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The influence of some aliphatic alcohols on the enzymic hydrolysis of methyl hippurate

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The hydrolysis of methyl hippurate under the influence of α-chymotrypsin and some aliphatic alcohols has been investigated. Methanol, ethanol, n-propanol, n-butanol and n-pentanol were found to inhibit the rate of hydrolysis. The dissociation constants K_I for an assumed enzyme-inhibitor complex and K_{SI} for an assumed (enzyme-substrate)-inhibitor complex have been evaluated. In all cases $K_{SI} > K_I$ with a ratio of 1·1 to 1 for methanol and 2 to 1 for other alcohols. From the relation between the pK values it is calculated that the free energy change accompanying loss of bound water from each $-CH_2-$ group was $-2{\cdot}1$ kJ mol^{-1}.

The lower aliphatic alcohols have been used frequently in enzyme catalysed reaction systems merely to increase the solubility of the substrate to a sufficient level so that its reactivity can be more easily measured (Laidler & Barnard, 1956). This practice is difficult to justify since aliphatic alcohols have been shown to produce both inhibitory and activation effects on enzyme catalysed reaction systems (McDonald & Balls, 1956; Bender & Glasson, 1960). In both reports the interpretation was that the alcohol becomes chemically involved in the reaction mechanism.

In recent years increased interest has been shown in the consequences and mechanisms of reversible binding of small molecules onto macromolecules. For enzymes, the tertiary structure, and thus the functional integrity in solution, is assumed to be under the influence of a structured solvation layer. For an enzyme to cause a catalytic effect in solution it is postulated that the reactants must penetrate and displace solvent, including structured solvent, in the vicinity of the active site. Similarly, reversible inhibitors of catalytic effects must penetrate and displace solvent around the enzyme surface. Thus, an alcohol may affect the catalytic activity of an enzyme as a result of becoming bound physically to the enzyme surface without reacting chemically.

The modification by aliphatic alcohols of the rate of hydrolysis of methyl hippurate catalysed by α-chymotrypsin has been examined. The first five members of the homologous series of normal aliphatic alcohols were investigated and the rate of hydrolysis was determined by continuous acid-base titration of reaction products at constant pH and temperature.

MATERIALS AND METHODS

Materials

Enzyme. α-Chymotrypsin (Seravac Grade IIa, 1,200 NF units/mg) was used throughout at a final concentration of $3{\cdot}2 \times 10^{-6}$M assuming a molecular weight of 25 000. Solutions of the enzyme in distilled water were prepared daily before use.

Substrate. Methyl hippurate was synthesized from hippuric acid (Koch-Light) and methanol (B.D.H. Analar grade) in a hydrogenator at 170° and 90 atmospheres (Nelson, Miles & Canady, 1962; Rinderknecht & Niemann, 1948). The residue was recrystallized from benzene to yield a crystalline product, m.p. 79–80° uncorrected (lit. m.p. 82–83°, Huang & Niemann, 1952). Found: C, 62·0; H, 5·7; N, 7·3, $C_{10}H_{11}N_1O_3$ requires C, 62·2; H, 5·7, N, 7·3. Later batches of methyl hippurate were recrystallized from cyclohexane, m.p. 79–80°. Solutions of substrate were prepared daily by accurately weighing 0·483 g of methyl hippurate and making up to 100 ml with distilled water.

Alcohols. The following alcohols were used as received: methanol (Fisons, Analar), ethanol absolute, propan-1-ol (lab. grade), butan-1-ol (BDH Analar), pentan-1-ol (Koch Lightpuriss). The purity of each alcohol was checked chromatographically using a Perkin-Elmer R.G.C.170 fitted with a Carbowax column. For each alcohol, peaks other than one corresponding to the nominal alcohol under test were either absent or insignificant.

Method

The rate of hydrolysis of methyl hippurate was measured by the pH-stat method (Alles & Hawes, 1940) using a Radiometer Automatic Titrator (Type TTT1C), equipped with a recorder (SBR2C) and syringe burette (SBU1A) supplied by Radiometer Copenhagen.

The reaction mixture contained enzyme, alcohol, substrate and 2M sodium chloride in a total volume of 25 cm³. To 19·0 cm³ of 2·63M sodium chloride in distilled water was added an appropriate volume of distilled water and 1·00 cm³ of enzyme solution. The pH was adjusted to 8·00 by addition of 0·01N sodium hydroxide during a 3 min period before either 5·00, 3·50, 2·50, 2·00, 1·50 or 1·00 cm³ of substrate solution was added to bring the reaction mixture volume up to 25·0 cm³. The pH was maintained constant at 8·00 by the controlled addition of up to 0·15 cm³ of 0·01N sodium hydroxide at the necessary rate. The rate of enzyme catalysed hydrolysis of methyl hippurate was determined at five substrate concentrations at each concentration of each alcohol investigated. All the components of the reaction mixture were maintained at 293·2 ± 0·1 °K before and during the reaction. A CO_2-free nitrogen atmosphere was maintained above the stirred reaction mixture in the water jacketed reaction vessel.

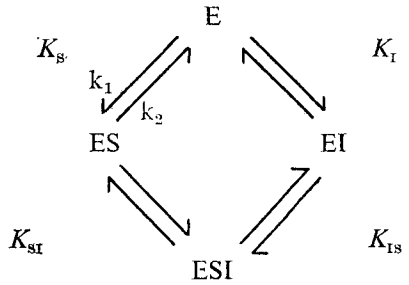
RESULTS

The results have been fitted to the simple classical models for Michaelis Menten steady state binding and reaction of enzyme and substrate

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} P + E$$

$$K_m = \frac{k_3 + k_2}{k_1}$$

and of enzyme, substrate and a reversible enzyme inhibitor



where $K_S = \frac{[E]\,[S]}{[ES]} = \frac{k_2}{k_1}$ $K_I = \frac{[E]\,[I]}{[EI]}$ $K_{SI} = \frac{[ES]\,[I]}{[ESI]}$

The experimental data have been fitted by least squares regression analysis to linear transformations of the equation derived directly from the above models:

$$\frac{V_{max}}{V} = \frac{K_m}{S}\left(1 + \frac{[I]}{K_I}\right) + \left(1 + \frac{[I]}{K_{SI}}\right)$$

where V is the observed reaction velocity at a substrate concentration S and V_{max} is the maximum velocity estimated by extrapolation to an infinite value of S.

DISCUSSION

Values obtained for the dissociation constants K_I and K_{SI} are given as negative logarithms (pK) in Table 1. Thus the pK values can be regarded as affinity constants

Table 1. *Values for the negative logarithms of the dissociation constants for the binding of alcohols to free enzyme (K_I) and enzyme-substrate complex (K_{SI}).*

Alcohol	Concentration(M)	pK_I	pK_{SI}
Methanol	0·09385	0·7174	0·6318
	0·1877	0·7271	0·6013
	0·2185	0·6378	0·6465
	0·3754	0·6778	0·6153
	0·4693	0·6427	0·6065
Ethanol	0·1955	0·6066	0·1981
	0·2932	0·5448	0·3268
	0·3910	0·5697	0·3671
	0·4887	0·5674	0·3202
	0·5864	0·6038	0·3017
Propanol	0·1526	0·8875	0·6575
	0·2034	0·9059	0·6386
	0·2543	0·9333	0·6037
	0·3052	0·9407	0·5832
Butanol	0·04142	1·3760	1·1463
	0·06213	1·3563	1·0643
	0·08284	1·3659	1·1502
	0·1035	1·3954	1·0938
	0·1243	1·4120	1·0922
Pentanol	0·01757	1·7445	1·0594
	0·03513	1·6703	1·3210
	0·04391	1·6654	1·3682
	0·05270	1·7040	1·3640

with higher values indicating greater affinity of the reactants in the formation of a reversible complex. The mean values of pK determined at several concentrations of each alcohol are shown in Fig. 1. It can be seen for all alcohols tested that $pK_I > pK_{SI}$ In the case of methanol the difference is statistically significant $P = 0{\cdot}05$, as determined by the Student t-test ($t_{calc} = 6{\cdot}66$, $t_{P=0{\cdot}05} = 2{\cdot}31$ and $t_{P=0{\cdot}001} = 5{\cdot}04$) and $K_{SI}/K_I = 1{\cdot}1$. The data for ethanol, propanol, butanol and pentanol is consistent with the generalization that $K_{SI}/K_I \approx 2$. Moreover, it can be seen in Fig. 1 that the pK values increase by a constant amount for successive members of the alcohol series with the exception of methanol.

The relative values of pK_I and pK_{SI} for any alcohol can be interpreted in terms of classical enzyme kinetics. For methanol the values are similar but distinguishable and the effect of methanol inhibition fits closely to a classical non-competitive mechanism with a slightly greater affinity of alcohol for complexation with free enzyme rather than the enzyme-methyl hippurate complex. The other alcohols are seen to exhibit a much greater affinity for the free enzyme rather than the enzyme-methyl hippurate complex so that the mechanism of inhibition can be regarded as partially competitive.

To use the pK values in an interpretation of the mechanism of action of the alcohols in molecular terms it is useful to consider the following reaction mechanisms for the catalytic hydrolysis of methyl hippurate by α-chymotrypsin.

$$EH + RCOOMe \rightleftharpoons EH : RCOOMe \rightleftharpoons EOCR + MeOH$$

$$EOCR + H_2O \rightleftharpoons RCOOH + EH$$

The data reported in this paper are consistent with the hypothesis that the aliphatic alcohols tested bind reversibly onto the enzyme surface to exert their inhibitory effects

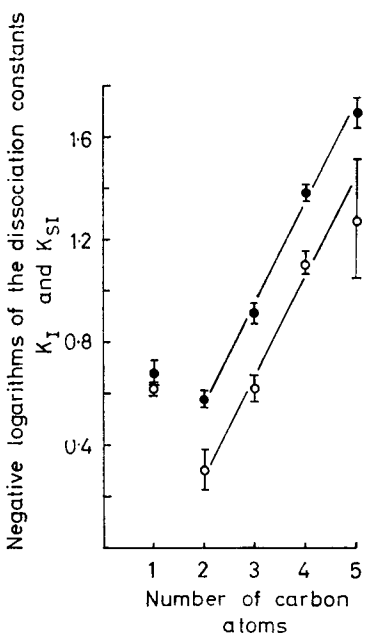


FIG. 1. Relation of the negative logarithms of the dissociation constants K_I ● and K_{SI} ○ to the number of carbon atoms in the alcohols. Vertical bars indicate 95% confidence limits.

and it can be assumed that the observed inhibitory action is due to one or more of the following mechanisms:

I. Competition between alcohol and methyl hippurate for the same binding site on the enzyme.

II. Initiation of an allosteric mechanism by the interaction of the enzyme with an alcohol thereby reducing either the affinity of binding of methyl hippurate to the catalytic site or the catalytic effectiveness of the binding site.

III. Competition between water and alcohol for a binding site that is related to the solvolysis of the enzyme-substrate complex. The effect of a reaction between an alcohol other than methanol would be to cause transesterification.

Bender & Glasson (1960) have quantitatively investigated transesterification in the α-chymotrypsin catalysed solvolysis of acetyl-L-phenylalanine methylester in the presence of aqueous methanol. Assuming a binding model they concluded that their kinetic results could not be interpreted in terms other than the independent binding of both ester and water (or methanol) onto the enzyme surface. Although it can be expected that mechanism III does operate to some degree in our system, the data are not consistent with the hypothesis that it is the dominant mechanism since in that case it would have been observed that $K_I > K_{SI}$ and classical uncompetitive kinetics would have been expected.

In consideration of mechanisms I and II it is useful to consider the work of Belleau (1968), who postulated that enzyme inhibitors can bring about their effects by causing a perturbation of the enzyme structure. He assumed that water molecules non-specifically bound to an enzyme surface play a definite role in the stabilization of the enzyme structure. Potentially adsorbable molecules modify the hydration layer around an enzyme allowing changes to occur in the spatial relations of constituent parts of the enzymes. Except with methanol, the values of pK_I and pK_{SI} were found to increase as the hydrocarbon chain of the aliphatic alcohols increased in length. It is known that alcohols can disturb water structure controlled by hydrogen bonding. It is also known that the affinity of alcohols for binding to hydrophobic sites increases as the length of the hydrocarbon chain increases. Thus an alcohol introduced into an aqueous solution of chymotrypsin may be expected to accumulate in the hydration layer around the enzyme surface and this locally enhanced concentration will be greater for alcohols with longer hydrocarbon chains. In the work reported here, very low concentrations of alcohol produced a significant reduction in the rate of hydrolysis of methyl hippurate; for example, pentanol was active at a concentration of 0·02M with water present at a concentration of 55M. However, it is probable that the concentration of alcohol in the vicinity of the enzyme surface was much greater than that in the bulk solution.

The free energy change (ΔG) accompanying the complexation of an inhibitor with an enzyme can be calculated from the expression

$$\Delta G = RT \ln K$$

According to Cammarata & Martin (1970) the total free energy change is approximately

$$\Delta G_v + \Delta G_h + \Delta G_p$$

where the subscripts refer to independent contributions from binding through van der Waals forces (v) hydrophobic bonding (h) and to change in conformation that the enzyme might undergo (p). From the work of Traube (1891), Ferguson (1939) and Aranow & Witten (1958) it can be assumed that for a homologous series of alcohols,

Me $(CH_2)_n$ OH, changes in the affinity of hydrophobic binding as a function of n can be expressed as a constant increment of ΔG_h for each additional $-CH_2-$ group; that is a linear relation between n and the free energy of binding due to hydrophobic interactions would occur. According to Aranow & Witten (1958), the typical free energy change accompanying loss of bound water from a methylene group is —2·41 kJ mol^{-1}. The slope of the linear free energy relations for pK_I and pK_{SI} shown in Fig. 1 for ethanol, propanol, butanol, and pentanol correspond to free energy changes of about —2·1 kJ mol^{-1} for each additional methylene group. Consistent with other work of Seydoux, Yon & Némethy (1969) and of Bender & Glasson (1960), it can be concluded that at least two properties of aliphatic alcohols determine the apparent values of the dissociations pK_I and pK_{SI} namely nucleophilicity and hydrophobic bonding: the relative importance of these two factors is probably not constant among the members of an homologous series of alcohols and will change as a function of n.

REFERENCES

ALLES, G. A. & HAWES, R. C. (1940). *J. biol. Chem.*, **133**, 375–390.

ARANOW, R. H. & WITTEN, L. (1958). *J. chem. Phys.*, **28**, 405–409.

BELLEAU, B. (1968). *Proceedings of the Third International Meeting, Pharmacological Meeting*, **7**, 207–236. London: Pergamon.

BENDER, M. L. & GLASSON, W. A. (1960). *J. Am. chem. Soc.*, **82**, 3336–3342.

CAMMARATA, A. & MARTIN, A. N. (1970). In *Mednl Chem.*, pp. 118–158, 3rd Edn, Editor: Burger, A. New York: Wiley-Interscience.

FERGUSON, J. (1939). *Proc. Roy. Soc. (Lond.), Ser.* B, **127**, 387–404.

HUANG, H. T. & NIEMANN, C. (1952). *J. Am. chem. Soc.*, **74**, 4634–4638.

LAIDLER, K. J. & BARNARD, M. L. (1956). *Trans. Farad. Soc.*, **52**, 497–502.

MCDONALD, C. E. & BALLS, A. K. (1956). *J. biol. chem.*, **221**, 993–1003.

NELSON, G. H., MILES, J. L. & CANADY, W. J. (1962). *Arch. Biochem. Biophys.*, **96**, 545.

RINDERKNECHT, H. & NIEMANN, C. (1948). *J. Am. chem. Soc.*, **70**, 2605–2606.

SEYDOUX, F., YON, J. & NÉMETHY, G. (1969). *Biochem. Biophys. Acta*, **171**, 145–156.

TRAUBE, I. (1891). *Ann. Chem. Liebigs*, **265**, 27.