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ON THE SUBSTRATE ACTIVATION OF LIVER ESTERASE

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SUMMARY

1. The apparent substrate activation of liver esterase (carboxylic-ester hydrolase, EC 3.1.1.1) has been further investigated with the *n*-butyryl, *n*-valeryl and *n*-caproyl esters of *m*-hydroxybenzoic acid as the substrates. Comparative studies were made of the crude and purified pig liver enzyme and also of the enzymes in liver extracts of various species.

2. Evidence is presented that crude liver extracts of pig, horse, cow, rat, rabbit, pigeon and chicken each contain only one enzyme that hydrolyzes the above substrates at a considerable rate. In each case the enzyme shows substrate activation with one substrate or another, that is, with increasing substrate concentration ([*S*]) the reaction rate increases more rapidly than is consistent with the Michaelis–Menten formulation.

3. At low [*S*], where the substrate activation does not manifest itself, the enzymes of all these species show similar specificities. For each enzyme the rate increases nearly exponentially with increasing acyl C-chain length of the substrate. However, at high [*S*] where substrate activation occurs, the specificities differ (particularly for the case of the pig liver enzyme), indicating that substrate activation is species dependent.

4. When the pig liver enzyme was purified, there was little or no effect on its kinetic behavior, confirming that the latter is associated with a single enzyme and that possible endogenous modifiers do not affect the substrate activation. These observations suggest that substrate activation is solely a manifestation of the structure of the enzyme protein.

5. The similar substrate specificities of these enzymes at low [*S*] suggest that the structures of their active centers, which determine the primary specificity and the reaction rate in this range of [*S*], are the same or similar. The more pronounced species differences at high [*S*] could mean that the structure of the additional substrate binding site (postulated by others to be involved in the substrate activation) differs by species, particularly for the case of the pig liver enzyme.

INTRODUCTION

The effect of substrate concentration ([*S*]) on the activity (v) of liver esterase

(carboxylic-ester hydrolase, EC 3.1.1.1) preparations of various sources often does not comply with the Michaelis formulation of enzyme action (see HOFSTEE[1] for a summary of the earlier literature). It has since been found[2–6] that this type of "anomalous" kinetic behavior is also manifested by pig liver esterase preparations apparently consisting of a single protein, which would confirm the conclusion[7] that the phenomenon, referred to here as "substrate activation", is related to a property of a definite enzyme species and is not the result of two or more enzymes simultaneously acting on the substrate. Evidence has been presented[6,8] to show that the phenomenon is independent of the strong tendency of the enzyme to form dimers under physiological conditions[5,8], *i.e.* the activation is a property of the monomeric form. STOOPS *et al.*[3] and also BARKER AND JENCKS[6] conclude that activation is caused by the substrate combining not only with the active site proper but also with an activator site present on the monomer (see also LEVY AND OCKEN[4]). However, more recent results with the pig liver enzyme suggest the possible presence of more than one enzyme species having similar physical but different kinetic properties (ref. 9 and W. P. JENCKS, personal communication).

Since substrate activation of a monomeric enzyme would be a rather unique phenomenon, it is of interest to investigate this matter further. The present approach is to compare the kinetic properties of: (1) the pig liver enzyme in different stages of purification and (2) the homologous enzymes from different species. It is shown that with the substrates employed the state of purity of the pig liver enzyme has no significant effect on its kinetic properties, including that of substrate activation. These results would support the contention that only one enzyme is involved and that substrate activation is not affected by endogenous modifiers[6]. It is also shown that the kinetic properties of the pig liver enzyme are atypical as compared to those of the homologous enzymes of 6 other species tested. Additional evidence is presented that substrate activation is connected with the monomeric form of the enzyme.

MATERIALS AND METHODS

Substrates

The substrates, *n*-fatty acid esters of *m*-hydroxybenzoic acid with varying acyl C-chain length and referred to as the C_4, C_5 esters *etc.*, were prepared as described previously[10]. They were converted to their soluble sodium salts through neutralization in ethyl alcohol by 50% NaOH and following the addition of several volumes of ether, were crystallized at $-15°$.

Enzymes

Liver acetone powders were either obtained commercially or were freshly prepared according to the method of HORGAN *et al.*[11]. Crude 10% aqueous extracts of these powders were used. Purification of pig liver esterase from the freshly prepared acetone powder was carried out by the method of HORGAN *et al.*[11] but with omission of the last step (chromatography on CM-Sephadex). Isoelectric focusing on polyacrylamide gels, carried out essentially by the method of CATSIMPOOLAS[12], showed only one major and one minor component, both with a p*I* near 5.0, which corresponds to the isoelectric point reported for this enzyme[2,5,13].

Activity determination

As described previously[10,14], initial rates of hydrolysis were determined spectrophotometrically in a Gilford spectrophotometer at substrate concentrations below the critical concentration of micelle formation[15]. For this purpose a 7-compartment cuvette (courtesy Pyrocell Company, New York) was used. Unless noted otherwise, the determinations were carried out at 30° in 0.01 M Tris–HCl buffer, pH 8.

Identification of the enzyme(s) in impure preparations by molecular size

For this purpose the preparations were eluted from a cooled (about 4°) Sephadex G-100 column. The eluate was run at room temperature through a mixing vessel having 3 outlets, through one of which an 0.05–0.1 M solution of either the *n*-caproyl or *n*-heptoyl ester of *m*-hydroxybenzoic acid was injected by means of a peristaltic pump. The rate of injection was about one tenth the flow rate of the column. The eluate–substrate mixture, which was magnetically stirred in the mixing vessel, was run through a flow-through cell in a Unicam double-beam recording spectrophotometer and the absorbance at 300–305 nm was recorded continuously. Increase in the absorbance of the eluate–substrate mixture, as compared to the absorbance during a blank run (in the absence of substrate), indicated the presence of enzyme. In the absence of substrate, absorbance of the eluate was usually negligible, at least at volumes greater than the void volume of the column.

RESULTS

Effect of substrate C-chain length on activity and substrate activation of pig liver esterase

Fig. 1A shows that nearly identical results were obtained with the purified enzyme and with the original crude extract of the freshly prepared acetone powder. These data are also very similar to those obtained with an extract of a commercial powder (Fig. 1B). The absence of an effect of purification and of the source of the enzyme is evidence against the involvement of two or more enzymes of different physical properties. Furthermore, chromatography of the crude extract on Sephadex G-100 failed to show any enzyme species of a molecular size other than that present in the purified preparation of the enzyme (see below). Therefore, these results support the contention that in each case one and the same enzyme is involved in the hydrolysis of the applied substrates.

Substrate activation is indicated by the discontinuity of the v *versus* $v/[S]$ plots for the C_5 and C_6 esters (Fig. 1). At low $[S]$ the relative rates of hydrolysis of the three substrates (*i.e.* the substrate specificity), are similar to those previously reported[10] for the horse liver enzyme. On the other hand, in the high $[S]$ region the specificities of the two enzymes are entirely different (see also below).

Results by others suggest that substrate activation, *i.e.* the increase in rate at high $[S]$, may depend on the presence of endogenous non-protein material in the enzyme preparations and even on the procedure of purification (see BARKER AND JENCKS[5]). However, as can be seen from the data in Fig. 1, under the present conditions purification of the enzyme has little or no effect on the relative rates of hydrolysis at high and low $[S]$ (see also ADLER AND KISTIAKOWSKY[2] and LEVY AND OCKEN[4]). Also, there is no significant effect on the relative rates of hydrolysis of the three substrates. Thus these results indicate that the phenomenon of substrate activation

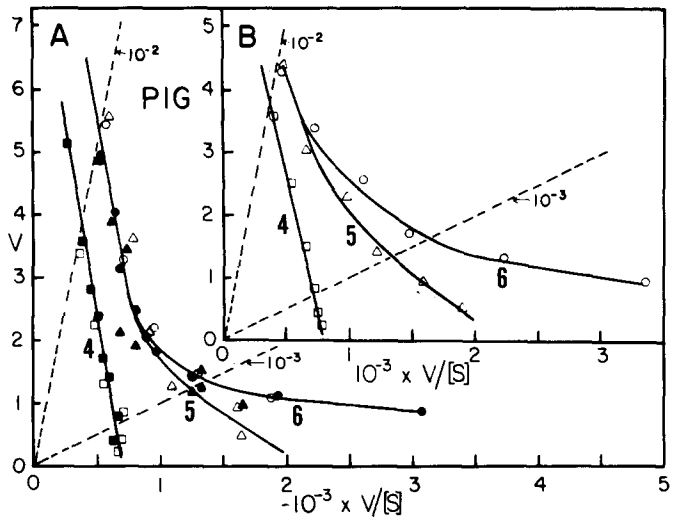

Fig. 1. Effect of concentrations ([S]) of substrate (n-fatty acid esters of m-hydroxybenzoic acid) and of substrate acyl C-chain length (4, 5 and 6 C atoms as indicated) on the activity (v) of pig liver esterase. (A) Purified pig liver esterase (solid characters), as compared with the original crude extract of freshly prepared acetone powder (open characters). (B) Same as A but with a crude extract from a commercial pig liver acetone powder as the enzyme source.

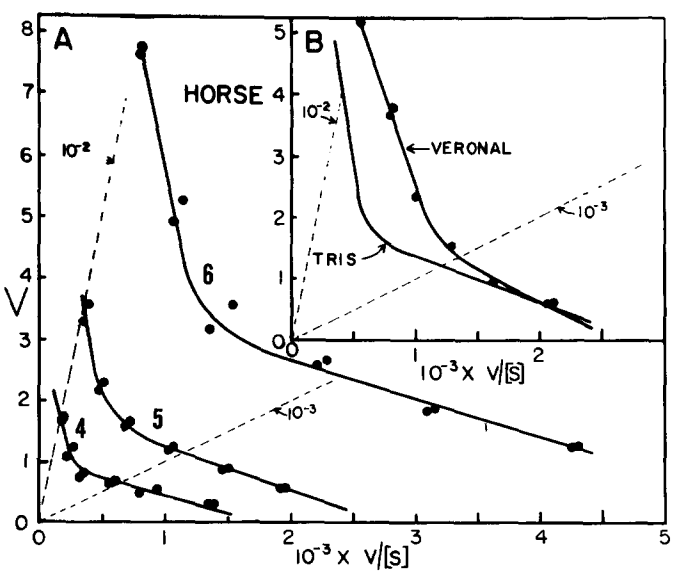

Fig. 2. Effect of substrate concentration ([S]) and of substrate n-acyl C-chain length (4, 5 or 6 C atoms) on the activities (v) of a crude aqueous extract of freshly prepared horse liver acetone powder. (A) The buffer used (0.01 M Tris–HCl, pH 8) and other conditions were the same as for Fig. 1. (B) Effect of the replacement of 0.01 M Tris–HCl by 0.0375 M veronal buffer (as used previously[10]) on the v *versus* $v/[S]$ curve of the caproyl ester.

as well as the specificity of the enzyme are not affected by endogenous dissociable modifiers. Such modifiers presumably would have been largely removed during the purification. The results would rather indicate that the unusual kinetic properties of the pig liver enzyme (see below) are expressions of its protein structure, the catalytic function of which is not affected by the applied purification procedure.

Differences in substrate activation and in specificity of the enzymes from horse and from pig liver

The *v versus* $v/[S]$ plots of Fig. 2 were obtained with a crude extract of freshly prepared horse liver acetone powder as the enzyme source. At least at high $[S]$, these results are entirely different from those obtained under identical conditions with either the pure or the crude pig enzyme as shown in Fig. 1. The major differences are: (1) the pig enzyme, in sharp contrast to the horse enzyme does not show substrate activation with the butyryl ester, at least not under the experimental conditions and (2) in particular at high $[S]$, the two enzymes exhibit different relative reaction rates with the various substrates, *i.e.* the specificity of the two enzymes differs. For the horse enzyme, the rate at all substrate concentrations increases by a factor of 2–3 for each additional methylene group in the acyl moiety of the substrate. In contrast, the increase in the rate for the pig enzyme at high $[S]$ is much smaller or even absent. However, the curves for the C_6 substrate are similar for the two enzymes.

With respect to the effect of the substrate C-chain length on the rate of hydrolysis, the data for the horse enzyme (Fig. 2) agree with those previously obtained with a partially purified preparation of this enzyme[10]. That is, as in the case of the pig enzyme, the relative rate of reaction of the horse liver enzyme with different substrates (the specificity) apparently is not affected by purification. On the other hand, the relative rate at high and low concentrations of a particular substrate (*i.e.* the extent of substrate activation) may depend on the composition of the reaction mixture as is shown in Fig. 2B for different buffers.

A decrease in the extent of substrate activation by acetone[6], benzene[3] and even serum albumin (J. P. Jencks, personal communication) has been noted by others. Such effects are consistent with the assumption that more than one enzyme site is involved[6]. Usually the additives are effective only in concentrations very much higher than those of the endogenous contaminants present in the small amount of crude enzyme which is required for an activity determination.

The data of Fig. 3 indicate that, under the applied conditions, the elution volume of the major component of the pig liver enzyme is larger than for the other species. The elution volumes from the calibrated column are consistent with the assumption that the pig enzyme consists mostly of the dimeric form whereas the horse liver enzyme occurs predominantly in the monomeric form. However, this difference in association cannot account for the difference in specificity of the two enzymes. With the monomeric fraction of the pig enzyme (chromatographically isolated and assayed at high ionic strength in order to promote dissociation[5]), the relative hydrolysis rates with 0.01 M concentrations of the C_4, C_5 and C_6 esters were similar to those of the dimeric enzyme (Fig. 1). Thus the unusual substrate specificity of the pig enzyme appears to be a property of the monomer.

Since the above results indicate that the observed differences in species specificity do not depend on the presence of modifying components in the crude prepara-

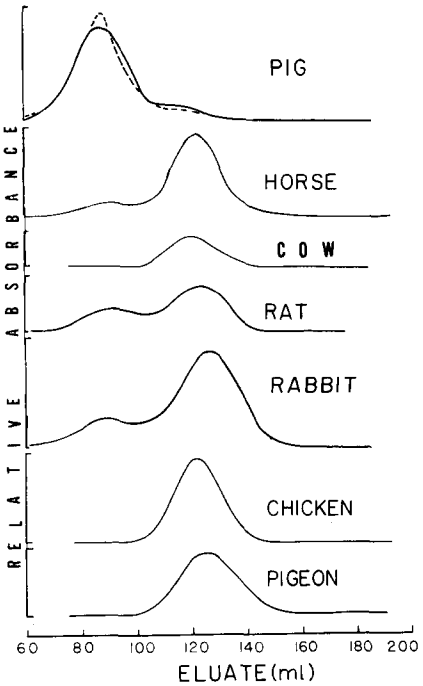

Fig. 3. Identification of esterases from various species by chromatography on a Sephadex G-100 column (2.5 cm × 40 cm). The substrate was continuously injected into the eluate (see MATERIALS AND METHODS). The curves represent relative light absorbances of the eluate–substrate mixtures, monitored continuously at a wave length in the 300–305-nm region. The solid curves refer to crude aqueous extracts of acetone powders and the dashed curve to the purified pig enzyme. The acetone powders of the pig and horse livers were freshly prepared whereas the others were obtained commercially. The column had a void volume of about 75 ml and was equilibrated and eluted with 5 mM Tris–HCl buffer (pH 8) containing 0.2 M KCl. The flow rate was 25–30 ml/h.

tion and cannot be ascribed to the difference in the relative amounts of monomer and dimer present, it would seem more probable that the difference in specificity is directly related to differences in enzyme structure (see DISCUSSION).

Comparison with the liver esterases from additional species

Since for the pig and horse enzymes discussed above, the kinetic results with the crude and the pure enzyme are not significantly different, suggesting that only one enzyme is involved in the hydrolysis of the present substrates, one might expect this to also be the case with crude liver esterase preparations from other species. In fact, the gel filtration patterns of crude aqueous extracts from the acetone powders of the livers of pig, horse, cow (and calf), rat, rabbit, pigeon and chicken (see Fig. 3), fail to reveal the presence of any enzyme in addition to the one in the purified preparation of pig liver esterase. Except for the pig liver enzyme, which under the experimental conditions occurs predominantly in the dimeric form (see also BARKER AND JENCKS[5]), the enzymes from all the other species appear predominantly monomeric. The greater tendency of the chicken enzyme to occur in the monomeric state has previously been noted by others (see AUGUSTEYN *et al.*[16]). That the esterases (with the

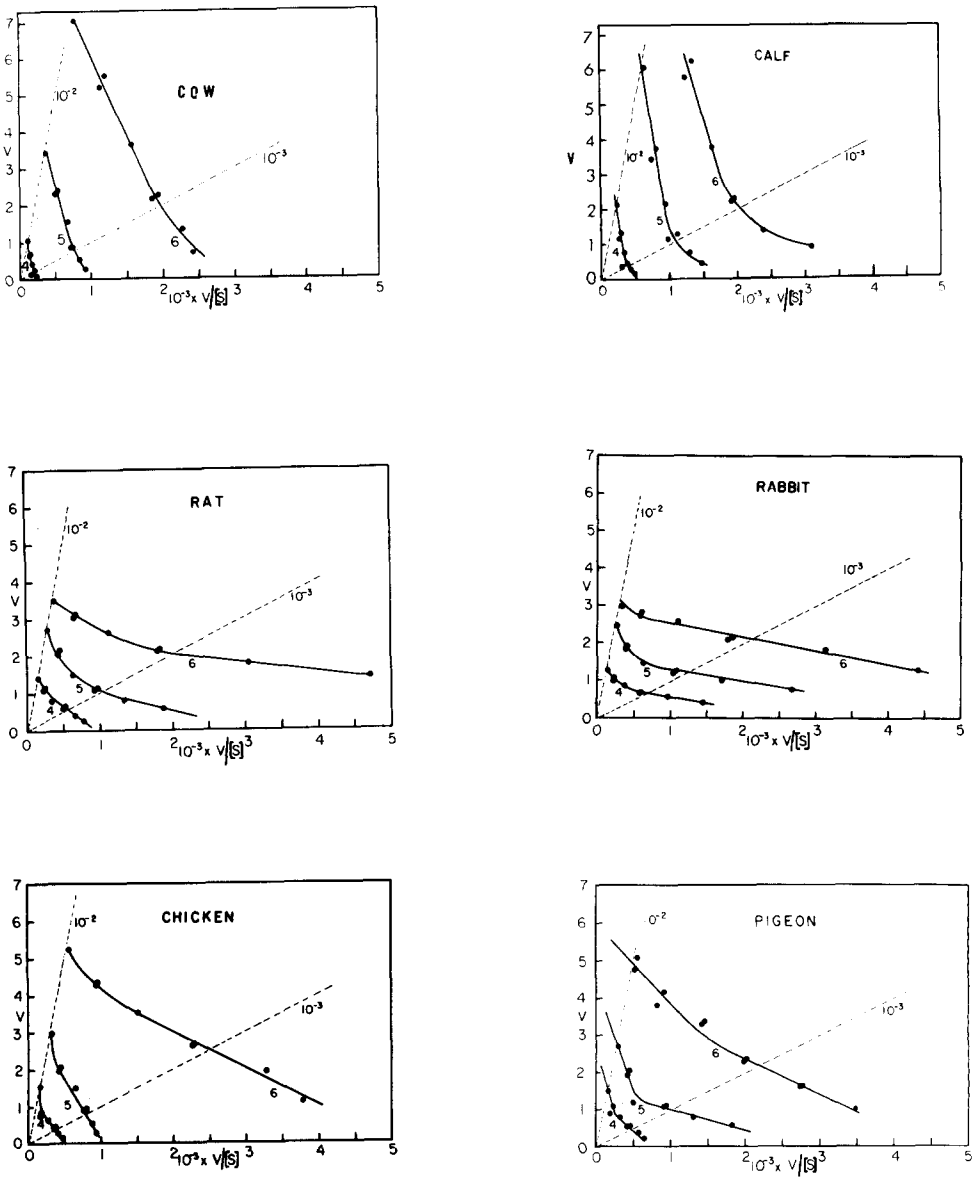

Fig. 4. Effect of the concentration [S] and of the n-acyl C-chain length of the C_4, C_5 and C_6 ester substrates on their rates of hydrolysis (v) by crude aqueous extracts of commercial liver acetone powders of various species. The conditions were the same as those for the data of Fig. 1.

exception of the pig enzyme) occur predominantly or entirely in the monomeric state (Fig. 3), but all show substrate activation (Figs. 1, 2 and 4) confirms the conclusion by BARKER AND JENCKS[6] that substrate activation is not dependent on the formation of dimers.

In view of the strong kinetic resemblance of the enzymes of all of these species, the specific dissimilarities (see Fig. 4), might indicate slightly different homologues

of the enzyme in each case. That the rate patterns obtained are characteristic for a particular species is shown, for instance, by the great similarity of the *v versus* $v/[S]$ curves for the cow and calf enzymes. In many cases the effect of the substrate C-chain length is solely or predominantly on v_{max} (intercept of the *v versus* $v/[S]$ plot with the v coordinate). This is observed (Fig. 4) for the enzymes from pig and cow at high $[S]$, for the rabbit enzyme at low $[S]$ and for the horse enzyme at both regions of $[S]$. On the other hand, for the rat, pigeon and chicken enzymes in the low $[S]$ region, the effect of the C-chain length also results in a change in K_m (slope of the plot), usually involving a decrease in this constant with increasing C-chain length. Since it has not been established that K_m is a dissociation constant, a change here does not necessarily denote a change in the enzyme–substrate affinity. On the other hand, the apparent absence of a change in K_m with increasing C-chain length, as in the case of the horse liver enzyme (Fig. 2), indicates that in this case K_m is an equilibrium constant (see HOFSTEE[17]).

The differences in the shapes of the curves for different species are largely due to the transition from the low into the high $[S]$ kinetic system occurring in a different range of $[S]$. As a consequence, the data for the chicken enzyme, for example, are predominantly in the low $[S]$ system, whereas those of the cow and the calf enzymes mostly pertain to the high $[S]$ system. In order to make valid comparisons it is therefore necessary to establish which kinetic system applies within a given substrate range (see also BARKER AND JENCKS[6]).

Comparison of the enzymes of all these various species emphasizes the above noted exceptional kinetic behavior of the pig enzyme with respect to the applied substrates. For $[S] \rightarrow 0$ this behavior apparently is not unlike that of the other enzymes. However, for the high $[S]$ system, the specificity of the pig enzyme is unusual since it is the only enzyme that hydrolyzes the C_5 and C_6 esters at about the same rate. Furthermore, relative to its C_5 and C_6 homologues, the C_4 ester is also hydrolyzed much faster than is usually the case.

DISCUSSION

Although evidence presented by others[9] indicates that two types of active site are involved in the activity of the pig liver enzyme, it has not been established as yet whether the different types of site are located on the same or on different enzyme molecules. The present results support the former supposition. Furthermore, as is also indicated by the present results, the kinetic behavior of the pig enzyme is atypical and may be connected with a particular property that is not common to all of these homologous enzymes. Thus, it would be of interest to make detailed studies of an enzyme, such as the one of horse liver, which shows more typical kinetic behavior than is the case with the pig enzyme.

On the basis of the assumption that substrate activation is connected with the monomeric form of a single enzyme which is endowed with an activator site situated outside the active center proper, the reaction rates for $[S] \rightarrow 0$ would be determined primarily by interaction of the substrate with this active center whereas the high $[S]$ kinetic system would additionally involve the activator site. Thus, for all the enzymes, the similarity of the specificities at low $[S]$ would suggest that the structures of the active centers proper and of the possible sub-sites therein (see KOSHLAND AND NEET[18])

would be the same or similar, whereas there would be more variation in the activator sites of the enzymes. This latter variation would result in a more variable specificity at high [S]. On this basis one may speculate from the present results that, of the enzymes tested, the structure around the activator site of the pig enzyme is the most atypical. The atypical kinetic behavior of this enzyme as well as its greater tendency to dimerize could both be manifestations of such an unusual structure. The isolation from the purified pig and horse enzymes of identical octapeptides which contain the active serine residues[16], would merely indicate that the differences in kinetic behavior and in dimerization are not related to that particular section of the molecule.

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