The Enzymic Hydrolysis of Alkyl Fluoroacetates and Related Compounds

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Methyl fluoroacetate has been reported by Boyarsky and his co-workers (Boyarsky, Postel & Rosenblatt, 1948; Boyarsky, Rosenblatt, Postel & Gerard, 1949) to block conduction irreversibly in isolated frog nerves. We have found this to be a common property of alkyl fluoroacetates, bearing a certain relationship to their general toxicity in animals (Bergmann, Gitter & Shimoni, 1953). Fluoroacetic acid, which has no effect on nerve conduction, is also less toxic than its esters. This difference between the acid and its esters could be due to better penetration of the latter or could be related to the fact that esters in general possess a higher affinity to esterases than the corresponding acids (Bergmann, Wilson & Nachmansohn, 1950*a*). The latter possibility made it desirable to investigate the behaviour of esters of fluoroacetic acid towards acetylcholine esterase, although the observation of Doty & Gerard (1949) and Gerard and Doty (1950), that methyl fluoroacetate inhibits respiration of nerve cells earlier than their conduction, points to interference with the oxidative metabolism of the nerve as the basis of the fluoroacetate effect. The fluoroacetates proved to be very good substrates of specific and unspecific esterases, in accordance with the observations of Adams & Whittaker (1950) on chloro- and bromoacetates, and revealed some new aspects of esteratic activity, which are dealt with in this and subsequent papers.

MATERIALS AND METHODS

Electric organ choline esterase. This was prepared from *Torpedo marmorata* (obtained through the generosity of Prof. Bacchi, Marine Biological Station, Naples, Italy) or *Electrophorus electricus*. The purified preparation diluted 3000 times produced 8 μmoles CO_2/ml./hr., with acetylcholine (ACh), $3{\cdot}3 \times 10^{-3}$ M, as substrate.

Liver esterase. This was prepared from dog's liver, as described earlier (Bergmann & Shimoni, 1952). The enzyme concentrate, diluted 100 times, produced 6·6 μmoles CO_2/ml./hr. with 0·43 M-diacetin as substrate.

Enzymic activity at 23° was measured by the Warburg manometric method, using gelatin-bicarbonate buffer in an atmosphere of 95% air and 5% CO_2. Since several substrates required the addition of ethanol for complete solution, the influence of ethanol on these enzymes was studied (Fig. 1). It is evident that the choline esterase is inhibited much more by a given percentage of ethanol in the medium than is liver esterase. When ethanol was present, enzymic activity was calculated by multiplying the experimental rate of hydrolysis (in μmoles CO_2/ml./hr.) by the factor 100/(100 − % ethanol inhibition). In the experiments to be described no concentrations higher than 1·5% (v/v) ethanol for choline esterase and 3% for liver esterase were used.

Substrates. The alkyl fluoroacetates were a gift of Blank & Bergmann (1952). Some of the relevant physical properties of these esters, many of which are new compounds, are summarized in Table 1. The densities were determined approximately by weighing on an analytical balance a sample, the volume of which had been measured with a pipette; this was done in order to avoid much handling of these volatile and extremely toxic substances. The substrates were dissolved first in the quantities of ethanol indicated in Table 1 whenever necessary, and then brought with buffer to a standard concentration; from this stock solution dilutions were made as required. The rate of enzymic hydrolysis was determined by allowing for spontaneous hydrolysis measured at each substrate concentration with a control solution devoid of enzyme, but of otherwise identical composition. Ethyl lactate was a commercial preparation (Stoney-Mueller Inc., N.J.). Ethyl glycollate and ethyl methoxyacetate were prepared according to Fischer & Helferich (1911), and methyl nitroacetate according to Bouveault & Wahl (1904). All other esters were Eastman-Kodak products and were purified by fractional distillation. Diacetin was a gift of British Industrial Solvents Ltd. and Mecholyl of Merck, Rahway, N.J. We synthesized ethyl trifluoroacetate according to Gilman & Jones (1943), but the action of esterases on this substrate could not be measured, since it undergoes very rapid hydrolysis in water.

RESULTS

Acetylcholine esterase

pS-activity curves for the hydrolysis of n-alkyl fluoroacetates by acetylcholine esterase are given in Fig. 2. As far as their solubility permitted us to cover a sufficient range of concentrations, they all show maxima, which become higher with increasing chain length, thus producing a steeper slope of the ascending part of the curves. At the same time the pS optimum shifts to higher values. For comparison of the relative affinity of the members of this series two properties are thus available: (*a*) the position of the pS maximum, (*b*) the height of this maximum.

Table 1. *Physical properties of alkyl fluoroacetates and related esters*

Compound	B.p. (°) at 760 mm.	Density at 26°	Maximum concn. in buffer (M)	% Ethanol added	Spontaneous hydrolysis
		(1) Fluoroacetates			
Methyl	103	1·15	$3·3 \times 10^{-1}$	None	Fast
Ethyl	119	1·08	$4·2 \times 10^{-1}$	2	Fast
n-Propyl	137	1·00	$1·6 \times 10^{-1}$	3	Appreciable
n-Butyl*	154	1·00	$3·1 \times 10^{-2}$	2	Negligible
n-Amyl*	171	0·96	$1·3 \times 10^{-2}$	3	
n-Hexyl*	189	0·96	$3·1 \times 10^{-3}$	None	
*iso*Propyl*	124	1·01	$2·2 \times 10^{-2}$	2	
*iso*Butyl*	150	1·01	5×10^{-2}	3	
tert.-Butyl*	133–135	0·98	$3·1 \times 10^{-2}$	None	
		(2) Other halogenoacetates			
(*a*) Chloroacetates					
Methyl	131·5	1·18	$3·1 \times 10^{-1}$	None	Very fast
Ethyl	144·2	1·16	$9·4 \times 10^{-2}$	2	Slow
(*b*) Bromoacetates					
Methyl	147	1·62	$2·1 \times 10^{-1}$	None	Very fast
Ethyl	159	1·51	$7·8 \times 10^{-2}$	None	Very fast
		(3) Acetates			
Methyl			2·5	None	Negligible
Ethyl			$7·8 \times 10^{-1}$	None	
n-Propyl			$1·6 \times 10^{-1}$	None	
n-Butyl			$4·2 \times 10^{-2}$	1	
n-Amyl			$1·2 \times 10^{-2}$	None	
		(4) Miscellaneous esters			
Ethyl glycollate	160	1·08	$3·1 \times 10^{-1}$	None	Slow
Ethyl lactate	154	1·03	$3·1 \times 10^{-1}$	None	
Ethyl methoxyacetate	140–141	0·97	$3·1 \times 10^{-1}$	None	
Methyl nitroacetate	82 (18 mm.)	1·21	$3·1 \times 10^{-1}$	None	

* New compounds.

In Figs. 3 and 4 both these properties are plotted as functions of N, the number of carbon atoms in the alkyl chain. Instead of the rate of hydrolysis at the pS optimum the rate at any fixed pS value can be used for comparison, provided this value is on the same side of the maximum for all substrates and the curves do not intersect. If these conditions are fulfilled, such a comparison will give at least the correct order of activities (see Fig. 4, curve 2).

Fig. 2 also demonstrates that ACh is hydrolysed about 60% faster than *n*-amyl fluoroacetate. We confirmed for one substrate, namely, *n*-butyl fluoroacetate, the linear relationship between rate of hydrolysis and enzyme concentration. This enabled us to compare activities, even when different enzyme concentrations had to be used for different substrates.

The sequence established in Figs. 3 and 4 is in contrast to the results of Mounter & Whittaker (1950) on *n*-alkyl acetates, reproduced in Fig. 4, curve 3, which show a maximum at $N = 4$. This difference may be due to the use of enzyme from a different source and of a different degree of purity by these authors. However, since so far erythrocyte choline esterase has shown identical properties with the enzyme from electric organ (Augustinsson, 1949), we are inclined to ascribe the difference in results to the method used. Mounter & Whittaker calculate the final 'effective' concentration of their substrates as 0·1 M, assuming that all of the added ester is in solution, which is by no means always the case (compare Table 1, 4). We have redetermined with our enzyme the pS-activity curves for alkyl acetates, using 'true' solutions. The limited solubility of these esters and their precipitating effect on high concentrations of the enzyme permitted only partial determination of these curves (Fig. 5). From our data it is clear that frequent crossing takes place and therefore comparison of activities is not too reliable. However, as far as can be judged from Fig. 5, at p$S = 1$ the order of activity will be identical with that of the fluoroacetate series and in any case will not show a maximum at $N = 4$, as claimed by Mounter & Whittaker (1950).

Branching has a profound influence on the speed of hydrolysis as shown in Fig. 6. Although the total number of carbon atoms increases, the maximal rate decreases in the order ethyl : *iso*propyl : *tert.*-butyl = 19 : 3·5 : 1. *iso*Butyl is split about half as rapidly as *n*-butyl. The decrease is less pronounced

in the series ACh : Mecholyl = 2 : 1. It is noteworthy that the latter compound exhibits a well-pronounced pS optimum, in contrast to the findings of Mendel, Mundell & Rudney (1943) on erythrocyte enzyme.

The chloro- and bromo-acetates behaved similarly to the fluoroacetates; especially the bromo derivatives which exhibited well-pronounced maxima (Fig. 7). However, the order of activity of the methyl and ethyl esters is reversed for bromoacetate. It may be mentioned that the optimal pS values for the three ethyl halogenoacetates are in the order F < Br < Cl, and the maximum rate decreases in the order F > Br > Cl.

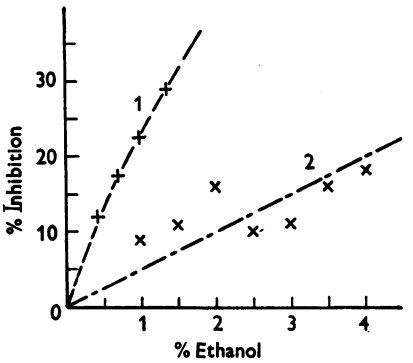

Fig. 1. Inhibition of esterases by ethanol. 1, Hydrolysis of ethyl fluoroacetate, 1×10^{-1} M, by acetylcholine esterase, 1:600; 2, hydrolysis of n-propyl fluoroacetate, $2{\cdot}7 \times 10^{-2}$ M, by liver esterase, 1:150.

Thus higher rate of hydrolysis is associated with a lower pS maximum. The methyl esters, however, follow a different order. Ethyl iodoacetate could not be measured very accurately, due to its rapid spontaneous hydrolysis, but the values obtained indicate a rather low degree of activity.

Inhibitory effects. Other electronegative substituents in the acetyl radical all produce a negligible hydrolytic rate (OH, OCH_3 and NO_2). This, however, is no proof of low affinity for the enzyme since these esters inhibit the hydrolysis of ACh. The same is true for *tert.*-butyl fluoroacetate, which is not split at all at an enzyme dilution of 1 : 600. Their I_{50} values are given in Table 2, including also butyrylcholine. This shows that an ester may form a rather stable complex with the enzyme, although this complex is not split at an appreciable rate. In accordance with previous observations (Bergmann & Shimoni, 1952), quarternary ammonium salts inhibit the hydrolysis of fluoroacetates.

In order to shed more light on the factors determining the rate of enzymic hydrolysis, a corresponding series of experiments was carried out with an unspecific esterase.

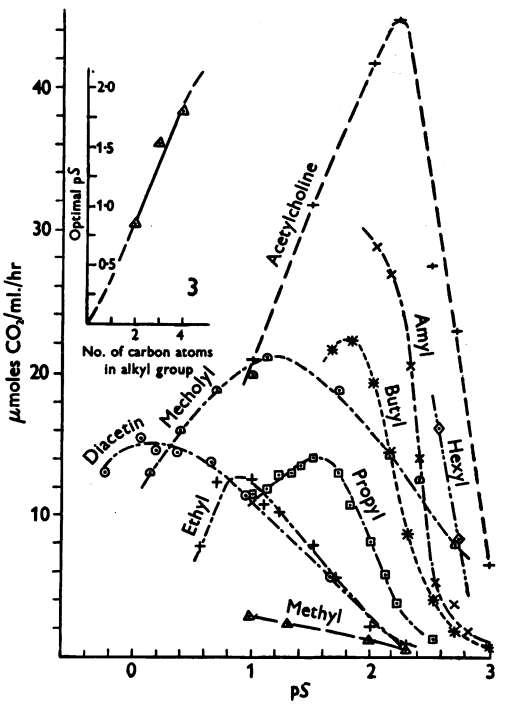

Fig. 2. Hydrolysis of n-alkyl fluoroacetates by acetylcholine esterase, 1:600. Mecholyl and diacetin were measured at the same enzyme concentration. For ACh an enzyme dilution of 1 : 1800 was used, and the rates then multiplied by 3 for comparison with the other esters.

Inset. Fig. 3. Optimal pS values of n-alkyl fluoroacetates as function of number of carbon atoms in the alkyl chain.

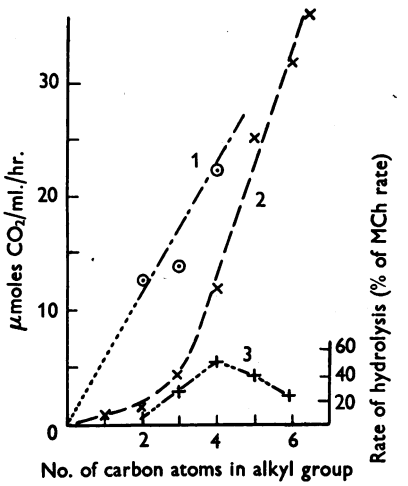

Fig. 4. Rate of hydrolysis of n-alkyl fluoroacetates as function of number of carbon atoms in the alkyl chain. 1, Rates at optimal substrate concentrations; 2, rates at pS 2·2; 3, relative rates of hydrolysis of n-alkyl acetates, according to Mounter & Whittaker (1950).

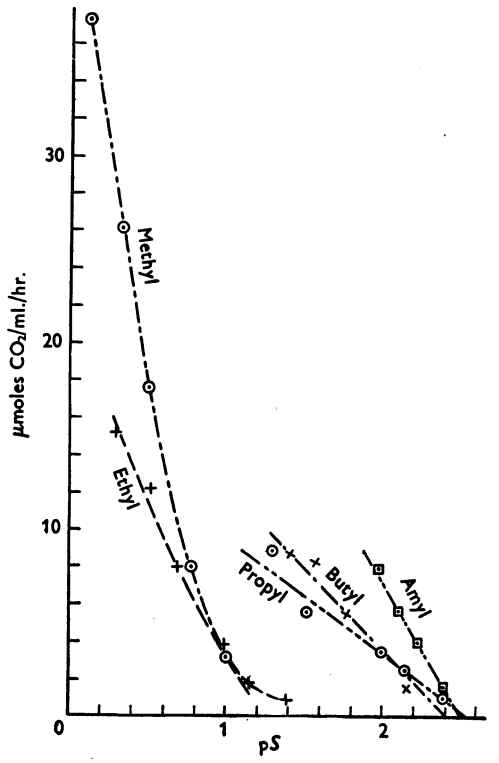

Fig. 5. Hydrolysis of *n*-alkyl acetates by acetylcholine esterase, 1 : 180.

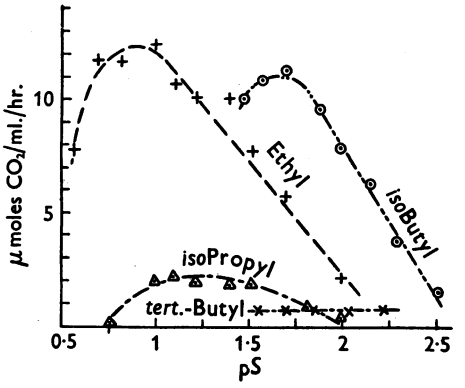

Fig. 6. Rate of hydrolysis of branched alkyl fluoroacetates by acetylcholine esterase, 1 : 600. The *tert.*-butyl ester was tested at an enzyme dilution 1 : 33 and the experimental values divided by 18 for comparison.

Liver esterase

The p*S* activity curves for the hydrolysis of fluoroacetates by liver esterase, as shown in Fig. 8, again present well-shaped maxima. It is very remarkable that whereas ACh possesses the highest maximum on choline esterase, it shows towards liver esterase an activity inferior even to diacetin so that a maximum can no longer be observed. We see in this fact a clear indication that two different principles are involved in the formation of ES_2- (or ES_n-) compounds in the case of fluoroacetates

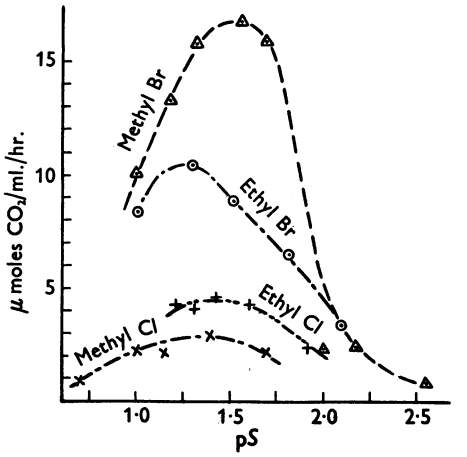

Fig. 7. Hydrolysis of chloro- and bromo-acetates by acetylcholine esterase, 1 : 600.

Table 2. *Inhibitory effect of various esters on acetylcholine esterase*

(The enzyme was incubated with the inhibitor for 1 hr. at 27°, then the substrate was tipped in and activity determined manometrically. For inhibitors 1–6, ACh, $3{\cdot}3 \times 10^{-3}$ M, was the substrate and final enzyme dilution was 1 : 3000. For inhibitor 7, *n*-propyl fluoroacetate, $1{\cdot}2 \times 10^{-2}$ M, was used as substrate and the enzyme was diluted 1 : 200. The concentration of inhibitor, required for 50% inhibition, was evaluated graphically.)

Inhibitor	I_{50} (M)
(1) Ethyl methoxyacetate	2×10^{-2}
(2) Ethyl glycollate	$3{\cdot}5 \times 10^{-1}$
(3) Ethyl lactate	2×10^{-1}
(4) Methyl nitroacetate	$2{\cdot}5 \times 10^{-2}$
(5) *tert.*-Butyl fluoroacetate	1×10^{-2}
(6) Butyrylcholine	$2{\cdot}5 \times 10^{-3}$
(7) Tetraethylammonium bromide	7×10^{-4}

on the one hand, and ACh on the other (see Discussion). Although it is impossible to demonstrate the presence of p*S* optima for the higher alkyl fluoroacetates due to limited solubility, it is apparent that the p*S* maxima are much more crowded and therefore the individual curves intersect frequently. Any comparison of rates becomes therefore arbitrary. From the limited data available it may be guessed that activity alternates between even and odd numbers of carbon atoms, since ethyl > methyl, *n*-butyl > *n*-propyl, *n*-hexyl > *n*-amyl and ethyl > *n*-propyl, *n*-butyl > *n*-amyl. However, for *n*-alkyl acetates no such regularity can be deduced from the curves in Fig. 9. But it can be stated that butyl appears to possess the smallest hydrolytic rate in

the homologous series. Ethyl chloro- and bromo-acetates show again pS maxima (Fig. 10), but the ethyl halogenoacetates exhibit now the following order of optimal pS values: Br > Cl > F, and the other than halogen give considerable activity and eventually show a pS maximum (Fig. 12). Methyl nitroacetate could be measured only at an enzyme dilution 1 : 10, which indicates low affinity for

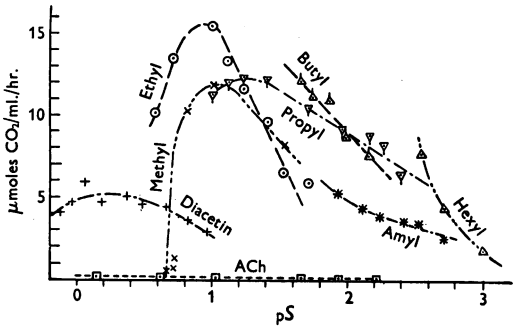

Fig. 8. Hydrolysis of *n*-alkyl fluoroacetates by dog's liver esterase, 1 : 150.

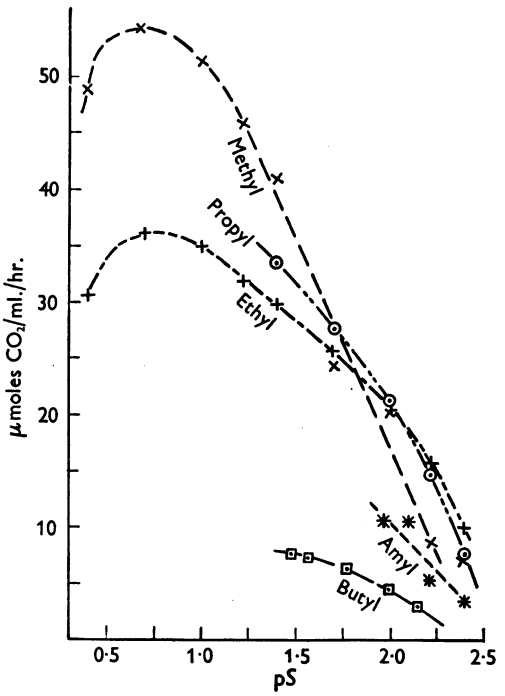

Fig. 9. Hydrolysis of *n*-alkyl acetates by liver esterase, 1 : 25.

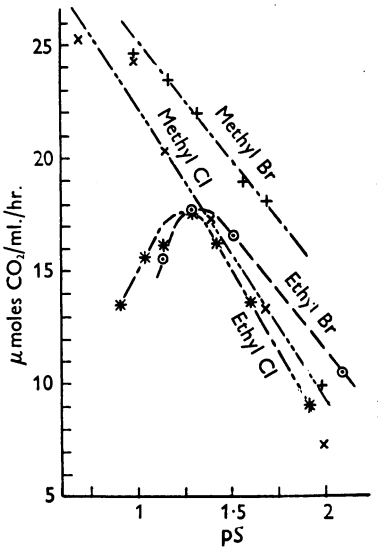

Fig. 10. Hydrolysis of chloro- and bromo-acetates by liver esterase, 1 : 150.

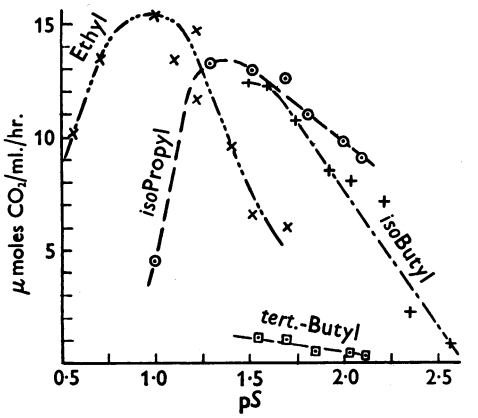

Fig. 11. Hydrolysis of branched alkyl fluoroacetates by liver esterase, 1 : 150. *tert.*-Butyl was measured at an enzyme dilution 1 : 10 and the experimental values divided by 15 for comparison.

order for the maximum rates is Br > Cl > F, although the absolute differences are very small. Again, ethyl iodoacetate shows a low rate of hydrolysis.

The effect of branching is demonstrated by the curves in Fig. 11. Activity decreases again in the order ethyl : *iso*propyl : *tert.*-butyl = 15 : 13 : 1, whereas *iso*butyl approximately reaches the rate of the *n*-butyl derivative. Mecholyl is hydrolysed at a very low rate (about $\frac{1}{4}$ of ACh). Substituents the methyl ester. Unfortunately, the ethyl ester was not available.

We have also investigated the inhibitory effect of *tert.*-butyl fluoroacetate on the hydrolysis of *n*-propyl fluoroacetate by liver esterase and found no inhibition up to a concentration of the tertiary ester of 2×10^{-2}M. The same is true for the system liver esterase-diacetin, where Mecholyl at 7×10^{-1}M does

not cause inhibition. For the liver enzyme, in contrast to choline esterase, negligible reaction rate appears to indicate that the *ES*-complex is not formed to an appreciable degree.

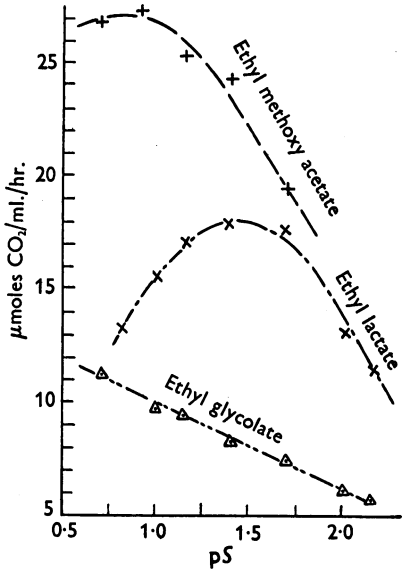

Fig. 12. Hydrolysis of miscellaneous esters by liver esterase, 1 : 150.

DISCUSSION

In a previous paper (Bergmann, Wilson & Nachmansohn, 1950*b*) we ascribed the occurrence of a Murray-Haldane curve for the system ACh-choline esterase to the presence of at least two negative groups in the neighbourhood of the esteratic site, leading to a sufficiently small value of K_2, the dissociation constant of Eqn. (2).

$$E+S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES; \quad ES \xrightarrow{k_3} ES^* \rightarrow E+P; \quad K_m=\frac{k_2+k_3}{k_1}, \quad (1)$$

$$ES+S \underset{k_5}{\overset{k_4}{\rightleftarrows}} ES_2; \quad K_2=\frac{k_5}{k_4}. \quad (2)$$

Since coulombic attraction cannot play a role in the combination halogenoacetate-choline esterase, the pronounced auto-inhibition of these esters must be ascribed to the presence of the halogen atom, which increases the electrophilic character of the carbonyl carbon. We therefore conclude that a bell-shaped p*S*-activity curve appears whenever substrates exhibit a high degree of affinity, affinity being expressed, e.g., by the magnitude of both K_m and K_2. It would appear at first glance that this statement invalidates the above conclusion on the combination of ACh with the enzyme surface. However, the experiments with liver esterase give a clear-cut answer: the halogenoacetates still exhibit a p*S* maximum, but not ACh. Therefore we conclude that two different principles are involved in the auto-inhibition of hydrolysis by acetylcholine esterase. In ACh the low affinity of acetates, as exemplified by diacetin or ethyl acetate, is overcome by the electrostatic attraction to the anionic sites. In halogenoacetates affinity must be mainly a function of the electronegativity of the substituent. Since ion-ion attraction is much stronger than ion-dipole or dipole-dipole interaction, we have a reasonable explanation for the outstanding affinity of ACh towards choline esterase. Since coulombic forces are also available for the combination with Mecholyl or butyrylcholine, this explains why Mecholyl is split half as fast as ACh, whereas *iso*-propyl fluoroacetate is hydrolysed at only one-fifth the rate of the ethyl ester. Similarly, the high affinity of butyrylcholine becomes evident from its I_{50} value (Table 2). Therefore the small rate of hydrolysis of butyrylcholine is due to the low rate of formation of an activated complex *ES** (small value of k_3, Eqn. (1)) from *ES*.

The auto-inhibition of neutral esters is difficult to reconcile with the original assumption of Haldane that two points of attachment are required in these cases for the combination enzyme-substrate. Since we assume that G_1, the nucleophilic group of the enzyme (Wilson & Bergmann, 1950*a*), combines with the carbonyl carbon and G_2, the electrophilic group, with the ether or carbonyl oxygen, the distance between G_1 and G_2 must be commensurable with the bond length of C—O or C=O (1·2–1·5 Å). Therefore, there is not sufficient space available to allow the combination of G_1 with one ester molecule and of G_2 with another one, since the van der Waals radii of atoms are at least twice as big as their bond radii.

Regarding the influence of halogen atoms, the following consideration will show that the halogenoacetates follow a simple rule on liver esterase. According to the procedure of Hammett (1940) a straight-line relationship should be expected between the dissociation constants of the halogenoacetic acids and the equilibrium constants of the enzymic hydrolysis. We have selected for comparison the Michaelis-Menten constants, which were determined approximately from a plot of s/v against S, neglecting the dissociation constants of the esteratic site (Wilson & Bergmann, 1950*b*). Although the K_m values are therefore not very accurate, they show for liver esterase linear proportionality as required by Eqn. (3) (Fig. 13, curve 2):

$$pK_m=A.pK_a+B. \quad (3)$$

The linear relationship is remarkable since the Hammett equation usually does not apply to aliphatic compounds. We may conclude that in the

systems under consideration proximity effects do not interfere seriously and the electron displacement due to the influence of the α-substituent is the decisive factor. Therefore the K_m values of the system halogenoacetate-liver esterase follow the order of electronegativity of the halogens (F = 4·0; Cl = 3·0; Br = 2·8; cf. Pauling, 1948). It should be mentioned that this result is in contrast to the conclusions reached by Adams & Whittaker (1950) for plasma choline esterase, for which van der Waals forces appear to play a decisive role.

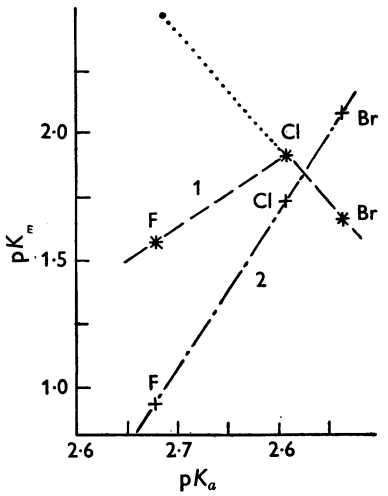

Fig. 13. Michaelis-Menten constants as function of acid dissociation constants of halogenoacetic acids. ($pK_m = A.pK_a + B$.) 1, for choline esterase; 2, for liver esterase.

Entirely different results were obtained with acetylcholine esterase. Curve 1 in Fig. 13 shows a maximum for chlorine. Since the negative sites introduce in this system ion-dipole interaction as a new force between enzyme and substrate, Adams & Whittaker (1950) calculated an affinity ratio for

$$\frac{\log K_{Cl}}{\log K_{Br}} = \frac{0{\cdot}40}{0{\cdot}55} = 0{\cdot}74.$$

However, their experimental figures, determined by the 'competition' method, did not confirm their calculation.* Since our K_m values are $1{\cdot}3 \times 10^{-2}$ for chloroacetate and $2{\cdot}2 \times 10^{-2}$ for bromoacetate, we find for the above ratio the value 0·87. Accepting this as a satisfactory agreement, we should expect fluoroacetate to possess a much smaller K_m, since the polarizability of a substituent is related to its molecular refraction. Actually the value is $2{\cdot}8 \times 10^{-2}$, i.e. greater than for the other halogenoacetates. Therefore fluorine does not occupy the position indicated by the dashed line in Fig. 13. A possible explanation for this abnormality will be given in a subsequent paper.

The description of the enzymic hydrolysis as a nucleophilic attack of the group G_1 in the active surface on the carbonyl carbon (Wilson & Bergmann, 1950*a*) explains the effect of changes in the alkyl chain to a certain degree. Since apparently there is enough space on the active surface of choline esterase for long alkyl chains, we should expect increased affinity due to larger van der Waals forces. This fact and, in addition, the decrease of activation energy, observed in the hydrolysis of homologous esters on cation-exchange resins (Davies & Thomas, 1952), both will explain the increase of rates with increased chain length. Apparently the effect of the dispersion forces on liver esterase is much smaller, since the observed pS maxima for the homologous series of fluoroacetates are crowded together.

Branching of the alkyl chain should decrease the rate of second-order hydrolysis (Hammett, 1940), as is observed for both enzymes. However, the effect of branching is less pronounced for liver esterase, suggesting again that steric hindrance is less effective here.

Our results contribute to a better definition of the specificity of acetylcholine esterase, for which a combination of factors is responsible: limited space for the acyl 'tail' of the substrate, the size of which determines the rate of conversion of ES into ES^*; large space for the alkyl group of the ester, as long as its side chains do not produce steric interference; the presence of negative charges in the neighbourhood of the esteratic site. All these factors are possibly various expressions of the influence of the large protein molecule on the active surface, which in 'unspecific' esterases is supposed to have a similar chemical constitution, but to be more free of environmental influences. Further studies, especially on serum choline esterase, will serve to define more sharply the differences between the various kinds of esterases and thus their specificity.

SUMMARY

1. Esters of halogenoacetic acids usually possess a bell-shaped pS-activity curve when hydrolysed by specific or unspecific esterases.

2. In the homologous series of n-alkyl fluoroacetates, when hydrolysis is effected by acetylcholine esterase, the pS optimum shifts to higher pS values with increasing chain length. Simultaneously the maximum rate of hydrolysis increases.

3. Fluoroacetates with a branched alkyl chain give a much lower rate on both types of esterases, the effect being greater on choline esterase than on liver esterase. This fact supports the assumption of

* It should be mentioned that this method is applicable only if the pS-activity curve is S-shaped. Our results show that no 'limiting' velocities exist for halogenoacetates.

nucleophilic attack of the group G_1 in the esteratic site on the carbonyl carbon of the ester.

4. Esters with a negligibly low rate of hydrolysis may prove to be efficient, reversible inhibitors of acetylcholine esterase. However, no such case has been observed on liver esterase.

5. The pK_m values (Michaelis-Menten constants) of the system ethyl halogenoacetates-liver esterase can be represented as a linear function of pK_a (acid dissociation constants), thus obeying the Hammett equation

$$pK_m = A \cdot pK_a + B.$$

The corresponding curve for choline esterase, however, possesses a maximum for chloroacetate. It is concluded that fluoroacetate behaves abnormally towards the latter enzyme.

REFERENCES

Adams, D. H. & Whittaker, V. P. (1950). *Biochim. biophys. Acta*, **4**, 543.

Augustinsson, K. B. (1949). *Arch. Biochem.* **23**, 111.

Bergmann, F., Gitter, M. & Shimoni, A. (1953). In preparation.

Bergmann, F. & Shimoni, A. (1952). *Biochim. biophys. Acta*, **8**, 520.

Bergmann, F., Wilson, I. B. & Nachmansohn, D. (1950*a*). *J. biol. Chem.* **186**, 693.

Bergmann, F., Wilson, I. B. & Nachmansohn, D. (1950*b*). *Biochim. biophys. Acta*, **6**, 217.

Blank, J. & Bergmann, D. E. (1952). *Bull. Israeli Res. Coun.* (in the Press).

Bouveault, L. & Wahl, A. (1904). *Bull. Soc. chim., Paris*, (3), **31**, 847.

Boyarsky, L. L., Postel, S. & Rosenblatt, A. (1948). *Fed. Proc.* **7**, 11.

Boyarsky, L. L., Rosenblatt, A., Postel, S. & Gerard, R. W. (1949). *Amer. J. Physiol.* **157**, 291.

Davies, C. W. & Thomas, G. Garrod (1952). *J. chem. Soc.*, p. 1607.

Doty, R. W. & Gerard, R. W. (1949). *Fed. Proc.* **8**, 35.

Fischer, E. & Helferich, B. (1911). *Liebigs Ann.* **383**, 81.

Gerard, R. W. & Doty, R. W. (1950). *Biochim. biophys. Acta*, **4**, 115.

Gilman, A. & Jones, R. G. (1943). *J. Amer. chem. Soc.* **65**, 1458.

Hammett, L. P. (1940). *Physical Organic Chemistry*. New York and London: McGraw-Hill.

Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.

Mounter, L. A. & Whittaker, V. P. (1950). *Biochem. J.* **47**, 525.

Pauling, L. (1948). *The Nature of the Chemical Bond*. Ithaca: Cornell University Press.

Wilson, I. B. & Bergmann, F. (1950*a*). *J. biol. Chem.* **185**, 479.

Wilson, I. B. & Bergmann, F. (1950*b*). *J. biol. Chem.* **186**, 683.

A Dynamic Osmometer for Accurate Measurements on Small Quantities of Material: Osmotic Pressures of Isoelectric β-Lactoglobulin Solutions

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The osmometer described in the first part of this paper can be used for accurate measurements on quantities of solution of the order of 0·5 ml. The results obtained with it on unbuffered solutions are presented in the second part; they are of considerably greater accuracy than any comparable measurements known to the author, except for one series of measurements which required about ten times as much solution.

Part I. Design and use of the osmometer

PRINCIPLE AND THEORY

A rigid semi-permeable sac is placed in one limb of a U-tube with protein solution on one side and diffusate material on the other. Flow across the membrane is measured by observing the movement of a droplet in a narrow section at the bottom of the U-tube and is controlled by means of a manometer which enables pressures, greater or less than the osmotic pressure, to be applied to one limb of the U-tube. The pressure corresponding to zero flow is the required osmotic pressure subject to corrections for the following effects: (*a*) any difference in levels of the solutions in the two U-tube limbs; (*b*) differences in densities of the protein solution and diffusate—a very small effect; (*c*) differences between the surface tension rises in the two limbs.

Fig. 1 illustrates the hydrostatics of the system in more detail. Let the pressures and densities be as shown, and the membrane rigid and semi-permeable.

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