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STUDIES OF SUBSTRATE HYDROLYSIS BY AN ESTERASE ISOZYME OF RAT SERUM

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SUMMARY

1. Studies of substrate hydrolysis have been carried out using a homologous series of *n*-alkyl aliphatic esters of α-naphthol on carboxylesterases (carboxylic-ester hydrolase, EC 3.1.1.1) of rat serum and on an esterase isozyme purified from the latter.

2. The results indicate that at identical substrate concentration the velocity of ester hydrolysis rises progressively with up to C_8 substrates in the case of whole serum and up to C_9 substrates in the case of the isozyme.

3. At increasing substrate concentrations, the kinetic behaviour of whole serum differs strikingly from that of its purified isozyme. While the esterases of whole serum observe the Michaelis–Menten kinetics, the isozyme exhibits a faster rise in the velocity of ester hydrolysis, particularly at higher substrate concentrations.

4. The probable underlying reasons are discussed in the light of available studies on substrate activation and subunit structure of carboxylesterases.

INTRODUCTION

Several kinetic studies are now available on the enzymic hydrolysis of esters and amides by purified preparations of carboxylesterases (carboxylic-ester hydrolase, EC 3.1.1.1). Deviation from the Michaelis–Menten kinetics, $v = V$ [ester]/(K_m + [ester]), has been reported by several workers [1–5]. A disproportionately faster rise in the rate of ester hydrolysis (v) has been observed to accompany a proportionate increase in substrate concentration [S]. Such anomalous kinetic behaviour, attributed by some to substrate activation, has been further demonstrated to be independent of the degree of purity of the enzyme preparation [1, 3, 4].

Most of the available data, however, concern liver esterases of different mammalian species and relatively little is known about the kinetic behaviour of serum carboxylesterases. The present study was, therefore, undertaken to obtain necessary correlates on rat serum esterases. Kinetic studies were performed on whole serum as well as on its most conspicuous isozyme after isolation and purification. As will be seen, the findings indicate that the kinetic behaviour of whole serum differs strikingly

from that of its purified isozyme. The significance of the present results is discussed in the light of available studies.

MATERIALS AND METHODS

Substrates

Commercially available α-naphthyl esters of *n*-alkyl aliphatic acids (analytical grade, obtained from Koch–Light Laboratories) with increasing carbon chain length from C_2 to C_{10} were employed. All substrate solutions were prepared in acetone.

Enzyme

(A) Whole serum. Blood, obtained from male Wistar rats by direct cardiac puncture, was allowed to clot overnight at 4 °C. Serum was separated from the latter by low speed centrifugation (5000 $\times$ *g* for 30 min).

(B) Isozyme. Starch gel electrophoresis of serum indicates that the most conspicuous and major esterase variant migrates to the terminal anodal region. This particular isozyme was isolated and purified from serum by successive chromatography on Sephadex G-100 and QAE A-50 (Sephadex). The detailed procedures have been outlined elsewhere [6]. Electrophoresis on analytical polyacrylamide gels showed a single component when stained for either enzyme or protein activity. Homogeneity of the isozyme was further established from immunodiffusion and immunoelectrophoretic analysis (Choudhury and McLean, unpublished observations).

Determination of enzyme activity

To determine the effects of increasing acyl carbon chain length of substrates, a simultaneous coupling technique, as described previously [7], was performed in spectrophotometer cuvettes with all substrates at identical concentration (0.00347 M). An 8-compartment cell holder with thermostat was used for this purpose and the initial reaction velocities with four different substrates at any one time were recorded simultaneously on a chart recorder using an automatic programme controller coupled to a spectrophotometer (Pye Unicam SP 1800).

For studying the effects of increasing substrate concentration on reaction velocities, a post-coupling technique [7] was employed. The capture of enzymatically liberated α-naphthol was effected with diazonium salt (fast blue B, 1 mg/ml) in 0.1 M phosphate buffer at pH 7.3. Velocity of ester hydrolysis in each case was determined from spectrophotometric measurements of optical density of the coloured diazotate at 560 nm.

RESULTS

Effect of acyl carbon chain length at fixed substrate concentration

At identical substrate concentration, the velocity of ester hydrolysis, in general, was seen to rise progressively with up to C_8 substrates in the case of whole serum and up to C_9 substrates in the case of the isozyme. Further lengthening of the acyl chain was accompanied by a decline in velocity. Whole serum, however, exhibited certain irregularities, particularly with the lower substrates. Thus, V for C_3 was higher than that for C_4 and was about the same as C_5. Values for the C_4 and C_2 substrates were

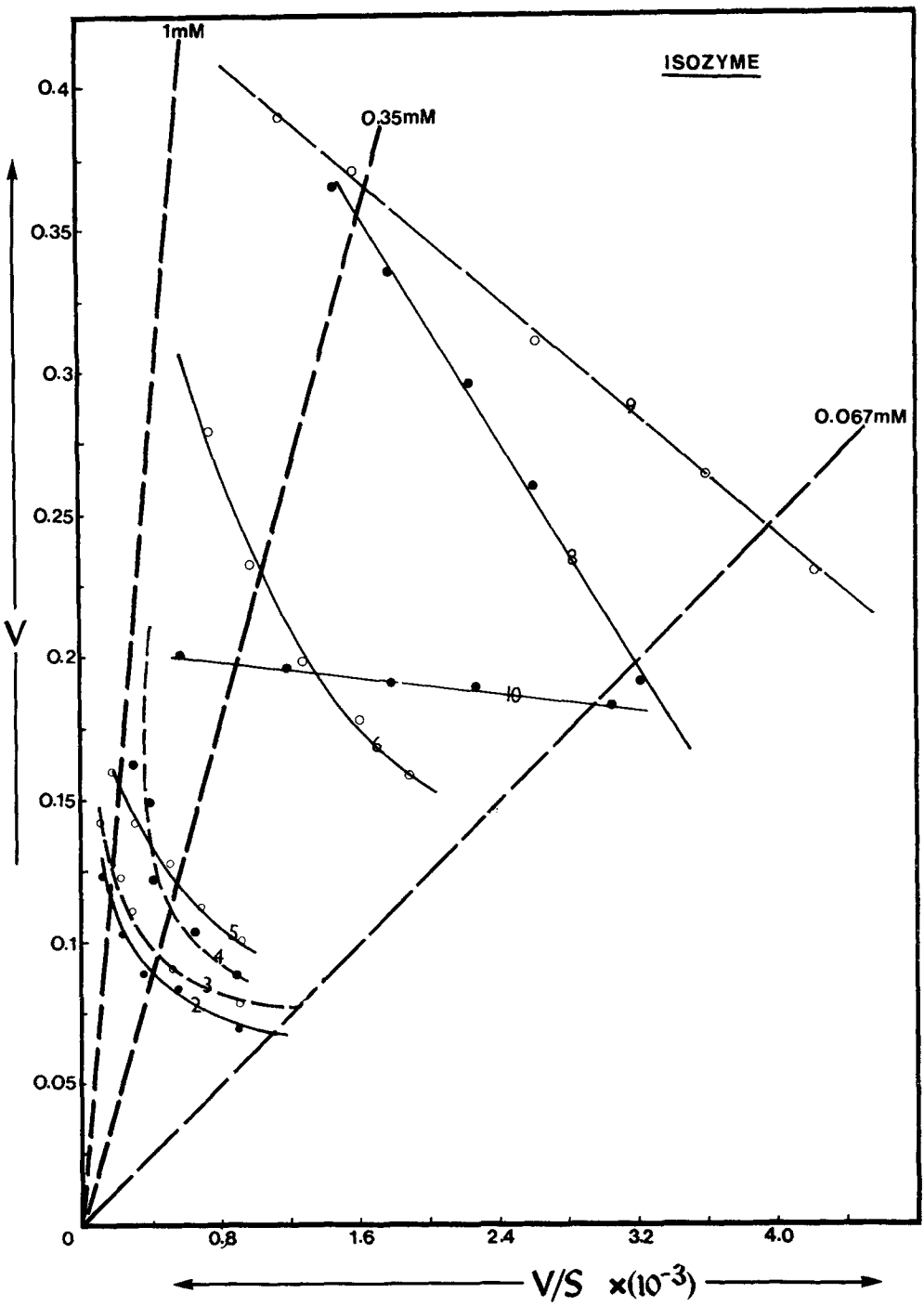

Fig. 1. v versus $v/[S]$ plots of isozyme catalysed hydrolysis of a series of α-naphthyl esters of n-alkyl aliphatic acids of increasing acyl carbon chain. The numericals on the plots correspond to the substrates bearing the same number of carbon atoms in the acyl chain. Velocity of hydrolysis exhibits a marked acceleration particularly at high $[S]$ with up to C_6 substrates. Further elongation of acyl carbon chain restores linearity in profiles at all substrate concentrations.

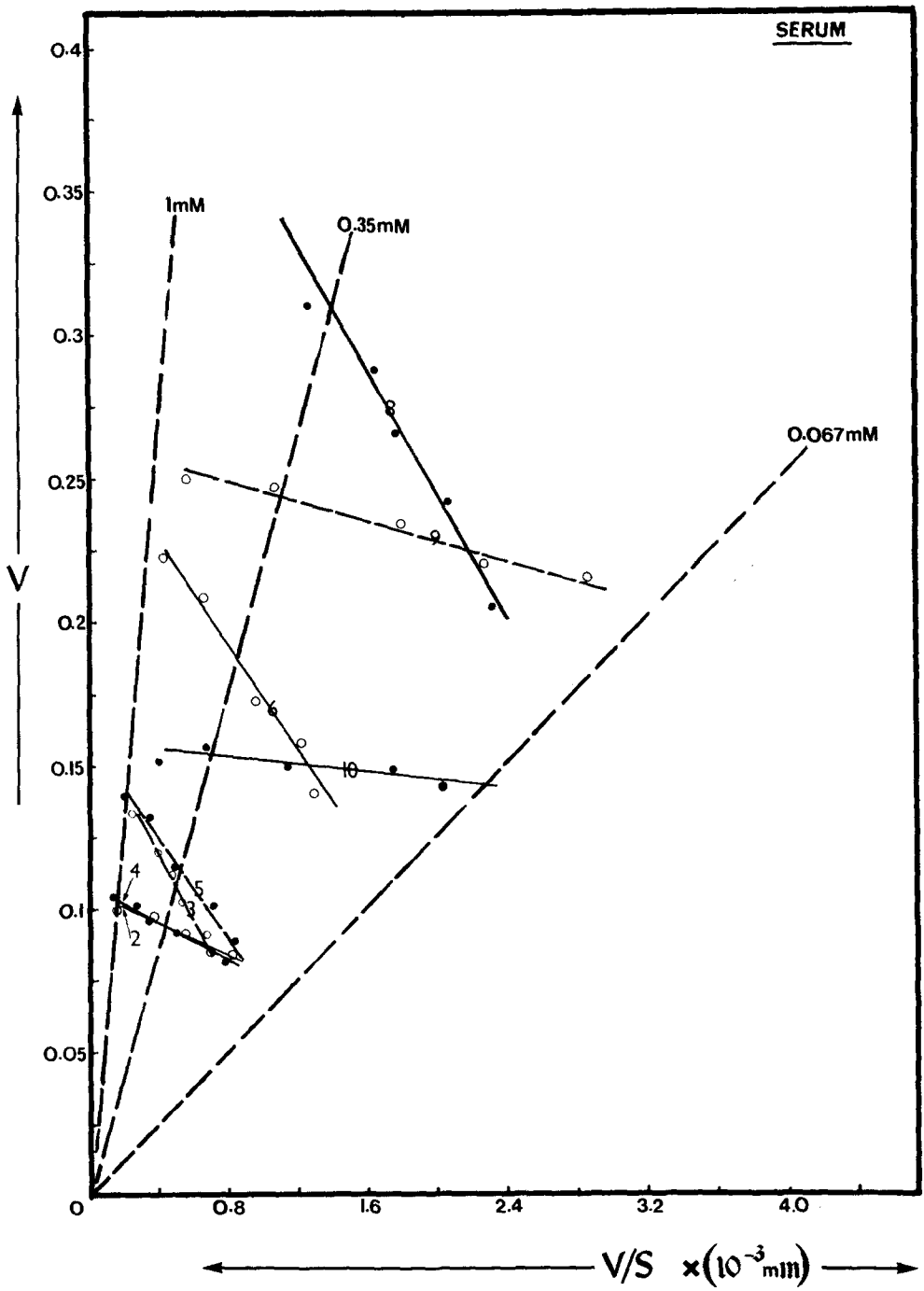

Fig. 2. v versus $v/[S]$ plots of hydrolysis of the same series of α-naphthyl esters (as in Fig. 1) by carboxylesterases of whole serum. The profiles are linear at all substrate concentrations.

the same. Less irregularities were noted with the isozyme. However, at higher substrate concentration ($S \rightarrow \infty$), V for C_4 and C_8 substrates were found to be higher than those for C_5 and C_9 substrates, respectively.

Effect of increasing substrate concentration

The activity of whole serum was found to be different from that of the purified isozyme over the same range of applied substrate concentration (10-fold). The latter showed marked acceleration in the velocity of ester hydrolysis at higher substrate concentrations. Whole serum, on the other hand, obeyed the Michaelis–Menten kinetics. The results are depicted graphically as v versus $v/[S]$ plots (Figs 1 and 2). It is evident that while the profiles for whole serum exhibit linearity throughout the entire substrate range studied (Fig. 2), those for the purified isozyme are essentially non-linear with up to C_6 substrates (Fig. 1). The steepness of the slopes at higher $[S]$ show a progressive rise with each additional carbon atom in the acyl moiety. With C_8, C_9 and C_{10} substrates, however, linear profiles are observed and their relative K_m values (slopes of the plots) are seen to decline with increasing carbon chain length. In serum catalysed hydrolysis (Fig. 2), the effect of carbon chain length also results in a change in relative K_m of the different substrates and from the slopes it is evident that the K_m values decline successively from that for C_8 through those of C_6, C_3, C_5, C_4, C_2, C_9 and C_{10} substrates.

DISCUSSION

It must be mentioned at the outset that very few reports are available on the use of naphthoic substrates in investigations of ester hydrolysis, although esters of monocyclic aromatic alcohols have been used in previous kinetic studies [3, 5, 8]. The present results indicate that the behaviour of α-naphthyl esters of unsubstituted fatty acids compares favourably with that of the corresponding esters of aliphatic or aromatic alcohols. Thus, an elongation of the acyl carbon chain up to C_8 or C_9 is seen, in general, to result in a progressive increase in the velocity of hydrolysis: a feature also demonstrated by homologous series of *n*-fatty acid esters of aliphatic or aromatic alcohols [5, 10].

The fact that the lengthening of the acyl carbon chain results in a progressive increase in the velocity of ester hydrolysis up to a certain limit, beyond which the latter declines, clearly indicates that the capacity for substrate binding of any given esterase is ultimately limited by the total number of carbon atoms in the acyl chain. Choudhury [11] suggested that esterases are each built on a subunit structure, exhibiting overlapping specificities through sharing of common subunits. The latter, in turn, vary in their capacity to hydrolyse esters of up to an optimal chain length. Such a concept is in agreement with the present observation of overlapping, but finite substrate cleaving abilities of ester hydrolases.

A closer analysis of the kinetic data of the serum catalysed reaction indicates that, unlike that generally observed in the case of the isozyme catalysed hydrolysis, V of the different substrates do not register a sequential change in response to a progressive increase in the carbon chain length of esters. These irregularities, as pointed out earlier, would appear to be due to the presence in the serum of multiple esterase

species having different substrate specificities. Such multiformity would further add to complexities in enzyme kinetics as are probably reflected in the lack of regularity in changes in relative K_m values observed with whole serum. It is interesting to note that although V declines beyond C_8 substrates, the lowest K_m values are observed with C_9 and particularly with C_{10} esters. The latter would thus appear to be the physiologically optimal substrate. However, as Hofstee [3] pointed out that K_m has not been established as a dissociation constant, any change, accordingly, in the K_m value may not necessarily imply a change in enzyme–substrate affinity.

The present studies on reaction velocities at increasing substrate concentrations conclusively demonstrate that the Michaelis–Menten kinetics is observed by the whole serum, but not by the isozyme. The results are in essential disagreement to those reported by Adler and Kistiakowsky [1], Hofstee [3] and Levy and Ocken [4] on the behaviour of liver carboxylesterases. As stated earlier, these workers demonstrated that the kinetic behaviour of liver esterases is independent of the degree of purification and that substrate activation is manifested by the crude and the purified preparations alike. It would appear from the present study that the serum esterases behave differently in this regard.

The underlying reasons for such behaviour are ill understood at present and must remain conjectural until more data become available. Several possible mechanisms, however, which can account for the observed deviations from the Michaelis–Menten kinetics may be considered. The presence of more than one enzyme serving as catalysts has been entertained as a possible causative mechanism [1]. This can be completely ruled out in the present case, since the isozyme is known to be homogeneous, consisting of a single enzymatically reactive protein. Moreover, the kinetics of whole serum, containing multiple esterase species, do not fail to comply with the Michaelis–Menten formulation of enzyme action. The presence of two or more non-identical active sites on the same enzyme molecule, also considered by Adler and Kistiakowsky [1] as another probable mechanism, can be discarded on the basis of the existing knowledge on the active centre of mammalian carboxylesterases [12].

The effects of organic solvents, particularly of acetone, should be seriously considered in the present case, as these chemicals are known to cause enzyme activation [2, 5]. Barker and Jenks [2] demonstrated that purified pig liver esterase is subject to activation up to 2.4-fold by acetone and that the latter increases the maximum velocity in both the low and high substrate concentration regions. In the present investigation, however, the role of acetone as an exogenous modifier appears doubtful, since the departure from normal kinetics is observed in the case of the isozyme alone. Activation by substrate itself, combining with the enzyme at the catalytic site as well as at a postulated activator site [2, 4, 5], clearly emerges as an important causative mechanism. However, it is difficult to understand why such a mechanism should selectively affect the isozyme only, unless endogenous modifiers present in the serum prevent combination of substrates with the activator site. The latter, of course, remains a distinct possibility. As an alternative, it is equally probable that the isozyme provides parallel but interactive pathways for substrate hydrolysis by dissociating into active subunits. The physiochemical processes to which the isozyme is subjected during isolation and purification may facilitate such a breakdown and, in fact, this isozyme is known to undergo dissociation into several active components [13]. In this context it is pertinent to point out that Adler and Kistiakowski [1] also failed to exclude the

possibility of the existence of two or more closely related, but interactive sites on the same enzyme molecule as a probable causative mechanism.

In conclusion, two possibilities exist that can account for the observed deviation from the Michaelis–Menten kinetics. Firstly, the possibility of substrate activation, acting selectively on the isozyme and secondly, that of the existence of two or more closely related but interactive sites on the same enzyme molecule, thus providing parallel but complementary pathways for enzyme mechanisms. In view of the possible subunit structure of esterases [2, 11, 12), the latter appears to offer a better explanation.

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