The Purification and Properties of Urocanase from *Pseudomonas testosteroni*

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Urocanase (urocanate hydratase, EC 4.2.1.49) purified from *Pseudomonas testosteroni* has a mol.wt. of 118000 determined by sedimentation-equilibrium analysis. Ultracentrifugation in 6M-guanidine hydrochloride and polyacrylamide-gel electrophoresis in sodium dodecyl sulphate show that the enzyme consists of two identical or very similar subunits. It is, like urocanase isolated from other sources, inhibited by reagents that react with carbonyl groups. Although urocanase from *Ps. testosteroni* is strongly inhibited by NaBH_4 , no evidence could be obtained for the presence of covalently bound 2-oxobutyrate as a prosthetic group; this is in contrast with findings elsewhere for urocanase from *Pseudomonas putida*. Urocanase from *Ps. testosteroni* does not contain pyridoxal 5'-phosphate as a coenzyme and in this respect is similar to all urocanases studied in purified form.

Urocanase (urocanate hydratase, EC 4.2.1.49) catalyses the conversion of urocanic acid (*trans*-imidazolylacrylic acid) into imidazolone-4-yl-5-propionic acid during the degradation of histidine.

The enzyme has previously been purified from several sources, including *Pseudomonas fluorescens* (Tabor & Mehler, 1955), *Klebsiella aerogenes* (Revel & Magasanik, 1958), ox liver (Rao & Greenberg, 1960; Hassall & Greenberg, 1971), chicken liver (Gupta & Robinson, 1961), cat liver (Swaine, 1969), *Bacillus subtilis* (Magasanik *et al.*, 1970) and *Pseudomonas putida* (George & Phillips, 1970; Hug & Roth, 1971).

The molecular weight of urocanase from cat liver was estimated to be 127000 by gel filtration (Swaine, 1969) and the value for the enzyme from *B. subtilis* was 120000 by ultracentrifugation on sucrose density gradients. The most rigorous studies of the molecular weight and subunit composition of urocanase are those by George & Phillips (1970) and Lynch & Phillips (1972) with the enzyme from *Ps. putida*, which has a mol.wt. of 110000 ± 4000 and consists of two identical or very similar subunits.

Because of the inhibition of urocanase by reagents specific for carbonyl groups, pyridoxal 5'-phosphate was first proposed as a coenzyme for the enzyme (Gupta & Robinson, 1961), but subsequently this was shown to be absent from active urocanase from *Ps. putida* (George & Phillips, 1970). It was then

suggested, primarily on the basis of inhibition and labelling with NaB^3H_4 and subsequent recovery of 2-[^3H]hydroxybutyrate, that the enzyme from *Ps. putida* contained one covalently bound molecule of 2-oxobutyrate per subunit that was essential for catalytic activity.

The investigations on urocanase from *Pseudomonas testosteroni* were carried out to determine to what extent the properties of this enzyme were similar to those of urocanase from *Ps. putida*, particularly with respect to the involvement of 2-oxobutyrate.

Materials and Methods

Chemicals

Urocanic acid and imidazolylpropionic acid were synthesized as described by Coote & Hassall (1973). Other chemicals were purchased from the following sources: imidazolylpyruvate from Calbiochem, London W.1, U.K.; L-histidine hydrochloride from Cambrian Chemicals, Croydon CR9 3QL, Surrey, U.K.; Tris, pyridoxal 5'-phosphate and imidazolyl-L-lactate from Sigma (London) Chemical Co., London S.W.6, U.K.; radioactive compounds from The Radiochemical Centre, Amersham, Bucks., U.K.; trypsin from Miles-Seravac, Holyport, Maidenhead, Berks., U.K.; DEAE-cellulose (DE-32) from W. and R. Balston, Maidstone, Kent, U.K. All other chemicals were purchased from BDH Chemicals, Poole, Dorset, U.K., and were of AnalaR grade where available. Guanidine hydrochloride was recrystallized from hot ethanol and benzene, and from methanol, before use (Nozaki & Tanford, 1967). Trypsin was

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treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (TPCK) as described by Carpenter (1967).

Buffers. Phosphate buffer consisted of KH_2PO_4 with the pH adjusted as required with 5M-NaOH. Tris/HCl buffer consisted of Tris of the required concentration adjusted to the desired pH with 6M-HCl.

Maintenance and growth of the organism

Pseudomonas testosteroni (N.C.I.B. 10808) was maintained on nutrient-agar slopes and grown in batches of 100 litres in a medium containing L-histidine as the source of nitrogen, and succinate as the source of carbon (Hassall & Soutar, 1974; Hacking & Hassall, 1975). The cells were harvested while in the exponential phase and stored frozen at -18°C until required.

Assay of urocanase

Urocanase was assayed at 30°C in a Gilford 2000 multiple-sample absorbance recorder fitted with a Unicam SP.500 monochromator. The reaction mixture contained $0.2\mu\text{mol}$ of urocanate, 2.8ml of 0.1M-phosphate buffer (pH 7.0), a suitable volume of enzyme preparation (usually 0.1 ml or less) and water to 3 ml. The rate of disappearance of urocanate was measured by following the A_{277} of the reaction mixture (ϵ_{277} for urocanate, $18800\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$; Tabor & Mehler, 1955).

No attempt was made to correct the activities for the A_{277} of the product. Imidazolonylpropionate has an ϵ_{277} of approx. $2350\text{ litre}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ at neutral pH, but decomposes by first-order kinetics with a half-life of some 25 min (Hassall & Greenberg, 1963). With relatively pure preparations of urocanase that are free from imidazolonylpropionate hydrolase activity, the true urocanate activity may be some 10–15% higher than that given. One unit of enzyme activity is defined as the amount of enzyme apparently transforming $1\mu\text{mol}$ of substrate/min under the conditions of the assay.

Detection of urocanase on polyacrylamide gels

Urocanase was located on polyacrylamide gels by using 2,6-dichlorophenol-indophenol (Hassall *et al.*, 1970) or methylphenazonium methosulphate and Nitro Blue Tetrazolium (Bell & Hassall, 1976).

Determination of protein

In crude systems, protein concentration was determined by the method of Lowry *et al.* (1951), with freeze-dried crystalline bovine serum albumin as the standard. The elution of protein from chromatography columns was monitored by measuring the A_{280} of the eluate.

Purification of urocanase

Step 1: preparation of a cell-free extract. The cells

were thawed and resuspended in 5vol. of 0.1M-potassium phosphate buffer, pH 7.0, at $0-4^\circ\text{C}$. The suspension was then passed three times through a Manton-Gaulin laboratory homogenizer (Manton-Gaulin Laboratory Homogenizer and Sub-micron Disperser, type 15-M8-BA, obtained from A.P.V. Co., Manor Royal, Crawley, Sussex, U.K.) at a pressure of approx. 55 MPa (8000 lbf/in^2) at the outlet. The homogenate was cooled again to $0-4^\circ\text{C}$ and centrifuged at $30000g$ for 45 min.

Step 2: protamine sulphate treatment. Protamine sulphate solution (15 mg/ml in 0.1M-potassium phosphate buffer, pH 7.0) was added dropwise and with stirring to the supernatant obtained from the previous step to give a final concentration of 2.5 mg of protamine sulphate/10 mg of bacterial protein. The suspension was equilibrated for 30 min at room temperature (20°C) and the precipitate then removed by centrifugation at $30000g$ for 20 min.

Step 3: precipitation with $(\text{NH}_4)_2\text{SO}_4$. $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant, with stirring, to give 40% saturation (24.3 g/100 ml) at 4°C . The pH of the solution was maintained at 7.0 by the addition of 2M- NH_3 . After equilibration at 4°C for 30 min the resultant precipitate was removed by centrifugation ($38000g$ for 20 min) and discarded. More $(\text{NH}_4)_2\text{SO}_4$ (14.2 g/100 ml) was then added to the supernatant to give 60% saturation. After equilibration of the suspension as before, the precipitate was collected by centrifugation as above and the supernatant discarded. The precipitate was dissolved in a minimum volume of 0.1M-potassium phosphate buffer, pH 7.0, and dialysed overnight against two changes of 2 litres of the same buffer.

Step 4: calcium phosphate gel treatment. Calcium phosphate gel was prepared by the method of Keilin & Hartree (1938) and used at a concentration of 12 mg dry wt./ml . Different batches of gel varied slightly in their binding capacity for urocanase and contaminating proteins. Pilot experiments were therefore carried out to determine the maximum amount of gel that could be added without adsorbing urocanase. A ratio of approx. 3:4 (w/w) of gel dry wt. to protein dry wt. was normally found to be correct. The gel suspension was added to the enzyme preparation at $0-4^\circ\text{C}$ with stirring and the mixture allowed to equilibrate for 30 min at this temperature. The gel was then removed by centrifugation at $10000g$ for 10 min and discarded.

Step 5: DEAE-cellulose chromatography. The supernatant from the previous step was diluted with water to give a buffer concentration of 0.01M and then applied to a column ($8\text{ cm}\times 5\text{ cm}$) of DEAE-cellulose that had been equilibrated with 0.01M-phosphate buffer, pH 7.0. The column was washed with 1 bed-volume of the same buffer, and a linear gradient of 0.01–0.15M-potassium phosphate buffer (pH 7.0) in a total volume of 1 litre was applied.

Urocanase was eluted at a concentration of approx. 0.08M-phosphate.

The fractions containing urocanase were pooled and dialysed overnight against two changes of 2 litres of 0.01M-pyrophosphate buffer ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$ adjusted to pH8.5 with 6M-HCl). This enzyme solution was then applied to a second column (10cm $\times$ 2cm) of DEAE-cellulose that had been equilibrated with the 0.01M-pyrophosphate buffer. A wash with 0.02M-pyrophosphate buffer, pH8.5, was used to elute the urocanase from the column. This process resulted in the removal of traces of contaminating proteins and also produced some concentration of the enzyme.

The purification procedure is summarized in Table 1.

Concentration of protein solutions

Protein solutions were concentrated by using an Amicon model 52 ultrafiltration cell with a Difco PM 30 membrane. The ultrafiltration was carried out at 4°C with stirring and under N_2 at a pressure of approx. 206kPa (30lbf/in²).

Electrophoresis of protein

Polyacrylamide-gel electrophoresis of native proteins was performed at pH8.6 as described by Davis (1964). Gels at a concentration of 7.5% (w/v) acrylamide were used and the proteins were stained with 1% (w/v) Amido Black in 7% (v/v) acetic acid. Approx. 45 μ g of protein was applied to each gel.

Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate was carried out by the method of Weber & Osborn (1969). Urocanase and the reference proteins were first carboxymethylated as described by Hassall & Soutar (1974) and electrophoresis was performed in the presence of 0.1% (w/v) sodium dodecyl sulphate and 0.1% (v/v) mercaptoethanol on gels containing 10% (w/v) acrylamide. A current of 8mA per tube was passed for 4h and, after staining, the mobility of each protein band was calculated relative to the mobility of Bromophenol Blue.

Analytical ultracentrifugation

Protein solutions were first dialysed for 24h against the appropriate buffer. Where 6M-guanidine hydro-

chloride, containing 0.1% (v/v) mercaptoethanol, was used as a denaturant, dialysis was for 3 days. Sedimentation-velocity and sedimentation-equilibrium studies were carried out in a Beckman model E ultracentrifuge fitted with schlieren and interference optical systems. Distances on photographic plates were measured in a projectorscope (Precision Grinding Ltd., Mitcham Junction, Surrey, U.K.).

Sedimentation-velocity experiments were done in a cell with a 12mm aluminium single-sector centre-piece in an An-D rotor. Photographs were taken with the schlieren optical system and sedimentation coefficients calculated from the rate of movement of the boundary.

Sedimentation-equilibrium experiments were done in a cell with sapphire windows and a 12mm double-sector centre-piece, one channel of which contained 0.1 ml of protein solution (0.3–0.5 mg/ml) and the other contained 0.1 ml of the diffusate from the dialysis bath; both sectors contained 0.01 ml of fluorocarbon oil. Rotor speeds were chosen that would result in sample depletion at the meniscus (Yphantis, 1964), and photographs were taken after 40h by using both schlieren and Rayleigh interference optical systems. With 6M-guanidine hydrochloride, at the higher rotor speeds necessary to achieve meniscus depletion, a blank run was carried out with the solvent in both sides of the double-sector centre-piece. The readings obtained for the protein samples were corrected for the slight window distortion observed during the blank run.

Buffer densities were obtained by weighing 10ml samples of the solutions and comparing these weights with those of similar volumes of water. The densities, at 20°C, of 0.15M-KCl in 0.05M-potassium phosphate buffer, pH7.0, and of 6M-guanidine hydrochloride containing 0.1% (v/v) mercaptoethanol, were 1.013 and 1.136g/ml respectively. The partial specific volume of urocanase ($\bar{v}$) was calculated to be 0.73 ml/g from the amino acid composition. This value was decreased by 0.01 ml/g when 6M-guanidine hydrochloride was used (Hade & Tanford, 1967).

Data from Rayleigh interference photographs obtained at equilibrium were used for the calculation of molecular weights by the method of Yphantis

Table 1. *Purification of urocanase*

One unit of activity is the amount of enzyme catalysing the utilization of 1 μ mol of urocanate/min at 30°C.

Step	Volume (ml)	Activity (munits/ml)	Protein (mg/ml)	Specific activity (munits/mg)	Yield (%)
Crude extract	730	82	10.25	8	100
Protamine sulphate	810	85	6.07	14	115
40–60%-satd. $(\text{NH}_4)_2\text{SO}_4$	125	496	21.56	23	104
Calcium phosphate gel	244	204	6.80	30	84
DEAE-cellulose chromatography	251	181	0.73	248	76

(1964). The schlieren photographs of the same experiments were used for the calculation of z -average molecular weights by the procedure described by Lamm (1929).

Amino acid analysis

Samples of urocanase were hydrolysed with 6M-HCl in sealed evacuated tubes for 24, 48 or 96 h at 110°C. Amino acids were determined quantitatively by using an automatic analyser (Beckman Unichrom amino acid analyser) fitted with a high-sensitivity flow cell. Values for serine and threonine were extrapolated to zero-time hydrolysis, and half-cystine was determined as cysteic acid in hydrolysates of performic acid-oxidized samples (Hirs, 1967).

Tryptic digestion of [^{14}C]carboxymethylated urocanase and the preparation of peptide 'maps'

The methods for the carboxymethylation of urocanase with iodo[^{14}C]acetate and its subsequent digestion with trypsin and the preparation of peptide 'maps' were those used by Hassall & Soutar (1974).

Chromatography and electrophoresis of tryptic peptides and acid hydrolysates of urocanase

The system used for the separation of tryptic peptides consisted of chromatography in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) (Waley & Watson, 1953) followed by high-voltage electrophoresis in pyridine/acetic acid/water (1:10:89, by vol., pH 3.5) (Katz *et al.*, 1959). High-voltage electrophoresis of acid hydrolysates of urocanase was also done in this buffer and at pH 6.5 in pyridine/acetic acid/water (100:3:900, by vol.) (Beale, 1969).

Measurement of radioactivity

Samples were assayed for ^3H in a multi-channel Beckman liquid-scintillation system LS-200B with a scintillation fluid consisting of 5 g of 2,5-diphenyl-oxazole and 100 g of naphthalene made up to 1 litre with 1,4-dioxan. Scintillation fluid (5 ml) was normally added to 0.1 ml of aqueous sample, except where radioactivity was counted in paper. Observed radioactivities (c.p.m.) were corrected to absolute radioactivities (d.p.m.) by using a calibration curve constructed from a plot of external standard ratio against efficiency.

The positions of ^3H -labelled compounds after high-voltage electrophoresis were determined by cutting the appropriate 2 cm-wide track into 1 cm strips. Each piece of paper was placed in a scintillation vial with 0.5 ml of water and left at room temperature for 1 h before 5 ml of scintillation fluid was added. Because of the uncertainty with respect to counting efficiency the data were expressed as c.p.m.

Analysis of protein hydrolysates for pyridoxal derivatives

The method used for the assay of pyridoxal derivatives was that described by Barton-Wright (1961) with *Kloeckera apiculata*. The assay system consisted of 1 ml of the assay medium and sample and water to give a total volume of 5 ml. This was then inoculated with 1 drop of a cell suspension of the test organism and incubated for 24 h in the dark at 25°C with vigorous shaking. The A_{540} of the culture was then measured and compared with readings obtained by using standard amounts of pyridoxal or pyridoxine. An A_{540} of 1.0 was given by 8–10 ng of standard.

Results

Homogeneity of the enzyme preparation

The purified enzyme gave only one band detectable with Amido Black after disc electrophoresis in 7.5, 10 and 15% (w/v) polyacrylamide gels. In each case, the stainable band coincided with the previously detected urocanase activity band.

The enzyme was stable for many weeks when stored frozen (–18°C) in 0.1M-phosphate buffer, pH 7.0. Under these conditions there was the slow formation of another molecular species that, judged by its mobility in polyacrylamide gels relative to the original enzyme form, was a dimer. This dimeric form still showed activity when the gels were assayed for urocanase, although from a visual comparison of the relative intensities of protein bands and activity bands it is probable that dimerization led to a decrease in specific activity. The dimeric enzyme could not be dissociated with thiols such as reduced glutathione, mercaptoethanol or thioglycollate.

The specific activity of the purified enzyme was less than that of the urocanase isolated from *Ps. putida* A.T.C.C. 12633 (George & Phillips, 1970) and of the photoactivated form from *Ps. putida* A 3.12 (Hug & Roth, 1971). Using the methods of Hug & Roth (1971), we could not observe any photoactivation of urocanase from *Ps. testosteroni* either during the purification procedure or after prolonged storage of the enzyme under the conditions described above.

Absorption coefficient

The $A_{1\text{cm}}^{1\%}$ for purified urocanase at pH 7.0 was found to be 9.22 at 280 nm by determining the dry weight of freeze-dried solutions of urocanase of known absorption and correcting for the weight of buffer salts present. The ratio A_{280}/A_{260} was 1.63. Some preparations were faintly yellow and gave a low broad peak at 400 nm ($A_{1\text{cm}}^{1\%} = 0.4$).

Sedimentation coefficient

A preparation of the enzyme, at five different concentrations between 3.0 and 15.0 mg/ml, was subjected

to analytical ultracentrifugation at 56000 rev./min. In each case, the enzyme sedimented as a single symmetrical boundary, giving linear plots of $\log r$ against time. The linear plot of s against protein concentration when extrapolated to zero concentration gave a value of 7.15S for s_{20}^0 , and a corrected value for $s_{20,w}^0$ of 7.6S.

Molecular weight by sedimentation equilibrium

Two separate meniscus-depletion sedimentation-equilibrium runs were carried out at different concentrations of the enzyme. After 40h, photographs were taken by using Rayleigh interference optics, and these were used for the calculation of weight-average molecular weights. For one of the runs, a schlieren photograph was also taken at equilibrium and used for the calculation of a z-average molecular weight. Fig. 1(a) shows a plot of $\log(\text{fringe displacement})$ [$\log(y_F - y_0)$] against r^2 [Yphantis (1964) plot] and Fig. 1(b) a Lamm (1929) plot for the same run. Weight-average mol.wts. of 118000 and 120000 were obtained with solutions containing 0.68 and 0.95mg of protein/ml respectively, and a z-average mol.wt. of 126000 was obtained with a protein concentration of 0.95mg/ml.

Subunit structure of urocanase

(i) *Sedimentation equilibrium in guanidine hydrochloride solutions.* Two meniscus-depletion sedimentation-equilibrium runs were carried out at 37020 and 33490 rev./min on urocanase at 0.89 and 1.15mg/ml respectively. The preparations had been previously dialysed for 3 days against 6M-guanidine hydrochloride containing 0.1% (v/v) mercaptoethanol. The Yphantis (1964) and Lamm (1929) plots of the data were linear over the whole range of values for r^2 and gave weight-average mol.wts. of 51950 and 50000, and a z-average mol.wt. of 49750.

(ii) *Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate.* Samples of the enzyme and reference proteins in various combinations (up to four per gel) were subjected to polyacrylamide-gel electrophoresis in 0.1% (w/v) sodium dodecyl sulphate. A plot of $\log(\text{molecular weight})$ against mobility relative to Bromophenol Blue was linear. The reference proteins, together with their molecular weights and mobilities, each value being the mean of four determinations, were as follows: bovine serum albumin (68000), 0.19; pyruvate kinase (57000), 0.25; ovalbumin (43000), 0.36; lactate dehydrogenase (36000), 0.44; chymotrypsinogen

Fig. 1. High-speed sedimentation-equilibrium experiment with native urocanase

Protein concentration was initially 0.95 mg/ml in 0.15M-KCl/0.05M-KH₂PO₄, pH 7.0. The sample was centrifuged at 19160 rev./min for 40h at 20°C. (a) Yphantis (1964) plot of data from an interference photograph. (b) Lamm (1929) plot of data from a schlieren photograph of the same run.

(25700), 0.56; β -lactoglobulin (18400), 0.67; ribonuclease A (13700), 0.76. Samples of urocanase gave a single band with a mobility (0.23–0.24) corresponding to a subunit mol.wt. of 58000–59000.

Amino acid analysis

The amino acid composition of urocanase from *Ps. testosteroni* is shown in Table 2.

Peptide 'maps' of urocanase

Denatured, reduced and carboxymethylated enzyme was digested with trypsin for 4h. During this time, the insoluble preparation was completely solubilized. Peptide 'maps' were then prepared by chromatography and electrophoresis as described in the Materials and Methods section. These 'maps' were generally reproducible and the proportion of tryptic cores appeared to be small, as evidenced by the small amount of material remaining on the origin. Some 55–60 ninhydrin-positive peptides were obtained.

When urocanase was fully reduced in 6M-guanidine hydrochloride with dithiothreitol, and carboxymethylated with iodo[14 C]acetate, eight radioactive peptides were located by radioautography of the subsequent peptide 'map'. No labelled peptides were detected on 'maps' of tryptic digests of urocanase that had been incubated in the native state with iodo[14 C]acetate before reduction and carboxy-

methylation with unlabelled iodoacetate in the usual way.

Optimum pH for urocanase activity

The activity of urocanase in the pH range 5.5–7.8 was measured in 0.1M-KH₂PO₄, adjusted to the appropriate pH with 5M-NaOH. Tris/acetate buffer (0.01M) was used over the range pH 7.0–9.0. Activities were 15% lower in this buffer than in 0.1M-phosphate buffer at the same pH. Tris, triethanolamine and aminomethylpropanediol buffers all caused inhibition of urocanase activity that was dependent on their concentrations. Activities in 0.01M-phosphate buffer were approx. 10–15% higher than in the same buffer at 0.1M. Measured rates were corrected for the variation of urocanate absorption with pH. The enzyme had a sharp pH optimum at pH 7.2.

Michaelis constant for urocanase

Six carbonyl-group reagents were studied here as possible inhibitors of the urocanase reaction. Because of the report of Hug & Roth (1973) on the inhibition of urocanase by Cu²⁺, 11 μ M-EDTA was present in all reaction mixtures unless otherwise stated.

For investigation of the effects of hydrazine, phenylhydrazine and semicarbazide, the enzyme was incubated with the inhibitor in the absence of substrate. At time intervals over several hours, samples of

Table 2. Amino acid composition of urocanase

Samples were hydrolysed for 24, 48 and 96h, and mean or extrapolated values are used. Cysteine and cystine were determined as cysteic acid after performic oxidation of separate samples; tryptophan was not determined.

	Urocanase from <i>Ps. testosteroni</i>		Urocanase from <i>Ps. putida</i> *
	Amount present (nmol/mg)	Residues/molecule (mol.wt. 118000)	Residues/molecule (mol.wt. 110000)
Aspartic acid	880	104	107
Threonine	540	64	50
Serine	345	41	47
Glutamic acid	860	101	100
Proline	385	45	49
Glycine	875	103	107
Alanine	1020	120	117
Half-cystine	135	16	12
Valine	400	47	61
Methionine	175	21	26
Isoleucine	415	49	40
Leucine	1055	125	91
Tyrosine	215	25	29
Phenylalanine	345	41	28
Lysine	545	64	42
Histidine	245	29	24
Arginine	620	73	58
Ammonia	Not determined	—	108

* Data of George & Phillips (1970).

the enzyme were removed and assayed in the usual way with the inhibitor present at the preincubation concentration. Hydrazine at 0.02M had no effect on the rate of the urocanase reaction. Phenylhydrazine (0.2M) inhibited the enzyme by only 14% after 3h, but semicarbazide (0.02M) was slightly more effective, giving 53% inhibition after a 4h incubation.

Hydroxylamine inhibited much more quickly and at lower concentration than did the other carbonyl-group reagents. Concentrations of 0.1 mM and 0.2 mM were used, and the enzyme was incubated for 15 min at 30°C with the inhibitor before being assayed over a range of substrate concentrations. Double-reciprocal plots of rate and substrate concentration were linear and showed the inhibition to be non-competitive, giving values of 0.20 mM and 0.18 mM for K_i at the two different concentrations of hydroxylamine. The inhibition was reversible. Hydroxylamine (10 mM) completely inhibited urocanase, but when this sample was diluted 300-fold (to give 0.033 mM-hydroxylamine during the assay) 53% of the initial activity was regained.

Results obtained with NaHSO_3 were similar, but the reversal of inhibition on dilution was not as great. Double-reciprocal plots of rate against urocanate concentration were again linear and non-competitive with 0.003 mM- and 0.01 mM- NaHSO_3 , giving values of 0.019 mM and 0.021 mM for K_i respectively. NaHSO_3 at 0.1 mM completely inactivated the enzyme, but after a 300-fold dilution there was a slight (6.6%) recovery of activity.

The enzyme was also inhibited by NaBH_4 , solutions of which were prepared in 0.02M- NaOH . The extent of the inhibition was 23% and 63% at 1 mM- and 5 mM- NaBH_4 respectively.

Analysis for pyridoxal derivatives as a coenzyme

By the microbiological assay, calibration curves were obtained with untreated pyridoxal and pyridoxine (0–12 ng per assay) and for similar samples after hydrolysis at 110°C under N_2 with 6M-HCl for 6h. Controls were also carried out with bovine serum albumin, β -lactoglobulin, ovalbumin and bovine serum albumin to which equimolar amounts of pyridoxal had been added. These samples, and the urocanase to be tested, were hydrolysed, as described above, for 6h. Samples of these hydrolysates were then tested in the microbiological assay system.

The standard pyridoxal was stable to hydrolysis for 42h, but only 30–40% of pyridoxine survived this treatment. The protein controls did not support growth in the assay system, but pyridoxal was completely recovered from the hydrolysate of the mixture of bovine serum albumin plus pyridoxal. The hydrolysates of urocanase gave completely negative results when assayed in amounts that would have given 8.6, 17.2 and 25.8 ng of pyridoxal had this been present at

a concentration of 1 mol/mol of enzyme subunit. It was concluded that no vitamin B-6 derivative acts as a coenzyme for urocanase from *Ps. testosteroni*.

Treatment of urocanase with NaB^3H_4 and the attempted isolation of 2-[^3H]hydroxybutyrate

The enzyme was treated with NaB^3H_4 by a similar method to that used by George & Phillips (1970).

A preparation of urocanase (7.5 mg) in 2.4 ml of 0.3M-phosphate buffer, pH 7.0, was incubated with five 0.2 ml volumes of 0.2M- NaB^3H_4 (125 $\mu\text{Ci}/\mu\text{mol}$). The additions of the NaB^3H_4 were accompanied by similar volumes of 0.2M-acetic acid and were made at 3 min intervals. After the final addition, by which time the nominal concentration of NaB^3H_4 was 45.5 mM, the reaction mixture was left at room temperature for 30 min. The enzyme was completely inactivated by this treatment.

The preparation was then dialysed for 2 days against 5×2 litres of 0.01M-phosphate buffer, pH 7.0, and freeze-dried. When redissolved in 2 ml of 0.1M-Tris/HCl buffer, pH 8.5, the sample was found to contain 26×10^6 d.p.m. of ^3H and to have regained 29.5% of its original activity. This indicated that complete inactivation of the enzyme could correspond to an incorporation of 4.4 μg -atoms of ^3H per subunit.

A sample of the ^3H -labelled protein was hydrolysed at 110°C *in vacuo* in 6M-HCl for 24h. After evaporation to dryness, the residue was redissolved in 0.2 ml of water and a portion was assayed for ^3H . Only 12.8% of the original radioactivity remained, none of which ran with 2-hydroxybutyrate when the hydrolysate was subjected to high-voltage paper electrophoresis at pH 3.5.

The remainder of the ^3H -labelled protein was dissolved in 6M-guanidine hydrochloride, reduced with dithiothreitol and carboxymethylated with iodoacetate. After dialysis for 3 days against 0.01M- NH_4HCO_3 , pH 8.5, 71.5% of the radioactivity remained. The sample was then freeze-dried, dissolved in 2 ml of 0.5M- NH_4HCO_3 , pH 8.5, and digested with two additions of trypsin (each 25 $\mu\text{g}/\text{mg}$ of enzyme protein) for a total of 6h. The digest was freeze-dried and suspended in 1 ml of water; 38.3% of the initial radioactivity remained at this stage. When portions of this tryptic digest were chromatographed in butan-1-ol/acetic acid/water/pyridine, or subjected to paper electrophoresis at pH 3.5, no single radioactive peak was detectable. Radioactivity was spread throughout the length of the chromatogram, but most of it remained at the point of application.

A sample of the tryptic digest, to which 2-hydroxybutyrate had been added, was then hydrolysed with 6M-HCl for 4h at 110°C under N_2 . The hydrolysate was evaporated to dryness and analysed by high-voltage paper electrophoresis at pH 3.5 and pH 6.5. Although the carrier 2-hydroxybutyrate was in each

Fig. 2. Paper electrophoresis of an acid hydrolysate of tryptic peptides obtained from urocanase labelled with NaB^3H_4 .

The radioactive peptides were hydrolysed with 6M-HCl in the presence of 2-hydroxybutyrate and the hydrolysate was then subjected to electrophoresis at pH 3.5 (a) and pH 6.5 (b) at 45 V/cm for 120 min and 105 min respectively, alongside amino acid markers. Radioactivity was measured as described in the Materials and Methods section. Numbers 1–4 show the positions and spread of the markers 2-hydroxybutyrate, aspartic acid, alanine and lysine respectively. The anode is to the left in each case.

case detected by chemical methods as a prominent spot, no radioactivity was associated with it (Fig. 2).

Inhibition by metal ions

Inhibition by Cu^{2+} was non-competitive. With CuCl_2 at $2\mu\text{M}$ and $3.3\mu\text{M}$, K_i values of $2.5\mu\text{M}$ and $3.2\mu\text{M}$ respectively were obtained.

The enzyme was too sensitive to inhibition by HgCl_2 for meaningful K_i values to be determined, since the concentration range of the inhibitor was similar to that of the enzyme. At $0.066\mu\text{M}$ - and $0.66\mu\text{M}$ - HgCl_2 the enzyme was 10% and 90% inhibited respectively. The concentration of the enzyme in the assay mixture was approx. $0.1\mu\text{M}$.

Discussion

Preparation of urocanase purified 30–35-fold from *Ps. testosteroni* showed one protein band after electrophoresis on polyacrylamide gels and sedi-

mented in the analytical ultracentrifuge as a single boundary with an $s_{20,w}^0$ of 7.6S. The molecular weight of undissociated urocanase, determined by the equilibrium method of Yphantis (1964), was 118000–120000. The plot of log concentration [$\log(\text{fringe displacement}, y)$] against the square of the radius (r^2), being linear for all but the last three points, can be taken as confirmation of a high degree of homogeneity, but with possible low-level contamination by a high-molecular-weight component. The treatment of the sedimentation-equilibrium data according to Lamm (1929) gave a z -average mol.wt. of 126000, the difference between this and the result obtained by the Yphantis (1964) method again being indicative of slight contamination by a high-molecular-weight impurity. The number-average answer, the value sought, will therefore probably be somewhat lower than 120000, because weight-averages themselves emphasize the contribution made by the heavier components in a mixture.

Molecular-weight determinations in 6M-guanidine hydrochloride in the presence of mercaptoethanol are inherently subject to uncertainty. The solvent is dense and viscous, meniscus levels in the ultracentrifuge need to be balanced very carefully and there is doubt about the validity of calculations of partial specific volumes. Finally, errors are introduced by neglecting the non-ideality of guanidine hydrochloride at this concentration and omitting virial coefficients in the calculation. Molecular weights should be expected to be low (Munk & Cox, 1972). Therefore the determination of a subunit mol.wt. of 49700–51000 by the two ultracentrifuge methods was taken to show that the native enzyme was composed of two subunits. The fact that the plot of log concentration against the square of the radius was linear throughout the cell indicated that the subunits were of identical or very similar molecular weight. Electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate and mercaptoethanol also suggested two identical subunits, samples of the enzyme running as a single band with a mobility corresponding to a mol.wt. of 58000–59000. This conclusion was supported by the finding that eight tryptic peptides were radioactive if the enzyme, which contained 16 cysteine residues per molecule, was first denatured, reduced and carboxymethylated with iodo[^{14}C]acetate.

The enzyme from *Ps. testosteroni* is therefore similar in molecular weight and its two-subunit composition to the enzyme from *Ps. putida*, for which a mol.wt. of 110000 ± 4000 was reported (George & Phillips, 1970; Lynch & Phillips, 1972). This similarity extends to the amino acid composition of the two enzymes, although here there do appear to be some significant differences, namely in the higher proportion of histidine, arginine and lysine found in the enzyme from *Ps. testosteroni*, which results in a

higher isoelectric point and accounts for the observed lower mobility of the enzyme during polyacrylamide-gel electrophoresis (Bell & Hassall, 1976).

No labelled peptides could be detected on 'maps' of tryptic digests of urocanase where the native enzyme was incubated with iodo[^{14}C]acetate, presumably because the thiol groups that might react with iodoacetate under these conditions are buried or involved in disulphide bonding. Although this is consistent with the lack of inhibition of the enzyme found with iodoacetate, iodoacetamide or *N*-ethylmaleimide (all at 3 mM), the extremely high sensitivity towards inhibition by Hg^{2+} and Cu^{2+} is probably due to the blockage of a thiol group.

The microbiological assay with *K. apiculata* conclusively showed that a vitamin B-6 cofactor was absent from urocanase of *Ps. testosteroni*. Urocanase from *Ps. putida* was analysed for pyridoxal 5'-phosphate by George & Phillips (1970) by two different methods, and the results were also negative. Urocanase from cat liver showed an absorbance peak at 415 nm (Swaine, 1969), but it was concluded that this was due to non-specific adsorption of liver pigments, since the yellow colour of the enzyme could be removed by treatment with thyroglobulin without loss of urocanase activity. Thus the initial report by Gupta & Robinson (1961) of the involvement of a pyridoxal phosphate or deoxypyridoxal cofactor has not been confirmed by other workers.

Urocanase from all sources studied is inhibited to various degrees by reagents considered specific for carbonyl groups, and in this respect is similar to those enzymes that have been shown to have a pyruvate residue as a cofactor, e.g. L-histidine carboxylase (EC 4.1.1.22) from *Lactobacillus* (Riley & Snell, 1968), S-adenosylmethionine decarboxylase (EC 4.1.1.50) from *Escherichia coli* (Wickner *et al.*, 1970) and D-proline reductase (EC 1.4.4.1) from *Clostridium sticklandii* (Hodgins & Abeles, 1969). All three of these enzymes are sensitive to carbonyl-group reagents such as cyanide, phenylhydrazine, hydroxylamine, semicarbazide and NaBH_4 . Urocanase from *Ps. testosteroni* differs markedly in its sensitivity towards carbonyl-group reagents compared with histidine decarboxylase from *Lactobacillus*, the most comprehensively studied of the enzymes containing pyruvate. *Ps. testosteroni* urocanase was not inhibited by phenylhydrazine and semicarbazide, and, although hydroxylamine, NaBH_4 and NaHSO_3 were good non-competitive inhibitors of urocanase from *Ps. testosteroni*, in each case the inhibition was partially reversible, whereas reaction of these reagents with a carbonyl group should be irreversible under the conditions used. Swaine (1969) found that hydroxylamine inhibition of the cat liver enzyme was completely reversed by gel filtration or dialysis and proposed that hydroxylamine inhibited urocanase by

competing with water for certain important active centre sites.

When urocanase from *Ps. testosteroni* was labelled with NaB^3H_4 complete inactivation resulted, but after dialysis approx. 30% of the enzymic activity was regained. At this stage at least 6 mol of ^3H /mol of enzyme was incorporated, but during the preparation of the reduced enzyme for analysis for 2-[^3H]-hydroxybutyrate, radioactivity was lost at every stage; after acid hydrolysis an amount corresponding to only 0.8 mol of ^3H /mol of enzyme remained. However, these are minimal values, since when NaB^3H_4 is used there is a considerable isotope effect and the true incorporation of hydrogen is greater than that calculated from the incorporation of ^3H .

The ^3H incorporated into urocanase did not migrate when acid hydrolysates or tryptic digests were subjected to high-voltage paper electrophoresis. The radioactivity was either associated with neutral compounds or compounds that were absorbed on the paper. Even when carrier 2-hydroxybutyrate was added to the tryptic digest of the labelled enzyme before acid hydrolysis, no migration of radioactivity occurred on subsequent electrophoresis of the hydrolysate at pH 3.5 or pH 6.5. Under these conditions the carrier 2-hydroxybutyrate was clearly detectable and was unlabelled. We were therefore unable to obtain any evidence for the existence of 2-oxobutyrate as a coenzyme for urocanase from *Ps. testosteroni*.

The observed inhibition of the enzyme by NaBH_4 and the incorporation of ^3H into protein from NaB^3H_4 may therefore arise by mechanisms other than the reduction of a carbonyl cofactor. NaB^3H_4 is a strong reducing agent and might be expected to reduce any accessible disulphide bonds. The reaction of NaBH_4 with proteins can give rise to various artifacts such as the formation of amino alcohols resulting from the reduction of peptide bonds (Paz *et al.*, 1970). Crestfield *et al.* (1963), in experiments with ribonuclease, had suggested that NaBH_4 was not a suitable reagent for reducing disulphide bonds in proteins because a small amount of peptide-bond cleavage occurred as a side reaction. Results obtained by treating proteins with a strong nucleophile and reducing agent such as NaBH_4 may require interpretation with caution.

Our inability to confirm the presence of 2-oxobutyrate in urocanase from *Ps. testosteroni* may be because this enzyme differs from urocanase from *Ps. putida* with respect to its prosthetic group. Although the two enzymes are similar with respect to molecular weight and total amino acid composition, they are readily separated by polyacrylamide-gel electrophoresis (Bell & Hassall, 1976), and there is no immunological cross-reaction between the urocanase from *Ps. putida* and antibodies raised against the enzyme from *Ps. testosteroni* (M. V. Bell & H.

Hassall, unpublished work). The difference in specific activities of the enzymes from these two organisms might also indicate a difference in mechanism.

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