STUDIES ON THE ENZYMIC DECOMPOSITION OF UROCANIC ACID

II. PROPERTIES OF PRODUCTS OF UROCANASE REACTION

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SUMMARY

1. Urocanase has been purified about 150 fold from beef liver by classical enzyme fractionation procedures. The purified enzyme is free of imidazolone propionic acid oxidase and hydrolase activities.

2. Evidence indicating that the product of urocanase reaction is the suspected 4(5)-imidazolone-5(4) propionic acid has been obtained by comparison of its spectral and chemical properties to other known imidazolones.

3. Imidazolonepropionic acid is labile to oxygen but no hydantoin-5-propionic acid is formed by this nonenzymic oxidative reaction. It is also nonenzymically converted to formylisoglutamine, which was isolated and characterized by its physical and chemical properties.

4. The enzymic formation of formiminoglutamic acid from imidazolonepropionic acid was demonstrated by a coupled assay procedure in crude extracts of liver of various animal species and also in bacteria.

INTRODUCTION

Previous studies[1-6] have established the probable sequence of reactions for the enzymic conversion of urocanic acid to formiminoglutamic acid and its nonenzymic conversion to formylisoglutamine as shown below:

$$\text{Urocanic acid} + H_2O \xrightarrow{\text{Urocanase}} \text{Imidazolonepropionic acid} \quad (1)$$

$$\text{Imidazolonepropionic acid} + H_2O \xrightarrow{\text{Imidazolonepropionic acid hydrolase}} \text{Formimino-L-glutamic acid} \quad (2)$$

$$\text{Formimino-L-glutamic acid} \xrightarrow{\text{Nonenzymic}} \text{Formylisoglutamine} \quad (3)$$

The present report deals with the purification and separation of urocanase from imidazolonepropionic acid hydrolase, the properties of the product of reaction (1) (imidazolonepropionic acid), the unequivocal identification of formylisoglutamine and the further study of enzyme reaction (2).

EXPERIMENTAL

Materials

Urocanic acid was purchased from the California Corporation for Biochemical Research. Formiminoglutamic acid was prepared by the method of RABINOWITZ AND TABOR[7]. Formyl-L-glutamic acid and formyl-L-glutamine were prepared by the method of BOREK AND WAELSCH[2]. Formyl-L-isoglutamine was kindly furnished by REVEL AND MAGASANIK. Isoglutamine was made from carbobenzoxy-L-glutamic acid by the method of BERGMANN AND ZERVAS[8], and carbobenzoxy-L-glutamic anhydride by the method of LEQUESENE AND YOUNG[9]. A sample of isoglutamine was also kindly furnished by Dr. G. WOLF. Tetrahydrofolic acid was prepared by the method of RABINOWITZ AND PRICER[10]. L-Hydantoin-5-propionic acid was prepared by the method of DAKIN[11].

Chromatographic methods

For the paper chromatographic comparison and identification of the various metabolites of urocanic acid, the solvent systems described by REVEL AND MAGASANIK[5] were adopted. Radioactivity on chromatograms was detected with a recording, thin end window Geiger-Müller Counter for scanning paper strips.

Glutamic acid, isoglutamine, and formiminoglutamic acid were detected on paper chromatograms by dipping the paper in a 0.2 % solution of ninhydrin in acetone and heating. Formiminoglutamic acid was also detected with a ferricyanide–nitroprusside reagent[12] and by the enzymic method of TABOR AND WYNGARDEN[13]. Imidazole compounds were located with the diazotized sulfanilic acid reagent of AMES AND MITCHELL[14]. N-substituted amino acids (*i.e.*, formylisoglutamine, formylglutamine and formylglutamic acid) were detected by the modified starch-iodide procedure of PAN AND DUTCHER[15].

Urocanic acid was separated from histidine by ion exchange chromatography with Amberlite IRC-50 (XE-64) resin, which was processed as described by HIRS[16]. An 18 × 2.5 cm column was packed under gravity with the Na^+ form of resin and buffered at pH 4.8 by passing through 250 ml of 1 *M* sodium acetate buffer, pH 4.8, then washing with water until the washings were neutral. A mixture of D-histidine and urocanic acid in 30 ml of solution was gradually applied to the column over a period of one hour, followed by 25 ml of water. A gradient elution device containing 0.1 *N* acetic acid in the reservoir and 250 ml of water in the mixing flask was attached to the column and 3-ml fractions at the rate of 0.6 ml/min were collected. Approximately 90 % of the urocanic acid was recovered as a single peak in fractions 40 to 50. Most of the D-histidine was later recovered by passing 1 *N* acetic acid through the column. The urocanic acid was chromatographically homogenous in several solvent systems[4,5] and showed only one radioactive spot.

For the separation of the enzymic and nonenzymic degradation products of the urocanase reaction, ion exchange chromatography on a Dowex 1-acetate column (200–400 mesh, X-8) was used. The gradient elution device described above, but containing 0.3 *N* acetic acid in the reservoir, was employed. Fractions from ion exchange chromatography and other reaction mixtures were routinely subjected to high voltage paper electrophoresis at pH 3.5 and pH 6.4 at 2 kV and 40–50 mA in an apparatus described by MICHL[17]*. Formylglutamic acid, formylisoglutamine, glutamic acid and

* We wish to thank Dr. D. GROSS of this department for various suggestions in the use of the electrophoresis apparatus.

isoglutamine were separable at pH 6.4. Isoglutamine could be distinguished from glutamine at pH 3.5 but not at pH 6.4.

Enzyme fractions used in the present study

Histidase was prepared from extracts of *Pseudomonas fluorescens* cells by the method of TABOR AND MEHLER[18], except that the heat denaturation of inactive protein was carried out at 70–72° for 5 min instead of 78–83° for 15 min. Formiminoglutamic transferase was prepared by the method of TABOR AND WYNGARDEN[13] with the slight modification (for greater reproducibility) that the first ammonium sulfate precipitate "step B" was dissolved in 0.05 *M* potassium phosphate buffer, pH 7.2, dialyzed against 10 volumes of the same buffer for 16 h and further fractionated with solid ammonium sulfate. Fractions containing most of the formiminoglutamic transferase activity were pooled, dissolved in 0.05 *M* potassium phosphate buffer, pH 7.2, frozen and kept at —20° until needed for further use. In some preparations these fractions contained substantial quantities of imidazolonepropionic acid hydrolase, as determined by a coupled assay procedure. The formiminoglutamic transferase activity of these fractions could be demonstrated even after three months of storage at —20°.

In the initial stages of enzyme purification protein was determined by the biuret method of ROBISON AND HOGDEN[19] and in the later stages by the u.v. absorption method of WARBURG AND CHRISTIAN[20]. Units of activity and specific activity of histidase and urocanase preparations are those of TABOR AND MEHLER[18].

All optical measurements were made in a Cary Model 14 recording spectrophotometer.

RESULTS

Purification of urocanase

The method of purification of this enzyme reported by FEINBERG AND GREENBERG[6] has been considerably modified as described below. All operations were carried out at 0–5°.

Two portions of 500 g each of fresh beef liver were homogenized with 1500 ml of 0.25 *M* sucrose containing 0.005 *M* EDTA (ethylendiaminetetraacetate) at pH 7.2 for 15 sec at medium speed and 45 sec at full speed in a large Waring blendor of 1 gallon capacity. The pooled homogenate was centrifuged at 2700 × *g* for 30 min in a Servall refrigerated centrifuge. The supernatant fluid was filtered through one fold of cheese cloth into a 4-l Erlenmeyer flask, yielding a turbid filtrate with a volume of 3500 ml. A small measured volume of this fluid was centrifuged at 25,000 × *g* for 30 min in a Spinco preparative ultracentrifuge and urocanase activity determined on the clear supernatant fluid. The total activity in the original homogenate was computed from this determination. In many instances the urocanase activity of various beef liver homogenates was extremely low but the activity increased and could be detected as purification progressed.

To each liter of the turbid filtrate 100 g of a 1:1 mixture of Bentonite (the brand appears immaterial) and Hyflo-Supercell (Johns Mansville Corporation) were added and thoroughly mixed at medium speed for 1 to 2 min in a large Waring blendor. The thick suspension was left to stand for 1 h at approx. 0° in an ice bath, then

centrifuged at 10,000 × *g* for 1 h and the opalescent supernatant fluids (volume 1800 ml) were pooled. 564 g of ammonium sulfate were added gradually over a period of 1 h or more. The pH was maintained at 6.8–7.2 by addition of 3 *N* NH_4OH. (pH measurements were made on a 1:10 sample dilution). The solution was left to stand for at least 12 h in an ice bath and later centrifuged at 10,000 × *g* for 1 h. The precipitate was dissolved in 0.1 *M* potassium phosphate buffer, pH 7.2, and dialyzed against 2 l of the same buffer containing 0.001 *M* EDTA. The buffer was changed after 8 to 10 h. After 16–20 h of dialysis, the protein solution was centrifuged at 15,000 × *g* to remove insoluble material and the supernatant fluid diluted with 0.1 *M* potassium phosphate buffer, pH 7.2, to give a protein concentration of 20 mg/ml. This solution was further fractionated with solid ammonium sulfate starting from 0.2 saturation and raising the concentration by intervals of 0.05 saturation. Most of the activity was found between 0.2 and 0.5 saturation of ammonium sulfate. Fractions having a specific activity of over 60 were pooled, dissolved in the same buffer and dialyzed as described above for 12 h.

The dialyzed protein was diluted approximately to a protein concentration of 10 mg/ml and subjected to fractional negative adsorption on calcium phosphate gel as described below.

An enzyme preparation in 90 ml containing 928 mg of protein was stirred with 930 mg of packed calcium phosphate gel[21] for 45 min in an ice bath and then centrifuged at 10,000 × *g* for 15 min. The supernatant fluid was assayed for activity and again stirred with an amount of gel equivalent to the total protein for 30 min and centrifuged. The process was repeated several times and the final supernatant fluid from the gel was fractionated with ammonium sulfate. A considerable quantity of inactive protein is retained on the packed gel. On elution with 0.5 *M* potassium phosphate buffer, pH 7.2, enzyme of low specific activity could be recovered.

Results of a typical fractionation procedure are shown in Table I.

TABLE I

PURIFICATION OF UROCANASE

Fraction	*Total protein mg**	*Specific activity*	*Total activity*	*Yield*
Homogenate	212,500	3.2	680,000	100
Bentonite supernatant fluid	15,400	32.5	500,500	73.6
1st ammonium sulfate precipitate	5,000	76.8	384,000	56.5
Pooled ammonium** sulfate fractions	3,100	92.0	285,200	42.0
Pooled ammonium*** sulfate fractions	930	70.0	65,100	100
1st gel supernatant	492	105.5	51,810	79.5
2nd gel supernatant	192	216.0	41,500	63.7
3rd gel supernatant§	82.5	273.0	22,530	34.6
0.3–0.4 saturation ammonium sulfate precipitate	32.0	440.0	14,080	21.6

* Protein of upper 4 purification steps was determined by biuret method and of lower 5 purification steps by u.v. absorption.

** Approximately 25 to 45% of the original activity is recovered at this stage, depending on the initial activity of the homogenate.

*** Pooled fractions of a different enzyme preparation from above, yield set at 100 for comparison.

§ Gel fractionation invariably gave 2 to 4 fold purification. The specific activity listed was obtained only once.

Highly purified enzyme fractions (about 150 fold enrichment, specific activity of 400 or more) show in addition to the usual absorption maximum at 280 mμ an inflexion at 290 mμ and end absorption in the spectral range of 350–450 mμ. These fractions are free of imidazolonepropionic acid hydrolase and imidazolonepropionic acid oxidase activities. Other properties of the enzyme are similar to those described earlier by FEINBERG AND GREENBERG[6]. The activity of the enzyme was unchanged after treatment with Norite or Dowex 1-acetate or EDTA. Considerable loss of activity occurred on treatment with protamine sulfate.

Preparation of [2-^{14}C]urocanic acid

[2-^{14}C]urocanic acid was prepared from DL-[2-^{14}C]histidine by a modification of the large scale preparative procedure of MEHLER, TABOR AND HAYAISHI[22].

Approximately 20,000 units of histidase in 3 ml of water was added to a mixture of 200 μmoles of DL-[2-^{14}C]histidine (dissolved in 10 ml of water and adjusted to pH 9.2), 140 μmoles of sodium thioglycolate and 400 μmoles of sodium pyrophosphate buffer, pH 9.2, in a total volume of 25 ml and left at room temperature for 3 h, with occasional stirring. The reaction mixture then contained 98 μmoles of urocanic acid, as determined spectrophotometrically. The reaction was terminated by adding 0.3 ml of glacial acetic acid and heating in a boiling water bath for 10 min. The denatured protein was removed by centrifugation and the supernatant fluid adjusted to pH 4.8–5.0. The urocanic acid was isolated from the solution by ion exchange chromatography on Amberlite IRC-50 (XE-64) as described previously.

Urocanic acid + H_2O → imidazolonepropionic acid

In order to determine whether or not this reaction proceeds to completion, radioactive urocanic acid was incubated with urocanase and reisolated at the end of the reaction by addition of carrier urocanic acid.

The reaction mixture contained 200 μmoles of potassium phosphate buffer, pH 7.2, 0.294 μmoles of urocanic acid (68,600 counts/min) and 260 units of urocanase of specific activity 400, in a total volume of 3 ml, in a spectrophotometric cuvette. The blank contained all components except urocanic acid. The O.D. at 277 mμ dropped to a constant value in 10 min. The contents of the cuvette were then quantitatively transferred to a chilled graduated centrifuge tube and rinsed with 3 ml of water. The reaction was terminated by addition of 0.4 ml of 10 % perchloric acid, the denatured protein removed by centrifugation, the pH adjusted to 7 with 1 *N* KOH, and the volume made up to 8 ml. Aliquots were taken for determination of radioactivity and 200 mg of urocanic acid·2 H_2O were added as carrier and dissolved by heating in a boiling water bath. The solution was left overnight in the refrigerator. The crystals were separated from the supernatant fluid and recrystallized several times from hot water. After two recrystallizations the specific activity remained constant. The results of several experiments showed that 98–99 % of the urocanate was utilized in the enzyme reaction.

Properties of imidazolonepropionic acid

Stability and spectral characteristics: In the beginning of these studies the only known characteristic of the product of the urocanase reaction was its absorption maximum of 264 mμ at pH 7.2 to 7.6. In order to isolate the "264 mμ product" it

was necessary to deproteinize the reaction mixture, as traces of protein seriously interfered with spectrophotometric measurements. Only deproteinization with perchloric acid was found suitable for the desired purpose and to give reproducible results. Even when purified enzyme preparations of urocanase were used, considerable interference in the u.v. region by acid-soluble nucleotides was noticed. Consequently suitable blanks were run in all experiments. A typical experiment is described below.

The reaction mixture in matched Quartz spectrophotometric cuvettes contained 100 μmoles of sodium phosphate buffer, pH 7.6, and 350 units of urocanase of specific activity 70 in a total volume of 2.9 ml. 0.4 μmole of urocanic acid in 0.2 ml of water was added to one of the cuvettes, while 0.2 ml of water was added to the blank cuvette. The O.D. at 277 mμ dropped to a constant value in 8 min. The spectrum of the product was obtained by differential u.v. spectrophotometry and the contents of each cuvette was quantitatively transferred to a chilled glass centrifuge tube containing 0.1 ml of 30 % perchloric acid. After centrifugation at 3° to remove denatured protein, 2.5 ml of the clear supernatant fluid was pipetted from each tube into separate cuvettes and diluted to 3 ml with water, or 1 ml of undiluted aliquots were directly used in 1.5 ml cuvettes. Approximately 15 min were required to reach this stage after termination of the reaction. Various additions were made to each cuvette and the difference spectrum recorded at short time intervals. The difference spectral changes observed in such an experiment are shown in Fig. 1.

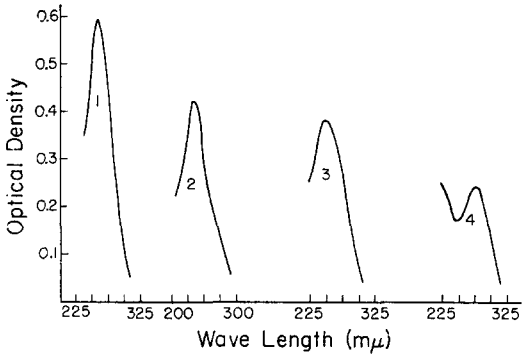

Fig. 1. Effect of acid and alkali treatment on product of urocanase reaction. Curve 1, spectrum of product of urocanase reaction, λ_{max}, 264 mμ; Curve 2, product after treatment with perchloric acid and dilution (pH 1.3), λ_{max}, 234 mμ; Curve 3, 30 sec after changing the pH to 9 with NaOH, λ_{max}, 250 mμ; Curve 4, 15 min after changing the pH to 9, λ_{max}, 275 mμ.

These experiments revealed that the absorption maximum of the "264 mμ product" changed to 234 mμ after perchloric acid treatment. In another experiment the perchloric acid extract was left under aerobic and anaerobic conditions for various periods at —20° and the absorption spectrum determined at different time intervals. Under aerobic conditions the product showed a 5 % decomposition in 26 h at —20°, whereas under anaerobic conditions there was no decomposition. After 76 h about 5 % decomposition was noticeable in the product preserved anaerobically. Attempts were made to recover the "264 mμ product" by increasing the pH to the original value of 7.2 to 7.6. For this purpose the perchloric acid supernatant fluid containing the product was adjusted to known pH values with measured volumes of 2 *M* $(NH_4)_2HPO_4$ and the spectrum scanned immediately. The results are shown in Fig. 2.

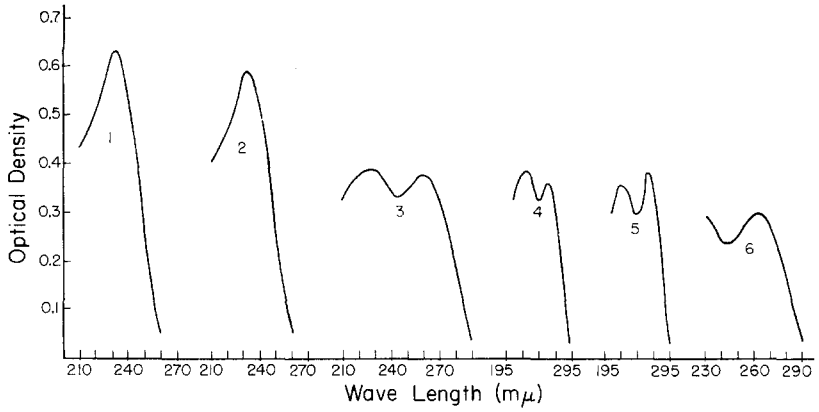

Fig. 2. Spectrum of product of urocanase reaction at various pH values. Curve 1, undiluted perchloric acid extract (pH 1.3), λ_{max}, 234 mμ; Curve 2, 30 sec after adjusting the pH to 2.9, λ_{max}, 234 mμ; Curve 3, 30 sec after adjusting the pH to 5.7, λ_{max}, 228 mμ, 260 mμ; Curve 4, after 5 min at pH 5.7, λ_{max}, 232 mμ, 265 mμ; Curve 5, 30 sec after adjusting the pH to 6.2, λ_{max}, 265 mμ; Curve 6, after 15 min at pH 6.2, λ_{max}, 260 mμ.

These experiments indicated that profound changes in the spectra took place, presumably because of the tautomerization of the imidazolone ring by acid-alkali treatment. The instability of the product with increasing pH values is possibly due to its susceptibility to nonenzymic oxidation and the irreversible opening of the imidazolone ring. Consequently kinetic data regarding the stability of the substance was obtained directly from the enzymic reaction mixture by determining the degradation of the "264 mμ product" from the disappearance of the absorption maximum at 264 mμ as a function of time. A log plot of the results is shown in Fig. 3.

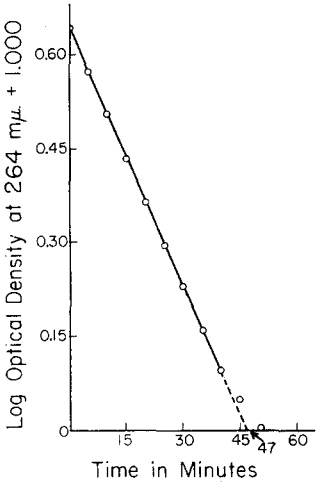

Fig. 3. Kinetics of nonenzymic degradation of "264 mμ product". The reaction mixture contained 200 μmoles of potassium phosphate buffer, pH 7.2, 400 units of urocanase of specific activity 123 and 0.4 μmoles of urocanic acid in a total volume of 3 ml. The blank contained all components except urocanic acid. The rate of nonenzymic degradation of "264 mμ product" was followed after the completion of urocanase reaction (*i.e.*, 12 min) for a further period of 1 h.

The nonenzymic degradation of the "264 mμ product" clearly follows first order kinetics with a velocity constant of 0.0309 min^{-1} and half life of 22 min at 27° at pH 7.2. The rate of nonenzymic degradation of the "264 mμ product" was independent of enzyme concentration, since addition of more enzyme did not cause a significant change in the rate.

Molar extinction coefficient: Isotope dilution experiments have indicated that virtually all the urocanate is utilized in the enzymic reaction and in previous work[6] it was shown that "264 mμ product" appears without time lag. On this basis a value for E_{max} of 3980 ± 300 was estimated from data of a number of experiments at pH 7.2. The E_{max} at 264 mμ did not vary significantly in the pH range of 6.3–8.6. In these experiments a large excess of enzyme was used, since the pH optima of the enzyme is between 6.8 to 7.6. No corrections have been made in the E_{max} value, for the nonenzymic degradation of the "264 mμ product" during its formation from urocanate. REVEL AND MAGASANIK[5] have also reported an approximate E_{max} value of 4000 for the product of the urocanase reaction.

Susceptibility of "264 mμ product" to oxidizing agents: The "264 mμ product" is extremely responsive to the action of oxidizing agents such as H_2O_2 or potassium persulfate. When small amounts of H_2O_2 or persulfate were added to the spectrophotometric cuvettes containing the product, the absorption maximum at 264 mμ disappeared instantaneously. No well defined products could be isolated from the oxidation reaction.

While the above investigations were in progress BROWN AND KIES[23] and BROWN[24] reported that hydantoin-5-propionic acid was a metabolite of urocanic acid in the rat. Previous experiments[6] have shown that the "264 mμ product" participates in a non enzymic reaction with oxygen in the presence of ferricyanide or dichlorophenol indophenol. An experiment to test whether hydantoin-5-propionic acid is formed in this reaction was carried out by the isotope trapping technique.

To spectrophotometric cuvettes containing 100 μmoles of sodium phosphate buffer, 400 units of urocanase of specific activity 50 in a volume of 2.8 ml, 0.3 μmole of urocanic acid (52,500 counts/min) in 0.2 ml of water was added to one of the cuvettes and 0.2 ml of water to the blank. The reaction was complete in 10 min. 0.9 μmole of dichlorophenol indophenol dye was added in portions of 0.3 μmole each. The color of the dye was bleached within 2 to 4 min during the first two additions of the dye. The mixture was then transferred to a centrifuge tube, deproteinized with an equal volume of acetone and centrifuged. The residue was extracted once with 3 ml of acetone containing 0.2 ml of 2 *N* HCl. The combined supernatant fluids were kept in an ice bath. Four experiments were performed similarly. The final supernatant fluids were pooled together and concentrated to near dryness at 35–40°. 200 mg of L-hydantoin-5-propionic acid in 5 ml of water were added, warmed to 50° in a water bath, the solution cooled to room temperature and centrifuged. Aliquots were taken for determination of radioactivity and the bulk of the solution left overnight in a refrigerator. The crystalline hydantoin-5-propionic acid was freed of the mother liquors and recrystallized several times from ice cold water. The results are shown in Table II. It is clear that hydantoin-5-propionic acid is not formed by nonenzymic oxidation of imidazolonepropionic acid.

Reaction of "264 mμ product" with functional reagents and attempts at the isolation of the product: Various attempts to form derivatives of the carbonyl group in the

TABLE II

FAILURE OF NONENZYMIC CONVERSION OF IMIDAZOLONE PROPIONIC ACID TO HYDANTOIN PROPIONIC ACID

Per cent total activity recovered in hydantoin propionic acid in Expt. 1 on basis of specific activity after six recrystallizations = 0.32 %. Recovery in Expt. 2 on basis of specific activity after five crystallizations = 0.40 %.

Expt. No.	*No. of crystallizations*	*Specific activity**	*Total activity in hydantoin propionic acid after correction for losses (counts/min)*
	3	24.3	4860
1	5	7	1400
	6	5	1000
	3	36	7200
2	4	7.5	1500
	5	6.2	1240

* Specific activity is expressed as counts/min/mg after correction for background.

"264 mμ product" (imidazolonepropionic acid) by addition of CN^-, hydroxylamine, semicarbazide or other reagents were without success. The possibility of trapping the lactim form of imidazolonepropionic acid with borate ions in analogy with the experiments of KNOX AND PITT[25] also was of no avail. No change in the spectral characteristics of the "264 mμ product" was noticed by addition of borate ions or by carrying out the urocanase reaction in borate buffer. Since previous experiments indicated that at an acid pH the 234 mμ absorption maximum remained fairly constant for periods of up to 76 h under anaerobic conditions, a large scale incubation using 20 μmoles of urocanic acid was carried out and the reaction terminated with glacial acetic acid.

After removal of the protein, the solution was lyophilized. Only a red material contaminated with inorganic salts was recovered, indicating that the product underwent decomposition.

Nonenzymic degradation of "264 mμ product": FEINBERG AND GREENBERG[6] have reported tentative evidence for the formation of formylisoglutamine from the product of urocanase reaction. Other investigators[26–28] have reported the formation of formylisoglutamine during the enzymic degradation of urocanic acid. The present experiments confirm and extend these observations.

In a representative experiment 200 μmoles of potassium phosphate buffer, pH 7.2, 0.294 μmoles of urocanic acid (27,400 counts/min) were incubated with 220 units of urocanase of specific activity 235 in a total volume of 3 ml. The reaction was terminated after 12 min by addition of 0.4 ml of 10 % perchloric acid and the denatured protein removed by centrifugation. The supernatant fluid was neutralized to pH 7 with 1 *N* KOH and the volume made up to 6 ml. Aliquots were taken for determination of radioactivity. 5 ml of the above solution was applied to a Dowex 1-acetate 30 × 1 cm column and washed with 25 ml of water. The gradient elution device described previously was attached to the column and 3 ml fractions at the rate of 0.3 ml/min were collected. The elution pattern is shown in Fig. 4. The radioactive fractions were individually lyophilized. Fraction C, which contained 53 % of the radioactivity, migrated on paper chromatograms in several solvent systems[4,5] as

authentic formyl-L-isoglutamine. Other fractions were not identified. None of the fractions contained formiminoglutamic acid as tested enzymically. In another experiment when formiminoglutamic acid was added as carrier and subjected to ion exchange chromatography in the above manner, most of the formiminoglutamic acid was found in the nonradioactive zones.

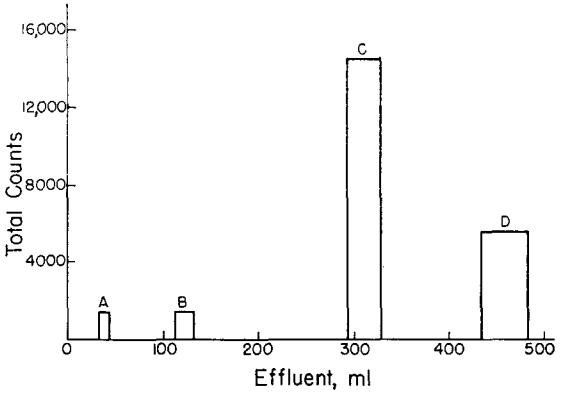

Fig. 4. Chromatographic separation of products of urocanase reaction on Dowex 1-acetate (30 × 1 cm) column.

Isolation of N-formyl-L-isoglutamine

Since sufficient amount of authentic material was not available to perform an isotope dilution experiment on fraction C, a large scale incubation was carried out as described below to secure sufficient N-formyl-L-isoglutamine to establish its identity unequivocally.

1 g of nonradioactive urocanic acid (5740 μmoles) and 15 μmoles of radioactive urocanic acid ($8.7 \cdot 10^6$ counts/min) were dissolved in water with aid of 7 ml of 1 *N* KOH. The pH was adjusted to 7.2 and the volume made up to 200 ml. 90 ml of 0.1 *M* potassium phosphate buffer containing $6.7 \cdot 10^4$ units of urocanase of specific activity 93 were added to the urocanic acid solution and incubated at room temperature for 10 h. The reaction mixture progressively turned yellow during incubation and 90 % of the urocanate disappeared. The reaction was terminated by addition of 5 ml of 60 % perchloric acid and the denatured protein removed by centrifugation at 3°. The supernatant fluid showed absorption maxima at 234 mμ and 260 mμ in the u.v. region and at 365 mμ in the visible region. The pH was cautiously adjusted to 7.2 with 20 % KOH. The spectrum now showed a single u.v. absorption maximum at 266 mμ, which disappeared rapidly with time and an absorption maximum at 398 mμ in the visible region, which did not change over a period of 12 h. The bulk of the solution, which was distinctly yellow, was left overnight at 3° in the refrigerator. The supernatant liquid was decanted from the $KClO_4$ crystals and concentrated to a volume of 35 ml in a rotary evaporator *in vacuo* at 40°.

Samples were taken for determination of radioactivity and the rest of the material was put directly on a Dowex 1-acetate, 33 × 2.5 cm column and washed with 50 ml of water. The radioactive components were eluted from the column as described previously. 8-ml fractions at the rate of 0.8 ml/min were collected. The

elution pattern is shown in Fig. 5. The yellow pigment in solution, which turned red during the concentration of the solution, was adsorbed as a band at the surface of the column. Increasing the concentration of the eluting acid gradually removes traces of the red pigment contaminating some of the fractions.

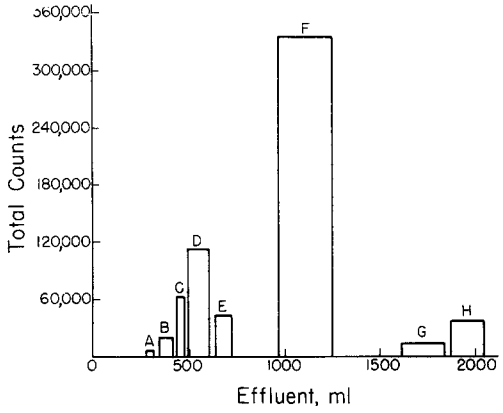

Fig. 5. Chromatographic separation of products of urocanase reaction (large scale incubation) on Dowex 1-acetate column (33 × 2.5 cm)—isolation of N-formyl-L-isoglutamine.

The radioactive fractions were examined on paper for u.v. absorbing material, diazo reagent-reacting substances, ninhydrin-reacting material and for N-substituted amino acids. The results are shown in Table III. The respective fractions were pooled and evaporated at 40° *in vacuo* in a rotary evaporator and 3 to 5 ml of absolute ethanol was added to all the fractions. Fraction "D", which contained approximately 13.4% of the total isotope added to the column, was crystallized from water and identified as unchanged urocanic acid by its u.v. absorption, color tests, chromatographic behavior and infrared spectra. Fraction "F", which contained approximately

TABLE III

RESULTS OF COLOR TESTS AND RADIOACTIVITY DETERMINATION ON VARIOUS FRACTIONS OBTAINED DURING ION-EXCHANGE COLUMN CHROMATOGRAPHY OF PRODUCTS OF UROCANASE REACTION

Explanation of signs: —, negative; ±, doubtful; +++, intense reaction.

Fraction	*Diazo reagent*	*U.v. absorption*	*Ninhydrin reaction*	*Starch–iodide reaction*	*Per cent of total radioactivity**
A	—	—	—	—	0.6
B	—	—	—	—	2.3
C	—	±	—	—	7.6
D	+++	+++	—	—	13.4
E	—	—	±	—	5.1
F	—	—	—	+++	40.8
G	—	±	—	—	1.3
H	—	—	—	—	3.4
Sum of total activity recovered in all individual fractions					74.5

* Approximately 9% of the radioactivity was distributed in various fractions in between the individual peak fractions. The rest of the radioactivity (16%) was held as a pigment on the surface of the column.

41 % of the radioactivity on the column, (or 67 % of radioactivity put on the column after correcting for unchanged urocanic acid), crystallized from ethanol yielding 150 mg of rosette crystals. The compound had a melting point of 133° and the mixed melting point with authentic N-formyl-L-isoglutamine was undepressed. The infrared spectrum of the unknown and authentic samples were identical in every respect (Fig. 6). However, when the sample was crystallized twice from boiling absolute ethanol, the melting point dropped to 128–130° and the optical rotation of a 2 % solution was 6° lower than that of synthetic N-formyl-L-isoglutamine[2]. The infrared spectrum was not significantly changed, but the solution used for optical rotation showed, in addition to large amounts of formylisoglutamine, significant amounts of isoglutamine and a trace of glutamic acid when examined by paper electrophoresis at pH 3.5.

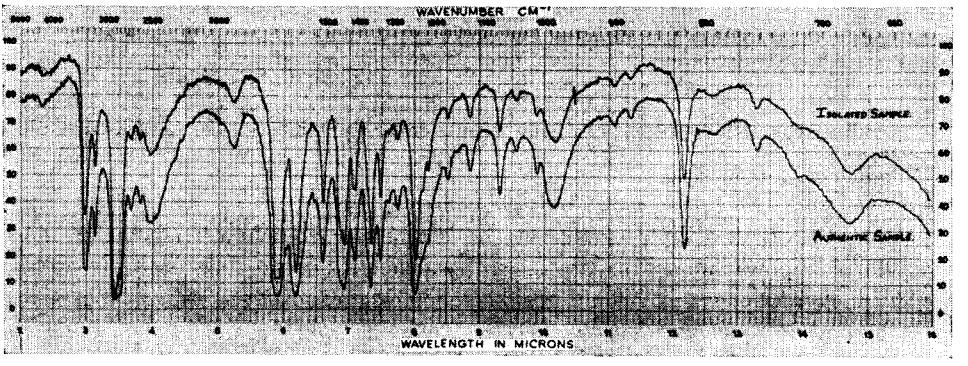

Fig. 6. Infrared spectrum of authentic and isolated samples of N-formyl-L-isoglutamine in Nujol mull. Beckman IR-5 infrared spectrophotometer was used. The peaks at wave lengths of 3.45, 3.52, 6.9, 7.35 represents the absorption of Nujol.

Since none of the original column fractions contained ninhydrin-reacting substances but contained significant amounts of N-substituted amino acids, it can be concluded that N-formyl-L-isoglutamine is the principal nonenzymic degradation product of imidazolonepropionic acid.

Imidazolonepropionic acid —→ formiminoglutamic acid

Because of the apparent instability of imidazolonepropionic acid and the failure of attempts to synthesize this compound, we had to resort to a multienzyme system to demonstrate the overall conversion of urocanic acid to formiminoglutamic acid. A typical experiment is described below.

The reaction mixture contained 200 μmoles of potassium phosphate buffer, pH 7.2, 0.2 μmoles of urocanic acid, 260 units of urocanase of specific activity 406 in a total volume of 1 ml. Exactly after 10 min incubation, an excess of formiminoglutamic transferase, and a source of imidazolonepropionic acid hydrolase (crude extracts of various tissues) were added. Finally 0.1 ml of folate·H_4 solution in 1 *M* potassium phosphate buffer, pH 7.2, containing 0.4 mg of folate·H_4 and 15 μmoles of mercaptoethanol were added. The final volume was 1.6 ml. The reaction mixture was incubated for a further period of 30 min at room temperature. The reaction was terminated by addition of 0.4 ml of 10 % perchloric acid and the contents heated

for 1 min in a boiling water bath to convert the formimino-FAH_4 to 5,10-methenyl-folate·H_4 and cooled in ice. It was centrifuged to remove denatured protein and $KClO_4$. The O.D. of the clear supernatant liquid was measured at 350 mμ in 1.5 ml cuvettes. Various combinations of the enzymic reaction mixture components were run as blanks to correct for nonenzymic and other side reactions. The results are shown in Table IV.

TABLE IV

ENZYMIC CONVERSION OF IMIDAZOLONEPROPIONIC ACID TO FORMIMINOGLUTAMIC ACID

Experimental details described in text.

Reaction components	*Net O.D. at 350 mμ*	*μmoles of folate·H_4 5,10-methenyl-FA·H_4 formed**	*Overall conversion of urocanic acid to formiminoglutamic acid (per cent)*
Complete system	0.77	0.0616	30.8
−crude extract	0.10	0.0080	4.0
−crude extract and FIGLU** transferase	0	0	0
−crude extract and urocanase	0.04	0.0032	1.6
−urocanic acid	0	0	0

* A molar extinction coefficient of 25,000 for 5,10-methenyl folate·H_4 has been used in these calculations.

** Formiminoglutamic acid.

Using the above assay procedure we have been able to demonstrate the presence of imidazolonepropionic acid hydrolase in homogenates of rat, beef, calf, sheep and hog livers. A trace of activity could be detected in extracts of *Pseudomonas fluorescens*. Liver homogenates of various species also caused a rapid disappearance of the absorption maximum at 264 mμ of imidazolonepropionic acid. Preliminary experiments indicate that the cleavage of the imidazolone ring does not appear to require divalent metal ions, since supplementation of the above multi-enzyme system with Mg^{++}, Mn^{++}, or Ca^{++} ions did not increase the overall yield of formiminoglutamic acid. Further, the presence of EDTA in the above assay system did not decrease the overall yield. Further studies on the enzyme system which converts imidazolone propionic acid to formiminoglutamic acid are in progress.

DISCUSSION

Previous work by FEINBERG AND GREENBERG[6], has indicated that the enzymic conversion of urocanic acid to formiminoglutamic acid proceeds through an intermediate having an absorption maximum at 264 mμ. These observations have now been confirmed and extended by isotopic experiments with highly purified beef liver urocanase. The enzymic degradation of urocanic acid by the purified enzyme yields a product whose spectral characteristics, stability, lability to oxygen, structure of nonenzymic degradation products (N-formyl-L-isoglutamine) and enzymic degradation product (N-formimino-L-glutamic acid) are strongly indicative of a substituted imidazolone. Evidence that no formiminoglutamic acid was formed in the urocanase reaction was obtained by isotope trapping experiments and also by the failure of the urocanase reaction to couple with the formiminoglutamic transferase system of TABOR AND WYNGARDEN[13]. However, when the same system was supplemented with crude extracts of liver of various species and tetrahydrofolic acid, the overall conversion of

urocanic acid to formiminoglutamic acid could be demonstrated. These experiments also demonstrate the existence of the enzyme imidazolonepropionic acid hydrolase, which catalyzes the formation of formiminoglutamic acid from imidazolonepropionic acid.

Highly purified fractions of beef liver urocanase (specific activity of 400 or more) show, in addition to the usual 280 mμ absorption maximum, an inflexion at 290 mμ and indications of another inflexion in the 400–425 mμ region. The cause of these anomalous spectra is not known at the present time. ROBINSON[29] has also reported similar observations with purified urocanase from chicken liver.

Evidence that the product of urocanase reaction is the suspected 4(5)-imidazolone-5(4)-propionic acid is based primarily on the striking similarity of its spectral characteristics at acid, alkaline and neutral pH to synthetic 4(5)-imidazolone, and 4(5)-imidazolone-5(4)-acetic acid. The last two compounds have been shown by FRETER, RABINOWITZ AND WITKOP[30] and KNY AND WITKOP[31] to be degraded to formiminoglycine and formimino aspartic acid by extracts of *Clostridium cylindrosporum* and *Pseudomonas fluorescens* respectively. Synthetic 2-methyl-5(4)-imidazolone-4(5)-propionic acid ethyl ester, which differs from 4(5)-imidazolone-5(4)-propionic acid in having an ester group on the propionic acid side chain and a methyl group on the 2 position of the imidazolone ring, shows very similar spectral characteristics to the compound under investigation*. However, these spectral characteristics are reversible with change in pH, in contrast to that of 4(5)-imidazolone-5(4)-propionic acid, which is presumably due to the stabilization of the imidazolone ring by substitution at carbon atom 2.

The instability of imidazolones unsubstituted in position 2 is well known[32]. The compound under investigation was labile to base or oxygen in analogy with the imidazolones (unsubstituted in position 2) mentioned above. The formation of colored products during attempted isolation of the enzymic reaction product is probably due to the formation of dimers, a property characteristic of imidazolones[32,33]. Isotopic experiments in the present study have clearly indicated that N-formyl-L-isoglutamine is the principal nonenzymic hydrolytic degradation product of 4(5)-imidazolone-5(4)-propionic acid and that isoglutamine is formed by decomposition of N-formyl-L-isoglutamine during the isolation process. The highly reactive nature of imidazolonepropionic acid may be of some significance in the formation of hydantoin-5-propionic acid, since this reaction involves the oxidation of carbon 2 of the imidazolone ring. It is interesting to note that the reverse reaction, namely, the reduction of hydantoins to imidazolones has been demonstrated by chemical methods[34]. Hydantoin-propionic acid is not formed nonenzymically from imidazolone propionic acid by oxidation with dichlorophenolindophenol. It is probably formed enzymically from imidazolonepropionic acid, although such a reaction has not been demonstrated so far in purified enzyme systems. Hydantoin acrylic acid[35] has been implicated as an enzymic oxidation product of urocanic acid in *Pseudomonas aeroginosa* and in rabbit liver[36].

ADDENDUM

After this paper was written the report of BROWN AND KIES appeared (*J. Biol. Chem.*, 234 (1959) 3182, 3188) in which the partial purification of 4(5)-imidazolone 5(4)-

* An alcoholic solution of 2-methyl-4-imidazolonepropionic acid ethyl ester had an absorption maximum at 230 mμ which was shifted to 256 mμ by addition of NaOH. The original 230 mμ maximum reappeared when acidified (D. R. RAO AND D. M. GREENBERG, unpublished results).

propionic acid on Dowex-50 ion exchange resin was described. They have made identical observations regarding its stability and also demonstrated its enzymic conversion to L-hydantoin 5-propionic acid.

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