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HYDROPHOBIC INTERACTIONS OF SOME ALCOHOLS WITH ACYL TRYPSINS

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SUMMARY

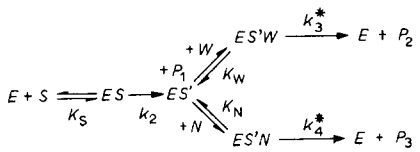
The variation of the apparent reactivity of added nucleophiles competing with water in the deacylation of some acyltrypsins has been studied with different ester substrates: Lysine ethyl and methyl ester, arginine methyl ester and α-*N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester. The efficacy of the primary aliphatic alcohols increases with the length of the apolar chain. For instance, the reactivity of heptanol toward lysyl trypsin is about 45 times greater than that of ethanol.

These data cannot be explained merely in terms of the chemical reactivity of the alcohols. It is necessary to assume that water as well as the aliphatic primary alcohols used as nucleophiles interact with a corresponding site of the enzyme.

The apparent reactivity of nucleophilic compounds is interpreted in terms of hydrophobic interactions between an apolar site of the acyl enzyme and the hydrocarbon chain of the alcohols. Results obtained with organic solvents such as dimethylformamide and isopropanol, an unreactive alcohol, are in good agreement with such an interpretation.

INTRODUCTION

In previous papers[1,2] it has been shown that the nucleophilic competition between the water molecule and some nucleophilic water analogues for the acyl enzyme intermediate in trypsin (EC 3.4.4.4) catalyzed reactions cannot be explained merely on the basis of relative nucleophilicities. It is necessary to assume the existence of another acyl enzyme–water (*ES'W*) or acyl enzyme–alcohol (*ES'N*) complex. The simplest sufficient scheme is the following:



E, S, ES, ES', are the enzyme, the substrate, the Michaelis complex and the acyl enzyme, respectively. $ES'W$ and $ES'N$ are non-covalent complexes between acyl enzyme and water or alcohol molecules, respectively; K_W and K_N are the corresponding dissociation constants. P_1 is the alcoholic fraction of the ester substrate S, P_2 is the carboxylate product, and P_3 is the ester product.

In the present work, the variation of the apparent reactivity of various aliphatic primary alcohols as nucleophiles has been studied. Some experiments were carried out in the presence of dimethylformamide and isopropanol.

THEORETICAL PART

Employing the usual steady-state assumptions, the apparent catalytic rates constants k_{app_1} and k_{app_2} are obtained, as defined by the following relationship:

$$k_{app_i} = \frac{1}{E_t}\frac{dP_i}{dt} = \frac{k_{cat_i}}{1 + \frac{K_m}{S}} \text{ with } i = 1 \text{ or } 2 \tag{1}$$

where E_t = total concentration of the enzyme and:

$$k_{cat_1} = \frac{k_2\left(k_3^* + k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}\right)}{k_2\left(1 + \frac{K_W}{[W]} + \frac{K_W}{K_N}\frac{[N]}{[W]}\right) + k_3^* + k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}} \tag{2}$$

$$k_{cat_2} = \frac{k_2 k_3^*}{k_2\left(1 + \frac{K_W}{[W]} + \frac{K_W}{K_N}\frac{[N]}{[W]}\right) + k_3^* + k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}} \tag{3}$$

$$K_m = K_s\,\frac{k_3^* + k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}}{k_2\left(1 + \frac{K_W}{[W]} + \frac{K_W}{K_N}\frac{[N]}{[W]}\right) + k_3^* + k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}} \tag{4}$$

k_3^*, k_4^* are the monomolecular rate constants of deacylation for the decomposition of $ES'W$ and $ES'N$ into P_2 and P_3 and E, respectively.

For the evaluation of k_4/k_3^* two methods could be used measuring either the rate of appearance of the P_1 product or that of the P_2 product.

The apparent constant k_4 used in this ratio is defined in a previous paper[1] and is used in order to allow comparison with the simpler reaction scheme proposed by BENDER *et al.*[3].

This apparent constant has a rather complex meaning:

$$k_4/k_3^* = k_4^*/k_3^* \cdot K_W/K_N \cdot 1/[W] \tag{5}$$

under conditions of excess water in the medium. This definition is used as a simplification.

(a) The rate constant corresponding to the appearance of P_1 is given by relationships (1), (2) and (4) under the conditions that

$$k_2\left(1+\frac{K_W}{[W]}+\frac{K_W}{K_N}\frac{[N]}{[W]}\right) \gg k_3^* \text{ and } [S] \gg K_m$$

(see ref. 1) could be written:

$$k_{app_1} = k_{cat_1} = \frac{k_3^* + k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}}{1+\frac{K_W}{[W]}+\frac{K_W}{K_N}\frac{[N]}{[W]}} \tag{6}$$

In addition, under conditions where $K_W/(W)$ and $K_W\,[N]/K_N\,[W]$ are very small with respect to the unity and in the concentration range of the present investigation, Eqn. (6) simplifies into:

$$k_{app_1}' = k_3^* + k_4^* \cdot \frac{K_W[N]}{K_N[W]} \tag{7}$$

Plotting $k_{app_1}/k_{app_{1.0}}$ *versus* the $[N]/[W]$ ratio, a straight line is obtained, the slope of which gives $\frac{k_4^*}{k_3^*}\frac{K_w}{K_N}$

Dividing the slope value by the water concentration $[W] = 55.5$ M, gives the k_4/k_3^* ratio in water.

(b) The rate constant corresponding to the appearance of the P_2 product, given by the relationship (1), (3) and (4) under conditions where $[S] \ll K_m$, simplifies into:

$$k_{app_2} = \frac{k_2 k_3^*[S]}{K_S\left(k_3^* + k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}\right)} \tag{9}$$

or

$$1/k_{app_{2.0}} = K_S/[S]k_2 + \frac{K_S\,k_4^*\frac{K_W}{K_N}\frac{[N]}{[W]}}{[S]k_2\,k_3^*} \tag{10}$$

Defining:

$$1/k_{app_{2.0}} = K_S/[S]k_2 \tag{11}$$

and dividing Eqn. (10) by Eqn. (11):

$$k_{app_{2.0}}/k_{app_2} = 1 + \frac{k_4^*}{k_3^*}\frac{K_W}{K_N}\frac{[N]}{[W]} \tag{12}$$

The k_4/k_3^* ratio, which is a measure of the relative reactivity of the alcohol with respect to that of water, can be obtained from the experiment by dividing the slope of the straight line in a plot of $k_{app_{2.0}}/k_{app_2}$ *versus* $[N]/[W]$ by the concentration of water (55.5 M). Experimentally, both methods are not absolutely equivalent. In the first case, K_S does not appear in the equation and therefore solvent effects[1,4–6] on this parameter will not be registered, whereas in the second case the K_S value is an integral part of the equations.

Comparing the results obtained by both methods, it is possible to evaluate the magnitude of such effects and to determine whether or not they can be neglected in the determination of k_4/k_3^* value by the second method. The existence of an adsorptive complex $ES'W$ and $ES'N$ can be demonstrated by introducing in the system an unreactive molecule, Q, for example, an organic solvent, which can compete only

with the alcohol molecule. Assuming that Q competes with the alcohol but not with the water or the substrate, the reaction pathway would involve an ESQ complex and an $ES'WQ$ complex. K_Q is the dissociation constant of the $ES'Q$ complex. The water site and the Q site are assumed to be independant. In this case Eqn. (5) becomes:

$$\frac{k_4}{k_3^*} = \frac{k_4^*}{k_3^*} \frac{K_W}{(W)} \frac{1}{K_N\left(1 + \frac{[Q]}{K_Q}\right)} \qquad (13)$$

giving a linear dependence of $(k_4/k_3^*)^{-1}$ upon $[Q]$.

MATERIALS AND METHODS

Materials

Enzyme: Twice recrystallized bovine trypsin (Worthington) was used. $MgSO_4$ present in the commercial preparation (about 50%), was removed by exhaustive dialysis against 0.01 M or 0.001 M HCl. Trypsin was purified by precipitation of the denatured fraction with 1 M NaCl (ref. 7), then the solution was dialysed against 0.001 or 0.01 M HCl to remove NaCl: the enzyme titration with α-*N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester and *p*-nitrophenylacetate indicates an active site concentration in trypsin[8] varying between 70 and 84%, according to the preparation and compared to the concentration spectrophotometrically measured at 278 mμ with $1/E^{1\,cm}_{1\,mg} = 0.63$. The absolute specific activity of trypsin on the α-*N*-benzoyl-L-arginine ethyl ester substrate, under the conditions $[S_0] = 1$ mM, pH = 8.0; temperature: 25°, NaCl = 0.1 M is thus: 24.5 ± 1 sec^{-1}.

Substrates: We have prepared L-lysine ethyl ester and L-lysine methyl ester[9,10]. The melting points are, respectively:

L-lysine ethyl ester · 2 HCl: 142° (lit.: 143–144°, ref. 9)

L-lysine methyl ester · 2 HCl: 212°–213° (lit.: 212°, ref. 10)

These ester substrates are chromatographically homogeneous with the solvent system:

n-butanol–pyridine–acetic acid–H_2O (30:20:6:24, by vol.). L-Arginine methyl ester is a "Mann Research Laboratory" product, α-*N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester is a "Cyclo Chemical Co." product. These products are used without further purification. The alcohols (methanol, ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-heptanol, isopropanol, benzyl alcohol) (Prolabo), have been freshly redistilled before use. Dimethylformamide has been also redistilled.

Kinetic measurements

The kinetics of the trypsin-catalysed hydrolysis of L-lysine ethyl ester, L-lysine methyl ester and L-arginine methyl ester were carried out by titrating P_2 with the potentiometric method at constant pH using a E.I.L. pH meter[1,2]. For accurate determinations of the reaction rate, it is necessary to determine with good precision the initial part of the reaction since the measurement is only meaningful for the initial 2% of the total hydrolysis. Beyond this range transesterification[2,11] interferes with the reaction unless the added nucleophile is the same as the alcohol moiety of the substrate. The kinetics of the trypsin-catalysed hydrolysis of α-*N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester were measured with a spectrophotometric method using

a spectrophotometer Cary 14 equipped with thermostated cell compartment. For this substrate we have found $\Delta\varepsilon_{320\ m\mu} = 8220$ in water, and 8700 in 6% isopropanol (v/v), the first value is in good agreement with that reported by BENDER *et al.*[8]. This substrate was kept in solution in 10^{-3} M HCl in the cold room, its exact concentration being determined by carrying out a total tryptic hydrolysis.

RESULTS

Effect of alcohols on the deacylation step

The effect of different primary aliphatic alcohols CH_3–$(CH_2)_{n-2}$–CH_2OH (n varying from 1 to 7) upon the deacylation of the following three acyl trypsins was analysed: L-lysyl trypsin (I), L-arginyl trypsin (II) and α-carbobenzoxy-L-lysyl trypsin (III). Isopropanol and benzyl alcohol were used to assess the influence of the steric hindrance of the isopropyl and aromatic groups respectively. The relative reactivity of the alcohols was experimentally obtained by plotting $k_{app_{2.0}}/k_{app_2}$(L-lysine methyl ester, L-lysine ethyl ester and L-arginine methyl ester) or $k_{app_1}/k_{app_{1.0}}$(α-*N*-

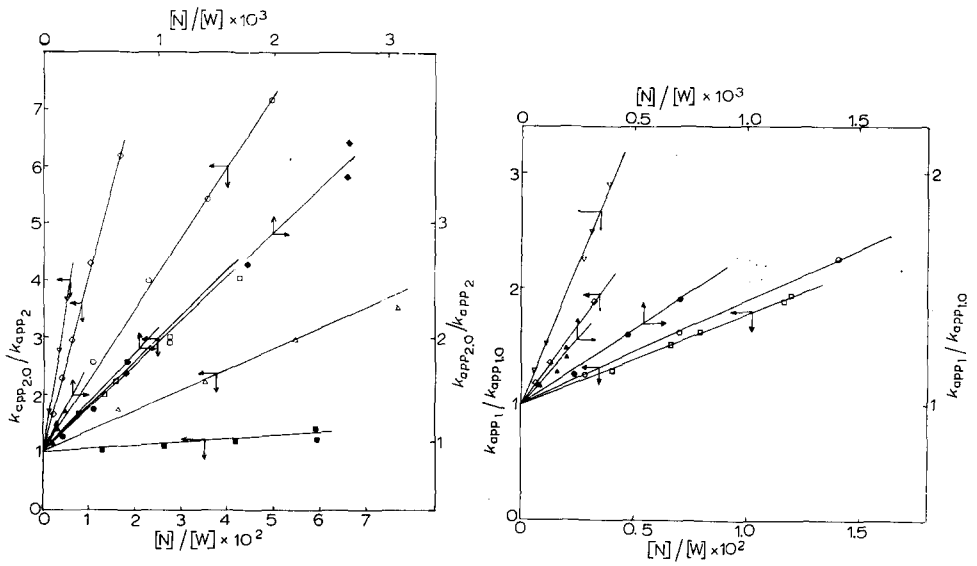


Fig. 1. Effect of primary aliphatic alcohols on the tryptic hydrolysis of L-lysine ethyl ester and L-lysine methyl ester, with $S \ll K_m$. The ratio $k_{app_{2.0}}/k_{app_2}$ is plotted against the alcohol–water ratio $[N]/[W]$, $k_{app_{2.0}}$ is the rate constant of the reaction without alcohol. The slope of each line is proportional to k_4/k_3^* for the corresponding alcohol. ○ = methanol, △ = ethanol, □ = *n*-propanol, ■ = isopropanol, ◇ = *n*-butanol, ▽ = *n*-pentanol, ● = *n*-hexanol, ▲ = *n*-heptanol, ◆ = benzyl alcohol. Experimental conditions: L-lysine methyl ester or L-lysine ethyl ester, 1 mM; $CaCl_2$, 25 mM; pH = 4.80 ± 0.01; temp. 25.0 ± 0.1°.

Fig. 2. Effect of primary aliphatic alcohols on the tryptic hydrolysis of α-*N*-carbolenzoxy-L-lysine *p*-nitrophenyl ester with $[S] \gg K_m$. The ratio $k_{app_1}/k_{app_{1.0}}$ is plotted against the alcohol–water ratio $[N]/[W]$, $k_{app_{1.0}}$ is the rate constant of the reaction without alcohol. The slope of each line is proportional to k_4/k_3^* for the corresponding alcohol. ○ = Methanol, □ = *n*-propanol, ◇ = *n*-butanol, △ = *n*-pentanol, ● = *n*-hexanol, ▲ = *n*-heptanol. Experimental conditions: α-*N*-carbolenzoxy-L-lysine *p*-nitrophenyl ester, 0.45 mM; $CaCl_2$, 25 mM; acetate buffer, 32 mM; pH = 4.79 ± 0.01; temp., 25.0 ± 0.2°.

carbobenzoxy-L-lysine *p*-nitrophenyl ester) *versus* $[N]/[W]$ for each alcohol. The linear relationship predicted by Eqn.7 and Eqn. 12 is followed accurately(Figs. 1 and 2). The slope gives $k_4\,[W]/k_3$*. The determinations were carried out under the following experimental conditions:

For esters of lysine or arginine: $CaCl_2$, 25 mM; temp., 25° ± 0.1°; pH, 4.80 ± 0.01; L-lysine ethyl ester* (or L-lysine methyl ester*), 1 mM; L-arginine methyl ester*, 0.62 mM.

For α-*N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester*, $[S]$ 0.45 mM; $CaCl_2$, 25 mM; acetate buffer, 32 mM; pH, 4.79 ± 0.01; temp., 25.0° ± 0.2°. Table I summarizes

TABLE I

RATIO OF THE APPARENT DEACYLATION RATE CONSTANTS OF L-LYSYL TRYPSIN (I), L-ARGINYL TRYPSIN (II) AND α-CARBOBENZOXY-L-LYSYL TRYPSIN (III) FOR THE REACTION WITH AN ALCOHOL (k_4) AND WITH WATER (k_3*)

Experimental conditions: see text. Standard deviations are indicated.

Alcohol	*Concentration range*	k_4/k_3* *values for acyl trypsins* (M^{-1})		
		I	*II*	*III*
Methanol	0.5 –4 M	2.05 ± 0.1	1.62 ± 0.1	1.64 ± 0.08
Ethanol	0.4 –4 M	0.76 ± 0.1	—	—
n-Propanol	0.7 – 2.5 M	1.41 ± 0.15	1.14 ± 0.10	1.48 ± 0.13
n-Butanol	0.1 –0.9 M	5.76 ± 0.5	4.65 ± 0.80	4.72 ± 0.3
n-Pentanol	0.1 – 0.3 M	9.2 ± 0.9	6.50 ± 0.50	8.5 ± 0.6
n-Hexanol	10 –40 mM	18.3 ± 2	14.1 ± 2.5	11.8 ± 0.8
n-Heptanol	3 –10 mM	34 ± 6	—	20.6 ± 4
Benzyl alcohol	50 –200 mM	20 ± 2	14.8 ± 2.2	11 ± 2
Isopropanol	0.65– 3.5 M	0.1 ± 0.05	—	—

the value of k_4/k_3* for the different alcohols and acyl trypsins I, II, III. It is seen that k_4/k_3* decreases from methanol to ethanol, but increases as the length of the aliphatic chain of the alcohol is further increased. The apparent effect of heptanol on the L-lysyl trypsin is about 16 times greater than that of methanol. Such a result cannot be explained in terms of inductive or steric effects on the nucleophilicity of the alcohol[12,13]. It is therefore necessary to assume the existence of some specific interaction between the enzyme and the alcohols.

The effect of dimethylformamide and isopropanol on the reactivity of the alcohols for the acyl enzyme

In the presence of dimethylformamide, at concentrations ranging from 0 to 4 M (0–30%, v/v), the ratio k_4/k_3* for lysyl trypsin remains constant for methanol, but decreases for the other alcohols. This effect is particularly clear for butanol and higher homologues. The plot of k_3^*/k_4 *versus* $[Q]$ for methanol and butanol is given in Fig. 3, showing the linear relationship predicted by Eqn. (13). This indicates a competition

* The kinetic parameters related to these substrates under the above mentioned conditions and referred to 100% fully active enzyme.

L-Lysine methyl ester: K_m = 26 mM $k_{cat_{2.0}}$ = 24.0 sec^{-1}; L-lysine ethyl ester: K_m = 49 mM; $k_{cat_{2.0}}$ = 21.0 sec^{-1}; L-arginine methyl ester: K_m = 7.2 mM $k_{cat_{2.0}}$ = 22.5 sec^{-1}; α-*N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester: K_m = 2.2 μM $k_{cat_{1.0}}$ = 2.46 sec^{-1}.

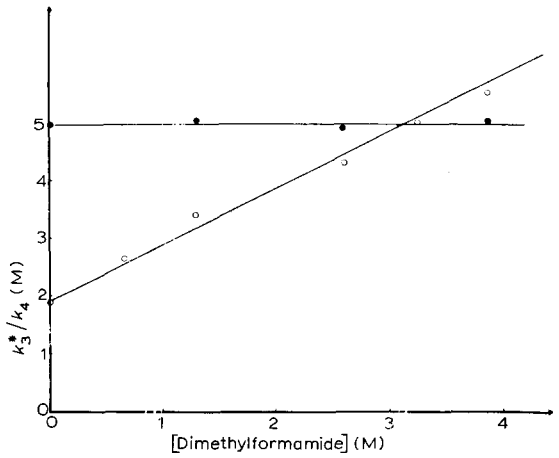


Fig. 3. The reciprocal of the ratio k_4/k_3^* for methanol (●) and for butanol (○) of L-lysyl trypsin in the presence of dimethylformamide, plotted as a function of dimethylformamide concentration. Experimental conditions: see Fig. 1.

between dimethylformamide and the apolar chain of the higher alcohols for binding of the enzyme. The apparent constant K_Q (defined by the solvent concentration needed to decrease the k_4/k_3^* ratio by a factor of two) can be obtained from the slope in Fig. 3. A very similar behavior is observed with isopropanol and α-*N*-carbobenzoxy-L-lysine *p*-nitrophenyl ester in the presence of butanol and pentanol. K_Q values of dimethylformamide and isopropanol for several alcohols are given in Table II.

TABLE II

THE K_Q VALUES FOR DIMETHYLFORMAMIDE AND ISOPROPANOL WITH VARIOUS ALCOHOLS AND ACYL TRYPSINS I AND II

Standard deviations are indicated.

Alcohol	*K_Q values (M) for:*	
	Isopropanol and acyl trypsin III	*Dimethylformamide and acyl trypsin I*
Methanol	—	>50
Ethanol	—	20 ± 8
Propanol	—	5.3 ± 1.1
Butanol	2.1 ± 0.5	1.8 ± 0.2
Pentanol	1.1 ± 0.2	1.2 ± 0.2

DISCUSSION

The presence of a hydrophobic binding site on the enzyme

The kinetic scheme previously given[1] adequately describes the experimental results obtained with various alcohols. In particular, it accounts for the observation that the apparent reactivity of the alcohol depends on both its intrinsic nucleophilic reactivity, k_4^*, and its affinity for a particular site on the acyl enzyme, given by K_N.

According to their behaviour in non-enzymatic catalysis[14,15] the first compounds of the aliphatic series CH_3–$(CH_2)_{n-2}$–CH_2OH should exhibit a decrease of k_4* as n increases to 3, reaching a constant value for the higher terms. In enzymatic catalysis this is observed only for the first two alcohols. For the higher alcohols, from propanol on (n > 3), the apparent constant k_4 increases with n. This effect cannot be due to an increase of the intrinsic reactivity k_4*. It is only possible to explain the behaviour of higher alcohols in the enzymatic reaction by a decrease of K_N as n increases. In the following derivation, it is assumed that k_4* is constant for n > 3, just as in non-enzymatic catalysis.

The increasing affinity of the higher alcohols for the enzyme suggests the occurrence of hydrophobic interactions[16] between a section of the hydrocarbon chain of the alcohol and a non-polar site of the enzyme. The ratio k_4/k_3* decreases for the first members of the homologous series and begins to rise only for the higher members. This leads to the conclusion that the non-polar site must be located at a definite distance from the reactive serine and histidine, so that a small alcohol molecule can not interact with it. The data agree best with the assumption that butanol interacts with the enzyme by means of its terminal methyl group only and pentanol, by its terminal ethyl group, as illustrated in Fig. 4. If this is true, then k_4/k_3* can be correlated with the

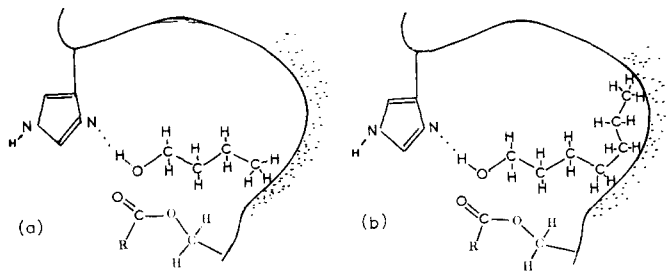


Fig. 4. Schematic representation of the hydrophobic interaction between an apolar site of the enzyme and the aliphatic chain of the alcohols. (a) *n*-butanol: only the terminal methyl group interacts with the enzyme. (b) *n*-heptanol: the terminal butyl group interacts with the enzyme (see text). The dotted area represents a non-polar region on the enzyme.

free energy of hydrophobic bonding between two hydrocarbons by rewriting Eqn. (5):

$$\log \frac{k_4}{k_3^*} = C + \frac{\Delta F^0{}_{H\Phi}}{2.303RT} \tag{14}$$

Where the constant C refers to the reaction of *n*-propanol:

$$C = \log \frac{k_4^*(\mathrm{pr})}{k_3^* K_N(\mathrm{pr})} - \frac{K_W}{[W]} \tag{15}$$

In Eqn. (14) it is assumed that the free energy of dissociation of butanol and of higher homologs from the enzyme is composed of two additive contributions, one corresponding to the free energy of dissociation of *n*-propanol, $\Delta F^0{}_N$ (pr), the other to the breaking of the additional hydrophobic bond, as described above, and in Eqn. (16):

$$\begin{aligned} -RT \log K_N(\mathrm{n}) &= \Delta F^0_N(\mathrm{n}) = \Delta F^0_N(\mathrm{pr}) - \Delta F^0_{H\Phi}(\mathrm{n}) \\ \Delta F^0_N(\mathrm{n}) &= RT \log K_N(\mathrm{pr}) - \Delta F^0_{H\Phi}(\mathrm{n}) \end{aligned} \tag{16}$$

TABLE III

COMPARISON OF THE FREE ENERGY OF HYDROPHOBIC INTERACTIONS BETWEEN ALIPHATIC ALCOHOLS AND ACYL TRYPSIN I, II AND III AS DETERMINED IN THIS WORK WITH THEORETICAL VALUES FOR THE INTERACTION BETWEEN TWO SIMILAR ALIPHATIC CHAINS, AS GIVEN FOR 25° BY SCHRIER, POTTLE AND SCHERAGA[17]

Alkyl chain	$\Delta F°$ *theoretical (kcal/mole)*	ΔF^0 *present work for acyl trypsin (kcal/mole)*			$\frac{\Delta F^0_{exp.}}{\Delta F^0_{theor.}}$		
		I	*II*	*III*	*I*	*II*	*III*
CH_3—	−0.70	0.835	0.835	0.685	1.19	1.19	0.98
C_2H_5—	−0.90	1.12	1.03	1.04	1.25	1.15	1.16
nC_3H_7—	−1.20	1.52	1.50	1.23	1.27	1.25	1.03
nC_4H_{10}—	−1.50	1.90	—	1.56	1.27	—	1.04
			means ± S.D.		1.25 ± 0.05	1.20 ± 0.07	1.05 ± 0.09

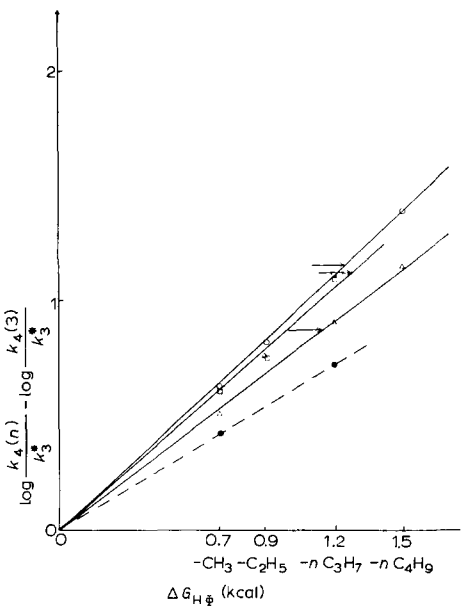


Fig. 5. Tryptic hydrolysis. Linear relationship between log $k_4(n)/k_3^*$ − log $k_4(pr)/k_3^*$ for aliphatic alcohols (n > 3) and the free energy of hydrophobic interaction between a pair of corresponding hydrocarbon chains, as explained in the text. The arrows indicate the ordinate value for benzyl alcohol. The free energies listed along the abcissa are theoretical values, cited from SCHRIER, POTTLE AND SCHERAGA[17]. ○, □, △ = data for lysyl, arginyl and *N*-carbobenzoxy-lysyl trypsin respectively (Table I). ● = data for furoyl chymotrypsin according to INWARD AND JENCKS[18].

The indices (pr) and (n) refer to *n*-propanol and to a higher homolog, respectively.

Theoretical values of $\Delta F^0_{H\Phi}$, the free energy of formation of a hydrophobic bond between two unbranched hydrocarbon chains, have been taken from the work of SCHRIER, POTTLE AND SCHERAGA[17]. They are listed in Table III and along the abscissa of Fig. 5.

$$\text{plotting } \log \frac{k_4(n)}{k_3^*} - \log \frac{k_4(pr)}{k_3^*}$$

against $\Delta F^0_{H\Phi}$ for the three acyl trypsins (I, II, III), a satisfactory linear correlation is obtained (Fig. 5). According to Eqn. (14), the slope of the straight line in Fig. 5 should equal $(2.303\ RT)^{-1}$, or 0.733 mole/kcal at 25°. The slopes actually observed for acyl trypsin I and II are 0.93 ± 0.02 (S.D.) and 0.90 ± 0.04 (S.D.) respectively. Thus it appears for acyl enzymes I and II that all free energy values used on the abscissa are too low by about 20% (see Table III). This apparent discrepancy can be rationalized easily if the non-polar site of the enzyme is not the equivalent of a linear hydrocarbon chain, but is more extensive. The interaction with the alcohol therefore results in a stronger hydrophobic bond[16] than that between two isolated hydrocarbon chains[17]. With α-*N*-carbobenzoxy-L-lysyl trypsin (II, Table I) a linear relationship is also obtained (Fig. 5). However, the slope of the straight line (0.76 ± 0.04) in this case is nearly equal to the theoretical slope. The difference of the acyl trypsins I and II on one hand, and of the acyl trypsin III on the other, could arise for two different reasons:

(a) If a solvent effect, modifying the K_S value, is added to the nucleophilic action of the alcohol, higher values of k_4/k_3* ratio could be expected for the acyl trypsin I and II, taking into account of the method used. The solvent effect modifying only the K_S value is not observed in the case of acyl trypsin III when the observed resulting values are smaller. Such an interpretation is very unlikely because the effect of dimethylformamide and isopropanol on the k_2/K_S ratio is quite negligible in the concentration range of the experiments.

(b) The active centre conformation of acyl trypsins I and II on the one hand, and of acyl trypsin III on the other hand, could be different with respect to their hydrophobic sites. The conformational differences could result in significant variations in the interaction energy between acyl enzyme and alcohol. Such variations could be related to the steric hindrance of the acyl radical of the substrate which would be quite similar for arginyl and lysyl trypsin but more important for carbobenzoxy-lysyl trypsin. The similar values of $\Delta F^0_{H\Phi}$ for acyl trypsin I and II (see Table III) are related to their similar kinetic properties (see footnote on page 150).

Similar results have been obtained by INWARD AND JENCKS[18] with furoyl chymotrypsin (EC 3.4.4.5) (see Fig. 5) and by MAC DONALD AND BALLS[19] with acetyl chymotrypsin.

We may ask now whether such a hydrophobic site also exists in the free enzyme. The transesterification analysed previously[2], *i.e.* lysine ethyl ester going to lysine butyl, pentyl or benzyl ester, are in accordance with the existence of such a site in the free enzyme. The observed increase of the ratio k_2/K_S on going from lysine ethyl ester to higher esters is probably the result of a decreasing K_S, corresponding to stronger hydrophobic interactions in the non-covalent complex. In such a situation competitive inhibition by the higher alcohol of the corresponding ester should be observed. However, a quantitative interpretation of the transesterification data is rather difficult, since the hydrophobic interactions could be different in the free enzyme and the acyl enzyme. The hypothesis might be verified by experiments with the corresponding synthetic esters.

As indicated by the high value of k_4/k_3*, benzyl alcohol also exhibits a strong interaction with the enzyme, although its intrinsic reactivity is probably close to that of methanol. No comparison of the data obtained here with those given by SCHRIER, POTTLE AND SCHERAGA[17] for benzyl alcohol is possible, because in the present model only a part of the benzene ring can participate effectively in the hydrophobic

interaction. Isopropanol reacts only very weakly with the acyl enzyme. This is in accordance with its behaviour in non-enzymatic systems: the methyl substitution on the α-carbon atom increases steric hindrance[12,13]. Such a difference between primary and secondary alcohols permits one to differentiate between a nucleophilic effect and simple competitive inhibition. In the latter case, a change in the reactivity of the alcohol should have little or no influence on the inhibition constant. This has, in fact, been observed for pepsin (EC 3.4.4.1)[20].

In the hydrolysis catalysed by trypsin, the added alcohol acting as a "water analog" not only behaves as a nucleophile, but it acts also as a second substrate of the enzyme, interacting more or less specifically with a particular site on the enzyme.

The interaction between unreactive organic solvents and the alcohol in the deacylation step

The experiments carried out in presence of dimethylformamide or isopropanol are in good agreement with the existence of a hydrophobic site in the enzyme molecule which can interact with alcohols having a chain length of three carbons or more. Therefore there is practically no effect of dimethylformamide or isopropanol on the hydrolysis of L-lysine ethyl ester and L-lysine methyl ester, as predicted by the model. The receptor sites for substrate and for alcohol are independent. The solvent effect on the alcohol binding to the enzyme is closely related to the observations of FINDLAY, MATHIAS AND RABIN[21] for the system ribonuclease (EC 2.7.7.16)—cytidine-2',3'-phosphate. These authors observe a decrease in the methanolyse/hydrolyse *rate ratio* in the presence of dioxane. For ethanol, propanol, butanol and pentanol, a fairly accurate linear correlation is observed between $1/K_Q$ and k_4/k_3*. The variation of K_Q with the number of carbon atoms of the alcohol indicates that K_Q is more complex than a simple dissociation constant.

The results reported in this paper suggest the existence of a hydrophobic binding site in the active centre of the trypsin molecule. This site is different from the site interacting with the non-polar part of trypsin substrates[22]. This interpretation could be related to the work previously reported by HEIDBERG, HOLLER AND HARTMANN[23] showing the existence in the active centre of trypsin of two hydrophobic sites divided by a polar region. One of these subsites could be identified with the hydrophobic site suggested by the present work.

The presence of some binding subsites near the catalytic site of trypsin is probably related to the polypeptidic structure of natural substrates, as it has been shown by SCHECHTER AND BERGER[24] with papain (EC 3.4.4.10). The existence of such subsites in all the intermediary complexes of the reaction pathway could explain the phenomena of non-competitive inhibition[25,26] and activation[26–28] observed during the tryptic hydrolysis of some substrates. A more complete investigation of these phenomena is now underway.

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