XXV. THE STEREOCHEMICAL SPECIFICITY OF ESTERASES.

I. THE AFFINITY OF LIVER ESTERASES FOR OPTICALLY ACTIVE ALCOHOLS.

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(Received January 1st, 1930.)

DAKIN [1904, 1905] first showed that lipases do not hydrolyse the two components of a racemic mixture with equal velocity. He found that liver esterase hydrolysed *d*-ethyl mandelate preferentially when allowed to act on the racemic mixture. Little further work was carried out on the subject until Willstätter and Memmen [1924] and Willstätter, Haurowitz and Memmen [1924] performed the corresponding experiments with pancreatic and gastric lipase. All these observations were confined to the behaviour of the enzyme to the racemic mixture. Rona and Ammon [1927] first investigated the behaviour of the enzyme to the pure optically active forms, and found that liver esterase actually hydrolysed the pure laevo-form with greater velocity than the dextro-form, although selecting the latter from a solution of the mixture. The explanation of this phenomenon was given by Willstätter, Kühn and Bamann [1928]. It was shown that the results were quite consonant with the theory of Michaelis and Menten [1913]. Briefly, the course of hydrolysis depends on the characteristic constants of the two stages of the reaction, which may be represented:

All the results may be explained on the single assumption that these constants may be different for the two optical isomers.

In the case of a single substrate, if the substrate concentration is sufficient to saturate the enzyme, the velocity, which is proportional to the concentration of enzyme-substrate-complex, is determined solely by the second constant, but if the enzyme is partitioned between two substrates, the relative velocities of hydrolysis are principally determined by the constants of the first equation, since these determine the relative concentration of the two enzyme-substrate-complexes. Such is the case of a racemic mixture. Mathematically the conditions may be expressed as follows. If K_D , K_L be the

constants of equation (I) (affinity constants or Michaelis constants), k_D , k_L the constants of equation (II), c_D , c_L the concentrations of the two enzyme-substrate-complexes, v_D , v_L the velocities of hydrolysis, A the initial amount of each substrate, y and x the amounts hydrolysed at any time, and ϵ the enzyme concentration, then

$$\frac{v_D}{v_L} = \frac{k_D c_D}{k_L c_L} = \frac{k_D K_L}{k_L K_D} \cdot \frac{\epsilon(A-y)}{\epsilon(A-x)} = K \cdot \frac{A-y}{A-x}, \text{ where } K = \frac{k_D K_L}{k_L K_D}.$$

But

$$v_D = \frac{dy}{dt} \text{ and } v_L = \frac{dx}{dt} \text{ so that } \frac{dy}{dt} / \frac{dx}{dt} = K \cdot \frac{A-y}{A-x},$$

$$\therefore \int \frac{dy}{A-y} = K \int \frac{dx}{A-x} \text{ or } K = \frac{\ln A - \ln(A-y)}{\ln A - \ln(A-x)}.$$

Weber and Ammon [1929] were able to show that the value of K obtained from observations on the racemate was equal to that obtained by multiplying the values of the constants measured separately, thus proving the correctness of the explanation.

It is obvious that experiments on the racemate alone will not give any information on the values of either the affinity constant or the breakdown constant of either form, but only a figure derived from all four. Thus many recorded observations of the "total specificity" of a particular lipase towards a racemic substrate are of little use for presenting a simple comparison between a series of related compounds.

It is now recognised however that enzymes are capable of forming reversible compounds of the Michaelis type with other substances than their substrates, certainly with the products of the reaction and probably with certain other types of quite unrelated compounds. In a system consisting of the enzyme, substrate and a third substance, the extent of formation of these compounds can be easily measured by observing the extent to which the hydrolysis is inhibited, hence the affinity of the enzyme for the substance can be readily measured. If the enzyme-inhibitor-complex is a compound of the Michaelis type, the two isomers of an optically active substance should show a difference in affinity comparable with the difference between the isomers of a substrate.

One of us [Murray, 1929] has already shown that secondary alcohols such as phenylmethylcarbinol inhibit lipase hydrolysis in this way. Such compounds possess the necessary asymmetry, and can be obtained in optically active forms. The three alcohols selected for the first investigation all had a methyl group attached to the carbinol on one side, the other group being in one case aliphatic, viz. *n*-hexyl, in one case aromatic, viz. phenyl, and in the third case mixed, viz. β -phenylethyl.

EXPERIMENTAL METHOD.

The choice of method lay between the gasometric and the direct titrational. The strict constancy of p_H gives to the former method a greater accuracy, but experiment showed that for the purpose sufficient accuracy

was obtained by the method of direct titration in a buffer-free fluid [cf. Bamann, 1929; Bamann and Schmeller, 1929].

The experiments were performed in test-tubes with a total initial volume of 8 cc. The tubes contained 3 drops of a suitable indicator (bromothymol blue) and whenever the solution became acid to the indicator it was brought back by a drop of $N/100$ NaOH added from a micro-burette which delivered about 40 drops per cc. The error of dilution during titration was found to be negligible. The maximum velocity of hydrolysis was regulated by the amount of enzyme added so that about 30 drops of NaOH solution were required during a half-hour period. All titrations were made at room temperature, and the velocities in a given series were always comparative to one or more control tubes (no inhibitor) in the same series. Usually 10 or 12 tubes were examined together for 30 minutes.

Preparation of material.

Preparation of enzyme. The enzyme extracts were made from preparations of liver, dried by acetone alone, or acetone and ether. The extracting agent was dilute ammonia. $N/40$ was suitable for sheep-liver extraction, but for rabbit-liver $N/10$ was found to be better. After standing for 3 to 4 hours with occasional shaking at about 20° , the residue was centrifuged off, and the solution acidified with acetic acid to precipitate the inactive proteins. The enzyme remained in solution after centrifuging, and, when neutralised with ammonia, was kept in this solution for use. One gram of dried sheep liver extracted with 30 cc. ammonia gives a very active preparation of enzyme, of which $1/20$ cc. is sufficient for each tube in this method. Rabbit-liver preparations are less active and contain more buffering matter, largely phosphate, which renders the colour change less easy to follow. A preliminary extraction of the powder with distilled water removes much of this. It has been shown by Willstätter, Bamann and Waldschmidt-Graser [1927] and Rona and Itelsohn-Schechter [1928] that the degree of purification does not affect the stereochemical specificity, so that the above type of preparation is quite suitable.

Preparation of the optically active alcohols. The alcohols were resolved by the method of Pickard and Kenyon [1911]. The method consists of converting them into acid phthalates which are then crystallised with an optically active base. We should like to express our indebtedness to Dr Kenyon who personally gave us the samples of the two forms of methylphenylcarbinol and methyl- β -phenylethylcarbinol.

Choice of substrate and optimal conditions.

Ethyl butyrate proved to be the most suitable substrate. The maximum velocity of hydrolysis with this substrate and sheep-liver esterase is reached at a concentration of about $0.005 M$. With ethyl propionate and this enzyme the maximum velocity is much smaller and is not reached until the concen-

tration is twice as great. Ethyl acetate is very slowly hydrolysed and the maximum is not reached at a concentration of ten times this figure, indicating a much lower affinity between the enzyme and the shorter chain fatty acid esters (Michaelis constant much larger). It is interesting to note that the value of the Michaelis constant is determined far more by the nature of the acid constituent than by the alcohol—thus *n*-butyl acetate was found to behave very similarly to ethyl acetate.

When the concentration necessary for maximum velocity is exceeded, the velocity falls again. Probably an excess of substrate favours the formation of a slowly hydrolysable complex with the enzyme. The curve relating velocity to substrate concentration is shown in Fig. 1, which also shows the effect of a small quantity of inhibitor on the concentration for maximum velocity. The competition of the inhibitor with the substrate for the active enzyme surface delays the inhibitory power of excess substrate.

Fig. 1. Effect of 0.00079 *M* methyl-*n*-hexylcarbinol on optimal substrate concentration.

Ordinates represent equivalents $\times 10^{-6}$ acid liberated in 10 min.

In all experiments the substrate concentration was that at which the maximum velocity is obtained in the absence of inhibitor. The degree of inhibition is indicated by the vertical drop to the lower curve in Fig. 1.

All the experiments unless otherwise stated were carried out at p_H about 7.0, the indicator used being bromothymol blue.

*Experiments with methyl-*n*-hexylcarbinol.*

The stock solutions of ethyl butyrate and ethyl propionate contained 8 g. per litre, so that when 1 cc. was present in the 8 cc. total fluid the concentration was 1 mg. per cc. For ethyl butyrate, 0.6 cc. was used in each tube, and for ethyl propionate, 1.2 cc., when working with sheep-liver enzyme.

Stock suspensions of the three forms of the sparingly soluble methyl-*n*-hexylcarbinol contained 0.2 cc. (0.165 g.) in 100 cc. Varying amounts of this were added to the substrate and made up to volume, the p_H being adjusted if necessary. The final concentrations of inhibitor were low enough to give complete solution. Enzyme was added to all the tubes and readings com-

menced. A typical result is given in the accompanying table, and the complete results are conveyed by Fig. 2, which shows the inhibition of the hydrolysis of ethyl butyrate, and Fig. 3, which gives the corresponding curves for ethyl propionate. The inhibition of the latter is much greater, corresponding with the fact that it has a much lower affinity for the enzyme.

Experiment. Inhibition of hydrolysis of ethyl butyrate by the three forms of methyl-*n*-hexylcarbinol. 0.6 cc. ethyl butyrate in all tubes.

Cc. suspension carbinol ...	Dextro-						Laevo-						Racemic					
	0.0	0.15	0.3	0.6	0.9	1.2	0.0	0.15	0.3	0.6	0.9	1.2	0.0	0.15	0.3	0.6	0.9	1.2
Drops <i>N</i> /100 NaOH	12	10	8	6	5	4	12	5	3	2	1	1	11	6	4	2	2	1
in 10 min. intervals	12	9	8	6	5	4	12	5	4	2	1	1	11	7	5	3	2	2
	12	10	8	6	6	5	11	6	4	2	2	1	11	6	5	3	2	2
Percentage inhibition		19	33	50	57	64		54	68	83	89	91		42	58	76	82	85

Fig. 2. Hydrolysis of ethyl butyrate inhibited by methyl-*n*-hexylcarbinol (concentration of substrate = 0.00517 *M*).

Fig. 3. Hydrolysis of ethyl propionate inhibited by methyl-*n*-hexylcarbinol (concentration of substrate = 0.01176 *M*).

The racemic inhibitor behaves as would be theoretically expected for a mixture of two inhibitors, namely, the concentration required to give 50 % inhibition (equal partition of the enzyme) is given by $2/C = 1/C_D + 1/C_L$, where C_D and C_L are the concentrations of the optically active forms similarly required.

Experiments with methylphenyl- and methyl- β -phenylethyl-carbinols.

Figs. 4 and 5 show the inhibitions given by the two optically active forms of methylphenylcarbinol and methyl- β -phenylethylcarbinol respectively. It will be seen that the general phenomenon of difference between the two forms of the alcohol occurs again, and to a similar extent; that is to say, the laevo-form inhibits from four to five times more powerfully than the dextro-form. The molar concentrations of these alcohols required to give the same degree of inhibition as was given by the aliphatic alcohol, methyl-*n*-hexylcarbinol, differ considerably, the requisite concentration of methylphenylcarbinol being nine to ten times, and that of methyl- β -phenylethylcarbinol about four times greater.

Fig. 4. Hydrolysis of ethyl butyrate inhibited by methylphenylcarbinol.

Fig. 5. Hydrolysis of ethyl butyrate inhibited by methyl- β -phenylethylcarbinol.

*Experiments with esters of methyl-*n*-hexylcarbinol.*

The acid phthalates of methyl-*n*-hexylcarbinol were prepared as intermediates in the resolution of the alcohol, and as they were found not to be hydrolysed by the enzyme, their inhibitory properties were tested. The sur-

prising result was obtained that the dextro-form inhibited more than the laevo-form as the following table shows:

	Molar concentration of inhibitor				
	0.0001	0.0002	0.00045	0.0009	0.0018
Percentage inhibition by dextro-form	16	33	49	60	75
Percentage inhibition by laevo-form	13	27	40	51	64

The difference is not so great as with the alcohols, but is well marked. No explanation of this phenomenon is suggested at the present time.

In view of this unexpected phenomenon, the acetates of the three forms were studied. Like the phthalates, they were not hydrolysed by the enzyme. The dextro-acetate was given to us by Dr Kenyon, the laevo- and racemic forms were prepared by ourselves. In this case the result was that all three forms gave nearly the same curve (Fig. 6). This curve differs however from all the others investigated in that it becomes flat at about 70 % inhibition, instead of continuing toward total inhibition. It is possible that this is due to the limit of solubility of the substances, but this view is rendered unlikely by an experiment in which ethyl propionate was used as the substrate. The curve of inhibition is again much steeper with this substrate, and flattens at about 85 % inhibition, but at a concentration where the curve for ethyl butyrate is still rising.

Fig. 6. Hydrolysis of ethyl butyrate inhibited by β -octyl acetate.

Comparison of sheep-liver and rabbit-liver esterases.

When rabbit-liver esterase is used in a similar manner to that described with sheep-liver esterase, the optimum concentration of substrate is higher [cf. Bamann and Schmeller, 1929] being about 1.5 mg. ethyl butyrate instead of 0.6, and the Michaelis constant is correspondingly larger. The inhibitory power of the two isomers of methyl-*n*-hexylcarbinol was found to be very nearly the same for both forms, the laevo-form inhibiting perhaps slightly more than the dextro-form. This was somewhat unexpected since Bamann [1929] found the enzyme to differentiate quite markedly between the two forms of ethyl mandelate, but the Michaelis constant was only determined for one form.

DISCUSSION.

The fact that the two optical isomers differ in the extent to which they inhibit hydrolysis tends to dispose of the objection that the inhibition is merely physical in nature. The similarity of the relation between the forms of the three alcohols further points to a chemical basis for the phenomenon. An asymmetry of the combining group of the enzyme itself probably raises the affinity for one configurational series and lowers it for the antipodal series. It may prove possible to relate this selectivity with that observed in the esters of the hydroxy-acid series. Of the esters, either ethyl or methyl, of mandelic acid, sheep-liver esterase has the greater affinity for the dextro-form. It is known, however [Freudenberg, Brauns and Siegel, 1923], that dextro-mandelic acid is configurationally related to the laevo-series, so that the same grouping in the enzyme structure may be the deciding factor of both phenomena.

Although the combination almost certainly occurs at the polar carbinol group, the remainder of the molecule plays an important part in the process, and appears to determine the measure of the affinity. The length of the non-polar hydrocarbon chain is an important factor, thus the phenyl group is very weak in comparison with the aliphatic six carbon chain, while the introduction of two $\text{—CH}_2\text{—}$ groups, between the phenyl and the polar group greatly increases the affinity. Preliminary experiments (not recorded in the text) show that one $\text{—CH}_2\text{—}$ group, as in methylbenzylcarbinol, is as effective as two, while ethyl-*n*-hexylcarbinol appears to be more active than the methyl compound. Further work will be carried out on the effect of substituted and non-substituted hydrocarbon chains of varying length.

SUMMARY.

1. The relative affinities of pairs of optically active secondary alcohols for liver esterases have been studied by observing the extent to which their presence inhibits the hydrolysis of ethyl butyrate or ethyl propionate.
2. The laevo-forms of methyl-*n*-hexylcarbinol, methylphenylcarbinol and methyl- β -phenylethylcarbinol inhibit sheep-liver esterase about four to five times more strongly than the dextro-forms, showing that their affinity is much greater.
3. With rabbit-liver esterase the inhibition caused by both dextro- and laevo-alcohols is practically the same, showing that any difference of affinity is very small.
4. The anomalous behaviour of esters of the alcohols is described.
5. The theoretical implications of the phenomena are discussed.

Our thanks are due to Dr J. Kenyon of Battersea Polytechnic, for supplying the compounds mentioned and some others. It is a pleasure to acknowledge the constant interest of Sir F. G. Hopkins and Mr J. B. S. Haldane in this investigation.

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