Mik'helson, M. Y. (1941). *Bjull. eksp. Biol. Med.* **11**, 230. Cited in *Chem. Abstr.* (1947), **41**, 6339.
Mik'helson, M. Y. (1943). *Farm. & Toksikol.* **6**, 49. Cited in *Chem. Abstr.* (1945), **39**, 129.
Mik'helson, M. Y. (1946). *J. Physiol. U.S.S.R.* **32**, 635.
Ord, M. G. & Thompson, R. H. S. (1950). *Biochem. J.* **46**, 346.
Plattner, F. & Galehr, O. (1928). *Pflüg. Arch. ges. Physiol.* **220**, 606.
Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
Seidell, A. (1941). *Solubility of Organic Compounds*, 3rd ed., vol. 2. New York: D. Van Nostrand, Co. Inc.
Stearn, A. F. (1949). *Advanc. Enzymol.* **9**, 25.

Composition of Ox Lens Capsule

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The behaviour and constitution of the ox lens capsule has been investigated to see whether it is made up of a single protein containing carbohydrate or of protein mixed with polysaccharide.

The lens capsule completely encloses the lens and is a non-cellular laminar membrane thought to be secreted by the lens epithelium. It is elastic and when cut rolls back on itself as if the outer layers were under tension. Mammalian lens capsules can easily be stripped off like a cellophan wrapper and are tough and withstand handling. The adult lens has a single layer of epithelial cells on the front face only and it is here that the capsule is thickest. The posterior capsule is thinner, being laid down in embryonic life when the lens epithelium extends to the back surface. The evidence that the capsule is secreted by the epithelium comes from the work of Shirmer (1889), who showed that injury to the capsule and epithelium of the lens in the adult rabbit was followed by repair and a thickening of the capsule over the site of injury where the epithelial cells proliferate. This was confirmed by Bakker (1936–7) who, after injuring the rabbit lens *in situ*, removed it and watched the repair of the capsule in a tissue culture of the whole lens.

Mörner (1894) showed that the ox lens capsule contained about 10% of carbohydrate. Krause (1933) found N, 14%, S, 0·84%, and considered that the capsule was a distinct insoluble protein unlike the other widely distributed insoluble proteins of the body. Hertel (1933) took X-ray diffraction photographs of the capsule and found the picture was like that of unoriented collagen. Washed lens capsule was used in all these analyses and was considered to be a single protein. If this assumption is correct the lens capsule protein is unique in giving the X-ray diffraction pattern of collagen and yet containing this large amount of carbohydrate. It is also peculiar if it is a type of collagen and yet is produced by epithelial cells.

METHODS

Preparation of lens capsule. Ox eyes were dissected within 1 hr. of death. The lens was removed in its capsule and this was then stripped off. The capsules were suspended in water and stirred for several days with many changes of water, in order to remove attached epithelial cells and lens fibres. Such treatment did not alter the appearance of the capsules, but would presumably remove any soluble substances present. The capsules were then either acetone-dried or used fresh.

Total nitrogen. This was estimated by micro-Kjeldahl followed by distillation and titration, as described by Markham (1942).

Hexosamine. Elson & Morgan (1933).

Total carbohydrate. The orcin colorimetric method described by N. W. Pirie (1936) was used.

Reducing sugars. Hagedorn & Jensen (1923).

Partition chromatography of amino-acids. The upward method described by Williams & Kirby (1948) was used.

Partition chromatography of sugars. The upward method was used with the developers and spraying solutions described by Jermyn & Isherwood (1949) and Partridge (1949).

RESULTS

Ox lens capsule, prepared as described, contained 14% N, 9–10% carbohydrate, estimated colorimetrically, or 9% reducing sugars after hydrolysis. It also contained about 1% hexosamine. This small amount is difficult to estimate owing to charring during hydrolysis. No phosphorus was found.

General properties. The properties of the capsule were studied to see how far they tallied with those of collagen. Lens capsule is insoluble in boiling water, but goes into solution readily at about pH 3. Such solutions, after dialysis, set to weak jellies at a concentration of about 1%. The capsule does not gel as readily as gelatin derived from skin or tendon.