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THE EFFECT OF SUBSTRATE ANALOGUES ON THE ACTIVITY OF CAT LIVER UROCANASE

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SUMMARY

1. A rapid reproducible method for the isolation of substantially pure urocanase from cat liver has been developed.

2. The enzyme has a molecular weight of 127 000 and is isoelectric at pH 5.4. The K_m for the urocanase–urocanate complex was 7.1 μM. No evidence could be found for a requirement for pyridoxal phosphate. The enzyme was inhibited by NH_2OH but complete activity was restored after dialysis or gel-permeation chromatography.

3. Only *trans*-urocanic acid was a substrate for the enzyme. Imidazolylpropionic acid was an effective inhibitor of urocanase; of other analogues tested only imidazolyllactic acid and α-chloroimidazolylpropionic acid inhibited the enzyme, and these were much less effective.

4. Possible reaction mechanisms for urocanase are considered, and a tentative picture of the urocanic acid binding site on the enzyme is developed. The contributions to the overall binding of different kinds of forces of interactions at various regions of the urocanic acid molecules are examined.

INTRODUCTION

Urocanase catalyses the addition of water to urocanic acid (3-imidazol-4 (or 5)-ylacrylic acid), the second reaction of the main degradative pathway for histidine. Most of the work on this enzyme has been concerned with the unstable product of urocanase activity, now identified as 4(5H)-imidazolone-5-propionic acid[1–5], but isolation procedures and the effects of general enzyme inhibitors have been reported[1,3,4,6–8]. The inhibition of urocanase from bacteria by imidazolylpropionate has also been noted[9,10].

In this paper a short method for the isolation of highly purified urocanase from cat liver is described, together with the results of an investigation into the effects of substrate analogues on the enzyme activity.

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MATERIALS AND METHODS

Preparation of liver homogenates

Freshly killed cats were obtained from local veterinary surgeons. Livers were removed as soon as possible, usually within 1 h of death, cooled in crushed ice and stored in polythene bags at −10° until required.

A frozen liver (90–110 g) was allowed to thaw in cold water, then was cut into small pieces and homogenised for 1 min with ice-cold 50 mM Tris–HCl buffer (pH 7.4). The homogenate, after straining, was centrifuged at 1250 × *g* for 20 min at 4°, and the supernatant solution was filtered through a thick pad of glass wool. After centrifuging at 20 000 × *g* for 1 h at 4°, the clear red supernatant solution was carefully decanted and stored in the refrigerator.

Definition of urocanase activity

A practical unit of urocanase activity was defined as that amount of enzyme which caused a decrease in absorbance at 277 nm of 0.001 units per min of a solution containing 0.1 μmole of urocanic acid in 3 ml phosphate buffer (pH 7.4; *I*, 0.1) in silica cells of 1-cm light path at 30°. One standard unit of activity (in terms of μmoles substrate transformed per min) was found to be equal to 6500 practical units.

The specific activities of enzyme solutions are given as practical activity units per mg protein.

Determination of urocanase activity

A silica cuvette of 1-cm light path, containing 0.5 M phosphate buffer (pH 7.4, 2 ml), enzyme (5–60 practical units) and water to give a total volume of 2.9 ml solution, and a reference cuvette containing buffer, enzyme and water to give a final volume of 3 ml, were incubated in the heated carriage of a Unicam S.P. 500 spectrophotometer at 30° for 15 min. Urocanic acid solution (1 μmole/ml, 0.1 ml) was then added to the test cuvette and the solution mixed with a polythene plunger. Values of absorbance at 277 nm were recorded at 30-sec intervals for a period of 10 min against the reference cell, and the rate of decrease of absorbance was obtained graphically.

Determination of protein concentration

The protein concentration of urocanase preparations was determined by the method of LOWRY *et al.*[11] with serum albumin as a standard.

Determination of Michaelis constant (K_m)

As the urocanase reaction in the normal assay system approaches completion, the absorbance due to imidazolonepropionic acid becomes predominant. By measuring alternately absorbance at the light-absorption maxima of urocanic acid (277 nm) and imidazolone propionic acid (264 nm) the amount of substrate remaining (U) can be calculated from the equation[12]:

$$U = 0.1639\ (A_{277\text{nm}} - 0.8508 \cdot A_{264\text{nm}})\ \text{nmoles/ml}$$

From the corrected curve for urocanic acid disappearance the reaction rate at any time could be obtained graphically. K_m was then determined from a double reciprocal plot of reaction rate against substrate concentration by the method of DIXON[13].

Effects of substrate analogues on urocanase activity

Each activity determination was carried out spectrophotometrically at pH 7.4 at the light-absorption maximum of the compound under examination. 0.1 μmole of potential substrate was incubated in the usual phosphate buffer (pH 7.4, 3.0 ml) with highly purified urocanase (200 practical units) in a silica cuvette of 1-cm light path at 30°. Readings of absorbance were taken at 3-min intervals for 30 min against a reference cuvette containing buffer and enzyme.

Effects of potential inhibitors on urocanase activity

Cuvettes containing highly purified urocanase (30 practical units) in the usual phosphate buffer (pH 7.4, 2.9 ml) were incubated with various amounts of potential inhibitor for 10 min at 30°. Urocanic acid solution (0.1 ml, 1.2 μmole/ml) was then added and the reaction followed at 277 nm against a reference cuvette containing enzyme and inhibitor in buffer (3.0 ml). Reaction rates at a substrate concentration of 33 μM were obtained graphically and compared with that of the noninhibited reaction.

Substrate and substrate analogues

The following imidazolyl compounds were synthesised: *trans*-3-imidazol-4 (or 5)-ylacrylic acid (*trans*-urocanic acid)[14] and its methyl ester[12], *cis*-urocanic acid[12,15], 1-methyl- and 3-methylurocanic acids[12], 2-methylurocanic acid[12], 4 (or 5)-vinylimidazole[16], imidazolylpropionic acid and its methyl ester[12], imidazolylpyruvic acid[17], α-benzoylamino urocanic acid[17], imidazolyllactic acid[18], and imidazolylbutyric acid[19]. Other compounds used included 5-hydantoinpropionic acid[20], 3-pyrrolylpropionic acid[21], furylpropionic acid[22], and thienylpropionic acid[22]. Furylacrylic acid and thienylacrylic acid were purchased from Koch-Light and Co., Colnbrook, and imidazolylacetic acid from Calbiochem, Calif.

RESULTS

Isolation of urocanase

Adsorption onto calcium phosphate gel. The homogenate from one cat liver was diluted with sufficient ice-cold water to give a solution of protein concentration 15 mg/ml, and brought to pH 5.0 with dilute acetic acid. The substantial precipitate was removed by centrifugation at 4°, and calcium phosphate gel[23] was added at a gel/protein ratio of 0.2. After 15 min the solution was centrifuged, the supernatant solution decanted and the packed gel drained by inversion of the centrifuge cups. Three more adsorption stages were carried out at pH 5.0 using the same quantity of gel. In each case adsorbed protein was removed from the gel by eluting twice with 0.1 M phosphate buffer (pH 7.4, 50 ml, then 30 ml) at 4°. Solutions with specific activity greater than 30 were combined.

$(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ (21 g/100 ml solution) was added at 4° with stirring and the solution adjusted to pH 7.0. The precipitate obtained after 30 min was removed by centrifugation and discarded, while the supernatant solution was brought to 50% satn. of $(NH_4)_2SO_4$ with a further quantity of salt (9.0 g/100 ml of solution). After standing for 30 min at 4° the solution was centrifuged at 2500 $\times$ g

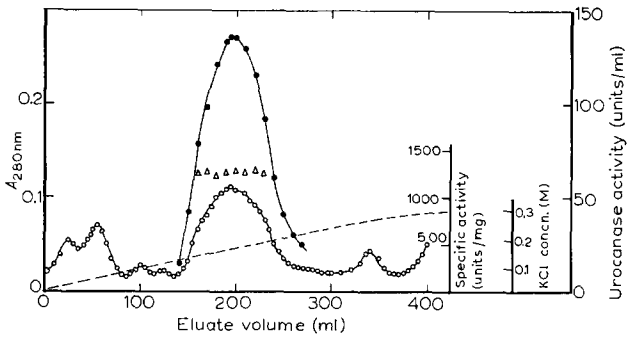

Fig. 1. Chromatography of urocanase on DEAE-cellulose. A solution of urocanase (45 ml; specific activity, 565 units/mg protein) purified by chromatography on Sephadex G-200 was applied to the column of DEAE-cellulose equilibrated with 10 mM Tris–HCl buffer (pH 7.4). The enzyme was eluted with Tris buffer containing increasing amounts of KCl. ○—○, $A_{280\ nm}$ of eluate; △ △ △, specific enzyme activities; ●—●, urocanase activities; — — —, KCl concentration.

for 30 min and the precipitate, containing the bulk of the enzyme, was dissolved in 0.1 M phosphate buffer (pH 7.4, 10 ml).

Chromatography on Sephadex G-200 and DEAE-cellulose. The solution was chromatographed on a column of Sephadex G-200 gel (60 cm × 3.4 cm) equilibrated with 10 mM Tris–HCl buffer (pH 8.0) at 4°, and eluted with this buffer at a flow rate of 40 ml/h. Protein was detected in the column eluate fractions (3 ml) by measuring extinction at 280 nm and urocanase activity (found in Fractions 52–76) assayed by the usual method. Solutions of specific activity over 500 were combined and applied to a column of DEAE-cellulose (25 cm × 1 cm) equilibrated with 10 mM Tris–HCl

TABLE I

ISOLATION OF UROCANASE FROM CAT LIVER

One cat liver (approx. 100 g wet wt.) was used in this experiment.

Stage	*Vol. (ml)*	*Activity (units/ml)*	*Protein (mg/ml)*	*Specific activity (activity units/mg protein)*	*Purification factor*	*Yield (%)*
Supernatant from low-speed centrifugation	260	348	84	4.1	—	(100)
Supernatant from high-speed centrifugation	240	340	46	7.4	1.8	91
Removal of proteins precipitated at pH 5.0	560	133	13	10.2	2.5	83
Adsorption onto calcium phosphate gel and subsequent elution	230	168	3.9	43	10.5	43
Fraction precipitated by $(NH_4)_2SO_4$ (35–50% satn.)	25	1120	6.5	171	43	31
Gel-permeation chromatography on Sephadex G-200	45	395	0.7	565	138	19
Chromatography on DEAE-cellulose	75	51	0.04	1275	310	4
Freeze-dried enzyme dissolved in water	2	1760	1.44	1202	298	—

buffer (pH 7.4). The column was eluted with a further amount of this buffer (50 ml) before urocanase was eluted with Tris buffer containing increasing amounts of KCl. Protein was detected in the column eluate fractions (5 ml) by measuring absorbance at 280 nm and urocanase activity assayed by the usual method (Fig. 1).

After freeze-drying, the urocanase was dissolved in a little water and the solution desalted on a small column of Sephadex G-25 previously equilibrated with distilled water. This solution was also freeze-dried and the material used to prepare standard solutions of urocanase.

The course of a typical purification procedure is shown in Table I.

General properties

The urocanase isolated is substantially pure. In experiments which gave a value for the iso-electric point of urocanase as pH 5.4 by electrophoresis on starch gels, the enzyme migrated as a single protein band over a range of buffers of pH 4.5–8.5. Electrophoresis using a discontinuous Tris–citrate–borate buffer system[24] failed to resolve the final preparations into more than one component. In addition, the protein solution eluted from DEAE-cellulose in the final stage of the isolation procedure had a constant specific activity throughout the central zone of urocanase activity.

The molecular weight of urocanase was estimated to be 127 000 by a comparative method[25] using gel-permeation chromatography on Sephadex G-200 columns previously calibrated with standard proteins.

The absorption spectrum of a pure urocanase solution was that of a typical simple protein, having a light-absorption maximum at 279 nm (pH 7.4) with no trace of the light-absorption maximum between 360 and 415 nm previously reported[3] for urocanase preparations. The enzyme-containing fraction from Sephadex G-200 chromatography had a light-absorption maximum at 415 nm, but this was almost certainly due to nonspecific adsorption of liver pigments onto the enzyme. When such fractions were rerun on the column with thyroglobulin, much of the coloured material was transferred to this protein without loss in activity of the urocanase.

The action of NH_2OH on urocanase was studied in some detail in view of the claims of GUPTA AND ROBINSON[8] that the activity of the enzyme could, after dialysis, be restored by incubation with pyridoxal phosphate. The enzyme was strongly inhibited by NH_2OH, but while 20% inhibition was obtained at NH_2OH concentrations as low as 2.5 μM complete inhibition was not observed until concentrations of 0.01 M were used. Such a wide range of inhibitor concentrations makes it most unlikely that NH_2OH inactivates urocanase by simple combination with a cofactor. In contrast to the earlier reports[8], the NH_2OH effect was easily reversed by gel-permeation chromatography or effective dialysis with no loss of enzyme activity. In addition, prolonged dialysis against 0.5 M Tris–HCl buffer[26] or isonicotinyl hydrazide, failed to inactivate urocanase. It thus seems unlikely that cat liver urocanase requires pyridoxal phosphate for its catalytic activity.

The effect of substrate concentration on reaction rate

The value for the Michaelis constant (K_m) of the urocanase–urocanate complex at 30° was determined graphically as 7.1 μM (Fig. 2). This may be compared with the value reported by FEINBERG AND GREENBERG[3] of 1.5 μM for beef liver urocanase which was determined at room temperature.

The effect of potential substrates on urocanase

The simplest explanation of imidazolone propionic acid formation from urocanic acid would appear to be 1,4 addition of a proton and a hydroxyl group to the conjugated system of urocanic acid. Neither sorbic acid (CH_3–CH=CH–CH=CH–CO_2H) nor acrylic acid derivatives with five-membered or six-membered aromatic rings were substrates for urocanase. Addition of even a methyl group to the imidazole ring gave inactive compounds. The normal substrate is the *trans* isomeric form of urocanic acid; the *cis* form proved completely inert. Removal of the carboxylate group (giving vinyl imidazole) resulted in a complete loss of activity, as did esterification. Substitution of the α-hydrogen with a benzoylamino group again was sufficient to prevent attack by the enzyme.

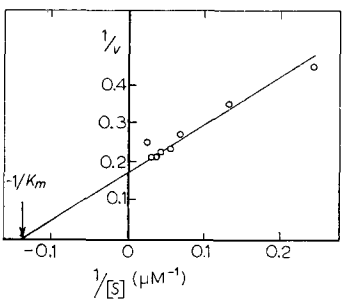

Fig. 2. Determination of Michaelis constant (K_m) for urocanase–urocanate complex. The reaction rate (v) in nmoles urocanic acid used per min was obtained graphically from curves corrected for the presence of imidazolone propionic acid as described in the text. [S] is the urocanic acid concentration.

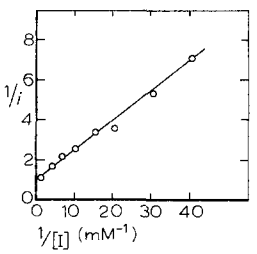

Fig. 3. Determination of inhibition constant (K_i)for imidazolylpropionic acid-urocanase. Cuvettes containing 30 units of highly purified urocanase and various amounts of imidazolylpropionic acid in 50 mM phosphate buffer (pH 7.4, 2.9 ml) were incubated at 30° for 10 min. Urocanic acid solution (0.1 ml, 1.2 μmoles/ml) was then added and the disappearance of the substrate followed spectrophotometrically at 277 nm. Reference cuvettes contained enzyme and imidazolylpropionic acid in buffer (3.0 ml). The reaction rates at a substrate concentration of 33 μM were obtained graphically. K_i was calculated from the slope of the figure, which for pure competitive inhibition[27] is $K_i = (1 + [S]/K_s)$. i = fractional inhibition; $[I]$ = inhibitor concentration.

No inhibition was observed when these unsaturated compounds were present in the spectrophotometric assay system at concentrations comparable to that of substrate. If these compounds do react with the active site, their affinity for this region must be less than that of the substrate.

The effect of imidazole propionic acid on urocanase

In the course of the urocanase reaction, the α, β double bond of the substrate becomes saturated; this bond is unlikely to be vital to the binding on the active site. Thus imidazolylpropionic acid proved to be a very effective inhibitor. The rates of disappearance of absorbance at 277 nm in the presence and absence of imidazolylpropionic acid showed that the degree of inhibition increased as the substrate concentration decreased, suggesting strongly the competitive inhibition to be anticipated from the close similarity between the inhibitor and the natural substrate. From the double reciprocal plots of fractional inhibition against inhibitor concentration at

constant initial substrate concentration[27], a value for K_i of the urocanase–imidazolylpropionate complex of 30 μM was obtained (Fig. 3). The ratio K_i/K_m is therefore of the order of 5.

In a short note HASSALL AND RABIE[10] found that urocanase from certain bacteria was inhibited by imidazolylpropionic acid. They state that this inhibition is competitive with K_i for urocanase–imidazolylpropionate of 0.47 mM. They also found a value of about 5 for the K_i/K_m ratio.

The effect of other substrate analogues on urocanase

Simple saturated aliphatic carboxylic acids such as propionic acid, butyric acid and valeric acid did not inhibit the enzyme at concentrations of 0.01 M, confirming the idea that the cyclic system was necessary for access to the active site. Formiminoglutamic acid formed *in vivo* from imidazolonepropionic acid and suggested as an inhibitor from analysis of metabolites during folic acid deficiencies[28] was also without effect on urocanase. However, the cyclopentane derivative of propionic acid was not active; neither were analogues in which the imidazole ring was replaced by the benzene, furan, thiophene or pyrrole nucleus. 2-Methylimidazolylpropionic acid and 5-hydantoinpropionic acid did not inhibit urocanase, and it appeared that the active site of the enzyme would only bind an unsubstituted imidazole ring. Imidazole itself however, even at concentrations of 0.01 M, was ineffective as an inhibitor, as was the combination of imidazole and propionic acid.

The methyl ester of imidazolylpropionic acid was inactive, confirming that a free carboxylate group was necessary for entry to the active centre. Imidazolylacetic acid and imidazolebutyric acid, even at concentrations as high as 0.01 M, were also without effect.

Interesting results were obtained with α-substituted imidazolylpropionic acids. α-Hydroxyimidazolylpropionic acid (imidazolyllactic acid) and α-chloroimidazolylpropionic acid were found to inhibit urocanase but were much less effective than imidazolylpropionic acid. The values of K_i for these acids (assuming the inhibition to be of the competitive type) obtained from double reciprocal plots of fractional inhibition against inhibitor concentration[27] was approx. 1.5 mM; the ratio K_i/K_m in this case is of the order of 200. α-Aminoimidazolylpropionic acid (histidine) and α-oxoimidazolylpropionic acid (imidazolylpyruvic acid), with side chains having charged groups, were without effect on the enzyme.

DISCUSSION

The urocanase reaction proceeds without oxygen[5] and as there is no evidence for the free occurrence of any intermediate compound between the substrate and imidazolonepropionic acid, the reaction is probably similar to those catalysed by the hydratase group of enzymes[29]. Most of these enzymes catalyse 1,2 addition of a proton and hydroxyl ion across an isolated double bond. In the urocanase reaction 1,4 addition to the conjugated double bond system of urocanic acid can be considered to be effected with the hydroxyl group entering the 4 position of the ring. A reaction mechanism, similar to that put forward for the hydration of α,β-enoyl thioesters[30] may be proposed (Fig. 4); the resulting structure would be expected immediately to rearrange to the enol form of imidazolone propionic acid[31]. In the enoyl-CoA hydratase

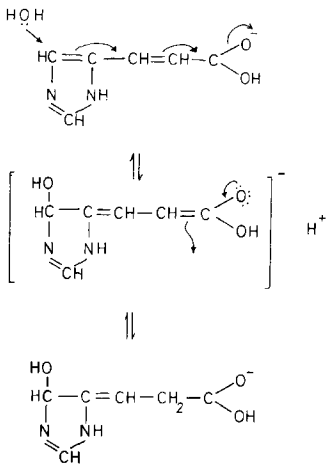

Fig. 4. Proposed reaction mechanism for urocanase.

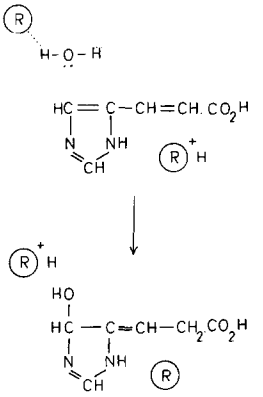

Fig. 5. Possible role of catalytic groups involved in the urocanase reaction .(R = a group able to donate and accept H^+).

reaction[36] the positive polarisation of the carbonyl carbon is aided by the presence of a carbon–sulphur bond. In the urocanase reaction, unmodified carboxylate group is essential for saturation of the substrate double bond to take place, and electrostatic binding at this point would result in increased polarisation. The electron shifts causing C-4 to become positively charged may well be aided by a withdrawal of electrons from the imidazole ring itself.

ALBERTY[32] proposed a model for the fumarase reaction according to which the substrate is bound by one of its carboxylate groups in such a way that the molecule comes under the influence of two groups able to donate and accept H^+. Kinetic data suggested that these might be imidazole groups of histidyl residues with water bound at one residue by hydrogen bonding. This type of catalysis may take place during the urocanase reaction (Fig. 5), and a possible explanation of the inhibitory effects of NH_2OH may be in terms of displacement of water from such sites.

The studies with analogues of urocanic acid have shown that urocanase is an enzyme with absolute substrate specificity. An unmodified carboxylate group *trans* to an imidazole ring free of substituents is necessary for saturation of the double bond system to be effected by urocanase. The enzyme binds imidazolylpropionic acid almost as strongly as its substrate and also accepts, with considerably less affinity, certain α-substituted imidazolylpropionic acids. There appear to be two major binding sites on the enzyme surface, one site combining with the imidazole ring system, the other interacting with the unmodified carboxylate group. Neither the ring system nor the carboxylate group acting alone interact sufficiently strongly to give effective binding, for neither imidazole itself nor acrylic, propionic or butyric acids inhibit urocanase. The combined binding energy of the double interaction is necessary, since a combination of imidazole and propionic acid was ineffective in inhibiting urocanase.

From the results of the studies with analogues of the substrate and from the probable reaction mechanism, a very tentative picture of the active site of the enzyme

may be drawn (Fig. 6). Since imidazolylpropionic acid is accepted it may be assumed that polarisation of the conjugated system is not necessary for entry to the active site. In this region of the enzyme a proton-donating group and a proton acceptor probably exist close to the α-carbon and C-4, respectively. Neither of these groups can impose a very rigid geometrical fit, since imidazolylpropionic acid, α-hydroxyimidazolylpropionic acid and α-chloroimidazolylpropionic acid are all accepted and involve modifications at the α-carbon. The reaction product, imidazolonepropionic acid, has an oxygen function in the C-4 position.

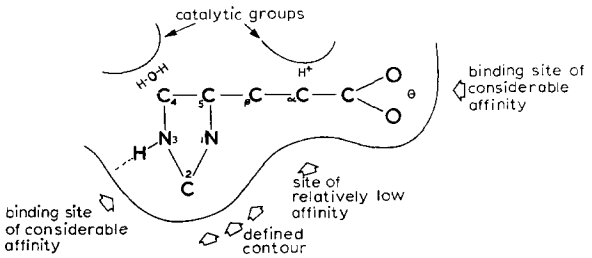

Fig. 6. Tentative picture of the active centre of urocanase.

The existence of a contour surrounding the carboxylate group may be inferred from the lack of activity of imidazolylacetic acid, imidazolylbutyric acid and the methyl esters of urocanic acid and imidazolylpropionic acid. This contour must include a site of considerable affinity, since vinyl imidazole had no detectable inhibitory effect at a concentration of 1 mM. 2-Pyrrolylpropionic acid is virtually identical geometrically to imidazolylpropionic acid and so is unlikely to be excluded from the active site, but gave no detectable inhibition at a concentration of 0.01 M. The differences in inhibitory effect between these compounds must therefore be one of differences in binding energy between inhibitor and enzyme. The sole structural difference between the compounds is the additional nitrogen atom of imidazolylpropionic acid, and one must suppose that a considerable attractive interaction occurs between this nitrogen and the neighbouring enzyme contour. This contour is closely defined, since the 3-methylurocanic acid is not accepted. At the pH optimum (pH 7.4 in phosphate buffer)[12] this nitrogen will be unionised but will carry a large electron excess[34] and offers the possibility of effective hydrogen bonding to a suitable atom in the protein.

Substitution at C-2 of the ring resulted in the loss of all substrate or inhibitor activity. The ineffectiveness of hydantoinpropionic acid must be due to the oxygen at C-2, since the rest of the molecule is identical to imidazolonepropionic aicd. The methyl groups of 2-methylurocanic acid and 2-methylimidazolylpropionic acid would have opposite effects to those of the C-2 oxygen function of hydantoinpropionic acid on the electron densities of the other atoms of the ring structure, and it would seem that the inability of these compounds to combine with the enzyme was due to steric hindrance rather than to reduction of binding forces.

The enzyme will accommodate the unsaturated side chain groupings of the substrate or the saturated side chains of imidazolylpropionic acids even when these

are substituted in the α-position. Therefore, a close lateral fit of the enzyme to this part of the molecule is unlikely. A study of the inhibitory effects of α- and β-alkyl substituted imidazolylpropionic acids might give further information on this point.

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