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Purification and Properties of a Diketo Acid Hydrolase from Beef Liver†

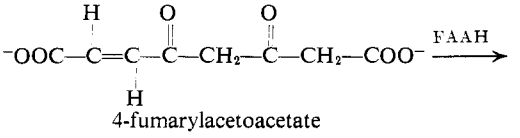
H. H. Hsiang, S. S. Sim, D. J. Mahuran, and Donald E. Schmidt, Jr.*

ABSTRACT: A diketo acid hydrolase which is probably fumarylacetoacetate fumarylhydrolase has been isolated from beef liver. The enzyme is homogeneous on Bio-Gel P-200 chromatography and on disc gel electrophoresis at two pH values. Acetopyruvic acid is cleaved by the enzyme into pyruvic acid and acetic acid. Substrate inhibition kinetics are found with acetopyruvic acid. The diketo acid hydrolase is sensitive to sulfhydryl-specific reagents but is insensitive to serine-specific reagents.

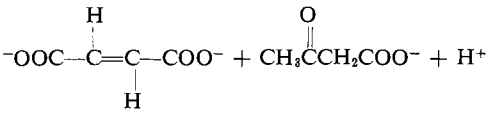
A few examples in which enzymes bring about the catalytic hydrolysis of carbon–carbon bonds are known. Oxaloacetase from *Aspergillus niger* catalyzes the hydrolysis of oxaloacetate to oxalic acid and acetic acid (Hayaishi *et al.*, 1956). Chymotrypsin is known to hydrolyze ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate to 3-(*p*-hydroxyphenyl)propionate and ethyl acetate (Doherty, 1955) and, in an analogous manner, trypsin cleaves ethyl 5-(*p*-aminophenyl)-3-ketovalerate (Roget and Calvet, 1962). L-Kynurenine hydrolase catalyzes the formation of anthranilate and alanine from L-kynurenine (Longenecka and Snell, 1955). In a bacterial system the pathway for the degradation of gentisic acid includes the enzyme fumarylpyruvate hydrolase, which hydrolyzes fumarylpyruvic acid to fumarate and pyruvate (Lack, 1961). The metabolic pathway for the degradation of tyrosine in mammalian systems utilizes the enzyme fumarylacetoacetate fumarylhydrolase (EC 3.7.1.2) to hydrolyze fumarylacetoacetate to fumaric acid and acetoacetic acid (Ravdin and Crandall, 1951). The mechanisms of these reactions have received little attention.

The study presented here describes the purification from beef liver and some properties of a diketo acid hydrolase which is probably fumarylacetoacetate fumarylhydrolase. Using this enzyme, a study of the mechanism of carbon–carbon bond hydrolysis will be undertaken.

Diketo acid hydrolases from several sources have been studied. A 2,4-diketo acid hydrolase had been isolated from rat liver (Meister and Greenstein, 1948). Other workers partially purified from beef liver a protein that hydrolyzed triacetic acid to acetoacetic acid and acetic acid (Connors and Stotz, 1949). Subsequent investigation showed that these two enzymes were probably the fumarylhydrolase (Ravdin and Crandall, 1951) which catalyzes the following reaction in rat and beef liver. Recently another diketo acid hydrolase



4-fumarylacetoacetate



fumarate acetoacetate

from rat liver has been purified 100-fold (Brock and Williamson, 1968). This enzyme hydrolyzed both triacetic acid and fumarylacetoacetic acid.

Experimental Section

Synthesis of Acetopyruvic Acid. Ethyl acetopyruvate was synthesized according to the method of Marvel and Dreger (1958). The ester was then hydrolyzed with 4 N sodium hydroxide to give the free acid (Lehninger and Witzemann, 1942). Three vacuum sublimations followed by recrystallization from carbon tetrachloride yielded colorless crystals that melted at 97.5–98.5° (uncorrected) (reported 98° (Lehninger and Witzemann, 1942)).

Enzyme Assay. A stock solution of acetopyruvic acid (1.22×10^{-3} M) was prepared by dissolving a weighed amount of the acid in 100 ml of 0.025 M sodium phosphate buffer at pH 7.2. This solution could be stored at 4° for a period of at least 1 week without significant decomposition.

Into a 3-ml cuvet was pipeted 2.6 ml of 0.025 M sodium phosphate buffer at pH 7.2 and 0.3 ml of the stock acetopyruvic acid solution. To this solution was added a measured

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amount of enzyme solution. Disappearance of substrate was recorded at 295 nm for 5–10 min at 25°. Optical density measurements were converted to concentrations by using a molar extinction coefficient of 7400 for acetopyruvic acid at pH 7.2 in 0.025 M sodium phosphate buffer. All kinetic data were obtained on a Gilford Model 2000 absorbance recorder attached to a Beckman DU monochromator.

All enzyme concentrations given in milligrams per milliliter are based on the 280 nm to 260 nm optical density ratio (Warburg and Christian, 1942). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 mmole of substrate per min under standard conditions.

Identification of Pyruvic Acid. Acetopyruvic acid (20 mg) was dissolved in 10 ml of 0.025 M sodium phosphate buffer. The pH of the resulting solution was adjusted to 7.2 by addition of 0.1 M sodium hydroxide. Enzyme was added and the solution allowed to stand for 24 hr. The breakdown of acetopyruvic acid after 1 day was shown to be complete by spectrophotometric measurement. The solution was then acidified to pH 2 with a 10% solution of trichloroacetic acid. The precipitated protein was removed by centrifugation.

The protein-free solution was treated with 1.5 ml of a 0.15 M solution of 2,4-dinitrophenylhydrazine dissolved in 15 ml of concentrated sulfuric acid, 20 ml of water, and 70 ml of 95% ethanol. The aqueous solution was extracted twice with 15-ml portions of ethyl acetate. Solid calcium carbonate was added with stirring to the combined portions of ethyl acetate. After removal of the calcium carbonate by filtration, the ethyl acetate solution was evaporated to a volume of about 1 ml. Toluene (20 ml) was added to the solution. The toluene solution was extracted twice with 5 ml of a cold solution of sodium carbonate (25%). Upon addition of concentrated hydrochloric acid to the combined sodium carbonate solutions, a yellow suspension formed. The yellow material was filtered and recrystallized from acetic acid. The melting points and mixture melting point of the 2,4-dinitrophenylhydrazone derivative of the product isolated from the enzymatic reaction and that of an authentic sample of the 2,4-dinitrophenylhydrazone derivative of pyruvic acid were identical (mp 217–218°, mixture mp 217–218°, uncorrected; reported mp 214° (Meister and Greenstein, 1948); 217–218° (Lack, 1959)).

Determination of Pyruvic Acid Produced during Hydrolysis. Pyruvic acid formed during the enzyme-catalyzed reaction was quantitatively determined using the 2,4-dinitrophenylhydrazine method of Lu (1939). Acetopyruvic acid which interferes with this determination was removed by boiling the solution for 1 hr after the addition of concentrated hydrochloric acid (Elgart and Nelson, 1941). The amount of pyruvic acid produced during the reaction was compared with the amount of acetopyruvic acid hydrolyzed during the reaction. Both sets of measurements were done using standard assay conditions.

Enzyme Kinetics. A stock enzyme solution was diluted with 0.025 M sodium phosphate buffer at pH 7.2 so that 0.02 ml of the final enzyme solution would give an initial rate of decrease in absorbance in the range of 0.10–0.25 OD unit per 5 min with 1 ml of 5×10^{-4} M acetopyruvic acid. The buffer 0.025 M sodium phosphate at pH 7.2 was used in all kinetic determinations.

Solutions of varying concentrations of acetopyruvic acid were incubated in a water bath at 37° before placing them in a 1-ml cuvet. The reaction was initiated by the addition of 0.02 ml of enzyme solution. The rate of the reaction was measured spectrophotometrically by recording the disappearance of the enol form of acetopyruvic acid at 295 nm. All experiments were conducted with enzyme of a specific activity not lower than 36. Kinetic points are the average of three determinations.

Effect of Specific Inhibitors. The experiments to measure the effect on the diketo acid hydrolase of potential inhibitors sodium iodoacetate, *p*-hydroxymercuribenzoate, *N*-ethylmaleimide, 5,5′-dithiobis(2-nitrobenzoic acid), and trinitrobenzenesulfonic acid were performed in the following manner. A portion of stock enzyme solution in 0.025 M sodium phosphate buffer at pH 7.2 containing 1×10^{-4} M dithioerythritol was placed in 2 ml of 0.025 M sodium phosphate buffer (pH 7.2) previously incubated at 25°. A portion (0.1 ml) of the resulting solution was then assayed under standard conditions at 25°. After the initial assay, the enzyme solution was transferred to a test tube containing a weighed amount of solid inhibitor. After 30 min at 25°, 0.1 ml of the solution containing enzyme and inhibitor was removed and assayed. The enzyme solution was found to be stable for at least 1 hr under these conditions in the absence of the inhibitors.

A slightly different technique was used for measuring the effect of the potential inhibitors phenylmethylsulfonyl fluoride and DFP. These inhibitors were dissolved in 0.6 ml of isopropyl alcohol. The alcohol solution was then diluted to 2 ml with 0.025 M sodium phosphate buffer at pH 7.2 which was previously incubated at 25°. A portion of the stock enzyme solution was added and 0.1 ml of the resulting solution removed immediately for assay. After 30 min at 25° another 0.1 ml was removed and assayed. Incubation of the diketo acid hydrolase under identical conditions in the absence of any inhibitor indicated the enzyme was stable for 1 hr.

Disc Polyacrylamide Gel Electrophoresis. The purified diketo acid hydrolase was examined for homogeneity by electrophoresis at pH 9.5, 8.9, and 4.2 (Smith, 1968).

Purification of the Diketo Acid Hydrolase. STEP I (EXTRACTION). A fresh beef liver in an iced container was brought directly from a slaughterhouse to a cold room (0°). All the subsequent steps were conducted at 0° with reagents at 0°. One-half of the liver (4–5 lb) was cut into small cubes. As much as possible of the white connective tissue was discarded. The cubes were passed through a meat grinder. Each 200-g portion of ground beef liver was homogenized for 2 min in a blender with 400 ml of 0.85% sodium chloride solution containing 1×10^{-4} M dithioerythritol. The mixed homogenates (about 9 runs or 1800 g of tissue) were allowed to stand for 3 to 5 hr.

STEP II (ETHANOL PRECIPITATIONS). To 800 ml of homogenate was added with stirring 890 ml of 95% ethyl alcohol to obtain a final concentration of 50% ethanol. This mixture was allowed to stand overnight. The solid material was discarded after centrifugation at 16,300*g* for 5 min.

To 1000 ml of the clear yellow or red solution was added 800 ml of 95% ethyl alcohol to produce a final alcohol concentration of 70%. This mixture was allowed to stand overnight while the enzyme precipitated. The supernatant fluid was carefully poured off. The precipitated protein was packed by centrifugation at 16,300*g* for 5 min. One-half of a beef liver would give about 30 g of gray paste. This crude enzyme paste could be stored at −20° for a period of 2 weeks without appreciable loss of enzyme activity.

STEP III (FIRST AMMONIUM SULFATE PRECIPITATION). Paste (3 g) was stirred for 30 min in 20 ml of 0.025 M sodium phosphate buffer at pH 7.2 containing 1×10^{-4} M dithioerythritol. The supernatant fluid was recovered after centrifugation of the suspension at 16,300*g* for 10 min.

Solid ammonium sulfate (8 g) was dissolved in the supernat-

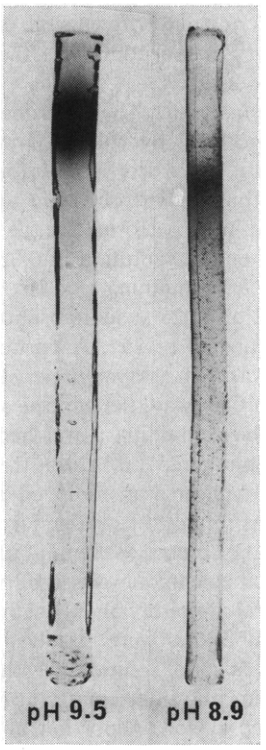


FIGURE 1: Acrylamide gel disc electrophoresis of the purified diketo acid hydrolase at pH 9.5 and pH 8.9 in 5% gels. The stock enzyme solution (5 μl) was placed in 10 μl of a 40% sucrose solution containing 1% Bromophenol Blue. The whole was then placed on a 4-cm gel tube and the electrophoresis was run at 5 mA per tube for 30 min. The gels were stained with Buffalo Black NBR (0.1%) in 7% acetic acid and were destained with 7% acetic acid.

TABLE I: Purification of the Diketo Acid Hydrolase from Beef Liver.

Preparation	Total Activity (Units)	Yield[a] (%)	Sp Act. (Units/mg of Protein)	Purification (-fold)
Crude extract	1200	(100)	0.26	(1)
Ethanol precipitation (50%)	660	55	0.98	3.8
Ethanol precipitation (70%)	160	13	1.2	4.6
First ammonium sulfate precipitation	13	11	9.8	38
G-25 Sephadex	11	9	9.8	38
TEAE-cellulose	8.5	7	23.5	90
Second ammonium sulfate precipitation	5	4	36.3	140

[a] Yields are based upon an activity of 0.1 of that shown in the first three steps as only 0.1 of the material from the 70% ethanol precipitation was carried through the remainder of the procedure.

ant fluid. This solution was allowed to stand for 1 hr. The resulting solid was compacted by centrifuging at 16,300*g* for 10 min.

The precipitated protein was dissolved in 7 ml of 0.02 M Tris-hydrochloride buffer at pH 8.5 containing 1×10^{-4} M dithioerythritol. This solution could be clarified if necessary by centrifuging at 16,300*g* for 10 min.

STEP IV (CHROMATOGRAPHY ON SEPHADEX G-25). The light yellow solution from the previous step was passed through a column (2 × 22 cm) containing 10 g of Sephadex G-25 equilibrated with 0.025 M Tris-hydrochloride buffer at pH 8.5 containing 1×10^{-4} M dithioerythritol. Fractions (10 ml) were collected at a flow speed of 100 ml/hr. The fractions containing enzyme activity were usually eluted in the third and fourth fractions.

STEP V (TEAE-CELLULOSE CHROMATOGRAPHY). Fractions from the Sephadex G-25 chromatography containing enzyme activity were combined and placed on a column (2 × 22 cm) containing 10 g of TEAE-cellulose that had been prepared by the method of Srere and Kosicki (1961). The TEAE-cellulose column had been equilibrated with 0.02 M Tris-HCl at pH 8.5 containing 1×10^{-4} M dithioerythritol. After placing the enzyme solution on the column, 200 ml of the 0.02 M Tris-hydrochloride buffer was passed through the column at a flow rate of 60 ml/hr. Fractions (10 ml) were collected. After 200 ml of effluent was obtained, an exponential gradient was started with 300 ml of Tris-hydrochloride buffer (0.2 M, pH 8.5, 1×10^{-4} M dithioerythritol) dropping into 100 ml of Tris-hydrochloride buffer (0.02 M, pH 8.5, 1×10^{-4} M dithioerythritol). All fractions having a specific activity of over 20, usually fractions 31 through 35, were combined and assayed.

STEP VI (SECOND AMMONIUM SULFATE PRECIPITATION). To the combined fractions from the TEAE-cellulose column containing enzyme activity was added sufficient ammonium sulfate to give a final concentration of 40% (w/v). A white precipitate formed that was compacted by centrifugation at 16,300*g* for 10 min. To the small amount of resulting precipitate was added 5 ml of 0.025 M sodium phosphate buffer at pH 7.2 containing 1×10^{-4} M dithioerythritol. After stirring for 5–10 min, the suspension was centrifuged and the supernatant fluid assayed for enzymatic activity.

Results and Discussion

Enzyme Purification. A 140-fold increase in the specific activity of the diketo acid hydrolase was achieved using this procedure (Table I). In about 20% of the purifications performed the specific activity of the crude extract of the fresh beef liver was only 0.13. In these cases, however, the final specific activity after proceeding through the entire purification procedure was always at least 36. The Sephadex G-25 chromatography was included to adjust the ionic strength to a level necessary for the TEAE-cellulose chromatography. Additional chromatography on CM-cellulose or calcium phosphate gels was unsuccessful in raising the specific activity of the enzyme.

The entire isolation procedure starting with 0.5 of a beef liver and then carrying through 0.1 of the material obtained in the ethanol precipitation step can be completed in 5 days. The diketo acid hydrolase obtained in this manner lost 50% of its activity in 1 week when stored at 0° in a 0.025 M sodium phosphate buffer at pH 7.2 containing 1×10^{-4} M dithioerythritol.

Purity. Polyacrylamide gel electrophoresis at pH 8.9 and pH 9.5 indicated that the purified enzyme has a protein component which migrates as a single band (Figure 1). All attempts at measuring any enzymatic activity in the protein band or in any other portion of the gel were unsuccessful. The enzyme preparation before the TEAE-cellulose chromatography step showed at least seven bands when analyzed by gel electrophoresis at pH 9.5. Gel electrophoresis at pH 4.2 indicated a

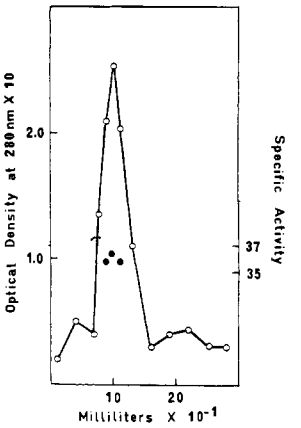


FIGURE 2: Bio-Gel P-200 chromatography of the purified diketo acid hydrolase. Stock enzyme solution (5 ml) in 0.2 M Tris-hydrochloride buffer, pH 8.5, containing 1×10^{-4} M dithioerythritol was mixed with sucrose to a final concentration of 5% (w/v). The solution was pumped onto the bottom of a Pharmacia K25/45 column packed with Bio-Gel P-200. The column was developed by an upward flow at a rate of 40 ml/hr. Degassed 0.5 M Tris-hydrochloride buffer (pH 8.5, 5 ml), containing 1×10^{-4} M dithioerythritol and 0.5 g of sucrose (10%), was pumped onto the column following sample application. Another 500 ml of degassed 0.5 M Tris-hydrochloride buffer (pH 8.5) containing 1×10^{-4} M dithioerythritol was used to elute the protein. –O–O–, optical density at 280 nm of the effluent. –●–●–, specific activity of the effluent.

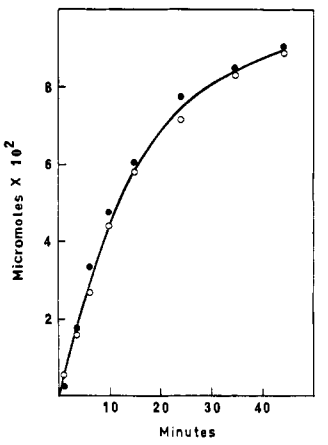
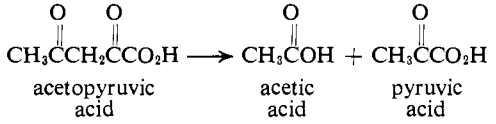


FIGURE 3: Hydrolysis of acetopyruvic acid by the diketo acid hydrolase as determined by chemical and spectrophotometric means in 0.025 M sodium phosphate buffer at pH 7.2; ● = micromoles of pyruvic acid appearing; ○ = micromoles of acetopyruvate disappearing.

single homogeneous band which appeared to be precipitated protein and could not be stained. When the preparation before the TEAE-cellulose chromatography step was analyzed by electrophoresis at pH 4.2, there appeared in the gel at least three bands of what again seemed to be precipitated protein.

The active protein emerged from Bio-Gel P-200 as a single symmetrical peak (Figure 2). In relation to Blue Dextran 2000 (molecular weight approximately 2×10^6) the enzyme was retarded during chromatography. The Blue Dextran was eluted from the column between 50 and 80 ml while the diketo acid hydrolase activity was found between 90 and 130 ml. A recovery of 95% of the active protein applied to the column was obtained and the specific activity was unchanged.

Determination of Pyruvic Acid. The hydrolysis of acetoacetate by the diketo acid hydrolase was found to occur in the following manner.



Pyruvic acid was identified as one of the products of the reaction. Acetopyruvic acid in 0.025 M sodium phosphate buffer at pH 7.2 was completely hydrolyzed enzymatically as determined spectrophotometrically. Then a 2,4-dinitrophenylhydrazone derivative of the hydrolysis product was isolated and shown to be identical with that of an authentic sample of the 2,4-dinitrophenylhydrazone derivative of pyruvic acid. The 2,4-diketo acid hydrolase isolated from rat liver hydrolyzed acetopyruvic acid in an analogous manner (Meister and Greenstein, 1948). The triacetic acid hydrolyzing enzyme from beef liver also hydrolyzed acetopyruvic acid although identification of pyruvic acid as a product was not reported (Connors and Stotz, 1949). In contrast to the enzymatic hydrolysis of acetopyruvic acid, hydrolysis of acetopyruvic acid in base produces only acetone and oxalic acid (Lehninger and Witzeman, 1942).

The rate of the enzymatic hydrolysis of acetopyruvic acid was conveniently followed spectrophotometrically by watching the disappearance of the enol form of acetopyruvic acid at 295 nm. The correspondence is excellent between micromoles of acetopyruvic acid hydrolyzed (measured spectrophotometrically) and the micromoles of pyruvic acid formed (measured after conversion to the 2,4-dinitrophenylhydrazone derivative) (Figure 3). The spectrophotometric method could probably be used to follow the hydrolysis of other 2,4-diketo and 3,5-diketo acids upon which this enzyme may act.

Kinetics of Acetopyruvate Hydrolysis. The kinetics of hydrolysis of acetopyruvic acid by the diketo acid hydrolase were studied spectrophotometrically over the concentration range of 5×10^{-5} M to 1.2×10^{-3} M acetopyruvic acid. When the initial velocity is plotted against the negative logarithm of the initial substrate concentration, a bell-shaped curve is obtained (Figure 4). Substrate inhibition by high concentrations of substrate may generally be fitted by eq 1 (Cleland, 1970).

$$v = \frac{a}{1 + b/[S_0] + [S_0]/c} \quad (1)$$

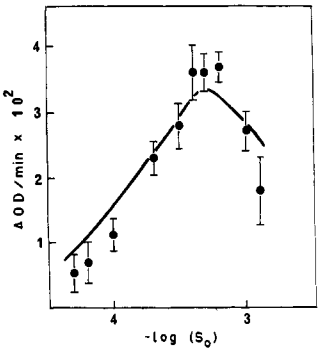


FIGURE 4: Initial velocity *vs.* $-\log [S_0]$ for the hydrolysis of acetopyruvic acid by the diketo acid hydrolase at pH 7.2 in 0.025 M sodium phosphate buffer. The solid line is generated from eq 1 by allowing $a = 0.092$ OD/min, $b = 5 \times 10^{-4}$ M, and $c = 6 \times 10^{-4}$ M.

SCHEME I

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k} E + P$$

$$E + S \overset{K_{S_1}}{\rightleftharpoons} ES_1$$

$$ES_1 + S \overset{K_{S_2}}{\rightleftharpoons} ES_2$$

An expression in the final form of eq 1 can be derived from Scheme I.

K_S, K_{S1}, and K_{S2} are dissociation constants for the respective ES, ES_1, and ES_2 enzyme–substrate complexes and k is the rate constant for the breakdown of the productive enzyme–substrate complex. If Scheme I is valid for this system then the constants a, b, and c from eq 1 may be expressed as $a = V_{max}K_{S1}/(K_{S1} + K_S)$ where $V_{max} = k(E_0)$, $b = K_SK_{S1}/(K_{S1} + K_S)$, and $c = [K_{S2}(K_S + K_{S1})]/K_S$.

Scheme I may be thought of as a form of substrate inhibition which involves two molecules of substrate simultaneously binding within a site which is designed for the binding of only one (Cleland, 1970). If the enzyme is to enzymatically hydrolyze a diketo acid molecule, then only one substrate molecule should be bound at the active site (ES). As acetopyruvic acid is undoubtedly not the natural substrate of the enzyme, a rational for the substrate inhibition is easily imagined. Since the natural substrate—fumarylacetoacetic acid—has two ionized carboxyl groups at pH 7, the enzyme probably has two positively charged binding areas in the active site for these two ionized carboxyl groups. In the case of substrate inhibition, an acetopyruvic acid molecule may become bound incorrectly at one of the positively charged areas in the active site (ES_1). After formation of the unproductive ES_1 complex, a second acid molecule may then become bound to the second positively charged area in the active site (ES_2). In such a circumstance neither molecule will be properly aligned for enzymatic hydrolysis and thus a dead end complex, ES_2, is formed.

An equally plausible and kinetically equivalent explanation for substrate inhibition assumes that a substrate molecule binds with the productive ES complex to form an ES_2 complex that is unproductive. As both explanations give final kinetic equations of the same form (eq 1), it is impossible to decide between the two models by kinetic means.

Reaction of Sulfhydryl-Specific and Serine-Specific Inhibitors. The reaction catalyzed by the diketo acid hydrolase is in some respects similar to the hydrolysis of an ester. This idea is reinforced when one realizes that chymotrypsin will also catalyze the hydrolysis of a carbon–carbon bond in ethyl 5-(*p*-hydroxyphenyl)-3-ketovalerate to give 3-(*p*-hydroxyphenyl)propionate and ethyl acetate (Doherty, 1955). As many esterases contain either a sulfhydryl group or a serine in their active sites, a study of sulfhydryl-specific and serine-specific inhibitors on the diketo acid hydrolase was conducted.

All the sulfhydryl-specific inhibitors except iodoacetate definitely inhibited the enzyme (Table II). Hydroxymercuribenzoate being the most effective inhibitor gave a totally inactive enzyme after 30 min. Further studies will be necessary to determine whether these inhibitors are reacting at the active site of the enzyme or are inhibiting the enzyme by reacting at sulfhydryl groups removed from the active site.

TABLE II: Effect of Sulfhydryl- and Serine-Specific Inhibitors on the Diketo Acid Hydrolase.

Inhibitor	Concn (M × 10^3)	Activity (%) Remaining after 30 Min[a]
Sodium iodoacetate	1	96
5,5′-Dithiobis(2-nitrobenzoic acid)	1	39
N-Ethylmaleimide	1	65
Trinitrobenzenesulfonic acid	1	24
Hydroxymercuribenzoate	1	0
DFP	1	100
	5	100
Phenylmethylsulfonyl fluoride	1	102

[a] See Experimental Section for details.

The serine-specific inhibitors DFP and phenylmethylsulfonyl fluoride (Table II) have no effect on the diketo acid hydrolase at pH 7.2. Thus, a serine esterase type activity for the diketo acid hydrolase seems unlikely.

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