The Degradation of L-Histidine, Imidazolyl-L-lactate and Imidazolylpropionate by *Pseudomonas testosteroni*

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1. Imidazol-5-ylpropionate and imidazol-5-yl-lactate are degraded by *Pseudomonas testosteroni* via inducible pathways. 2. Growth on either compound as the sole source of carbon results in the induction of the enzymes for histidine catabolism. 3. The pathway of histidine degradation in this organism, a non-fluorescent *Pseudomonad*, is shown to be the same as that operating in *Pseudomonas fluorescens* and *Pseudomonas putida*. It consists of the successive formation of urocanate, imidazol-4-on-5-ylpropionate, *N*-formimino-L-glutamate, *N*-formyl-L-glutamate and glutamate. 4. Whole cells of *P. testosteroni* accumulate urocanate in the reaction mixture when incubated with imidazolylpropionate, but only after an adaptive lag period which is removed by previous growth on imidazolylpropionate as the source of carbon. 5. Imidazolyl-lactate is oxidized to imidazolylpyruvate, which then gives rise to histidine by specific transamination with L-glutamate. 6. Cells grown on histidine, urocanate or imidazolylpropionate are also able to degrade imidazolyl-lactate. 7. Mutants lacking urocanase are unable to grow on imidazolylpropionate, imidazolyl-lactate, histidine or urocanate. One with impaired histidase activity cannot utilize histidine or imidazolyl-lactate, but grows normally on imidazolylpropionate or urocanate. A mutant unable to grow on imidazolylpropionate is indistinguishable from the wild-type with respect to growth on histidine, imidazolyl-lactate or urocanate. 8. Thus it is established that imidazolyl-lactate is metabolized via histidine whereas imidazolylpropionate enters the histidine degradation pathway after conversion into urocanate.

Imidazol-5-ylpropionate and imidazol-5-yl-lactate are normally occurring metabolites of histidine that are excreted in the urine of a number of mammals (Baldrige & Tourtellotte, 1958). Imidazolylpropionate is formed by the reduction of urocanate (Sen *et al.*, 1962) predominantly, if not exclusively, by gut bacteria (A. V. Emes & H. Hassall, unpublished work), and imidazolyl-lactate arises from the reduction of imidazolylpyruvate, which is formed from histidine by transamination (Spolter & Baldrige, 1963). These metabolites are therefore not intermediates of the main pathway of histidine catabolism and they do not appear to be degraded further by mammalian systems.

In bacteria, Westley & Ceithaml (1956) found that imidazolyl-lactate and imidazolylpyruvate would satisfy the growth requirements of a large number of histidine auxotrophs of *Escherichia coli* W, and concluded that endogenously supplied imidazolyl-lactate was converted via imidazolylpyruvate into histidine. The reverse of this reaction sequence was shown to be responsible for the formation of imidazolyl-lactate by *E. coli* B grown under conditions of nitrogen limitation in a medium containing histidine (Hedegaard

et al., 1966). Since the imidazolyl-lactate that accumulated in the medium was subsequently used by the organism, these authors suggested that it was possibly metabolized via a pathway involving ring-opening and the formation of one or several acyclic compounds.

In studies with other bacteria, imidazolylpropionate has been of interest because of its ability to function as a gratuitous inducer for the enzymes of the histidine degradative pathway in *Klebsiella aerogenes*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Magasanik, 1963; Schlesinger & Magasanik, 1965; Lessie & Neidhardt, 1967).

The pathway of histidine degradation in fluorescent species of *Pseudomonas* (*Pseudomonas fluorescens* and *P. aeruginosa*) has been shown to proceed via urocanate, 3-(imidazol-4-on-5-yl)propionate, *N*-formimino-L-glutamate and *N*-formyl-L-glutamate to L-glutamate (Tabor & Mehler, 1954; Lessie & Neidhardt, 1967). A similar pathway operates in *K. aerogenes* and *S. typhimurium*, but in these organisms formiminoglutamate is hydrolysed directly to glutamate without the intermediate formation of formylglutamate (Scheme 1).

By using enrichment culture techniques, we have been able to isolate a number of organisms that are able to use either imidazolylpropionate (Hassall &

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Scheme 1. Bacterial pathways for the degradation of L-histidine

Rabie, 1966) or imidazolyl-lactate (Hassall, 1966) as the sole source of carbon and energy for growth; all of these organisms are non-fluorescent species of *Pseudomonas*. The present paper describes the degradation of imidazolylpropionate and imidazolyl-lactate by one of these organisms, *Pseudomonas testosteroni*. Our results show that both compounds are adaptively metabolized and that, apart from the initial steps, their degradation is brought about by the enzymes normally concerned with the catabolism of histidine.

Histidine degradation in this organism follows the pathway used by the fluorescent species of *Pseudomonas*. A preliminary account of some of these findings has been published (Coote & Hassall, 1969).

Materials and Methods

Characteristics of the organism

The organism used was isolated from soil by enrichment culture in a medium containing imidazolylpropionate as the sole source of carbon. It was originally identified as *Pseudomonas acidovorans* (Hassall, 1966), but a more detailed investigation, by using the criteria of Stanier *et al.* (1966), has shown it to be the closely related species, *P. testosteroni*. It has the following characteristics: colonies grey, circular,

entire and slightly convex on nutrient agar; producing no pigment on King A or King B media; Gram-negative, motile rods with variable polar flagellation; indole-negative, gelatinase-negative, oxidase-positive; will utilize glucarate, glycollate, adipate, testosterone, benzoate, *m*-hydroxybenzoate, *p*-hydroxybenzoate as carbon source; oxidizes protocatechuate by *meta* cleavage after growth on *p*-hydroxybenzoate; growth not supported by glucose, mannitol, maleate, phenylacetate or 5-aminovalerate; no growth at 4°C or 41°C.

Methods

Growth of the organism. Cells were grown at 30°C in media containing (g/litre): KH_2PO_4 , 2; $(\text{NH}_4)_2\text{SO}_4$, 1; carbon source, 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; the pH was adjusted to 7.0 with 5M-NaOH. Small crops of cells, of 500ml or less, were grown on a rotary shaker. Larger cultures of cells were grown in flat-bottomed flasks of up to 10 litres capacity and were aerated by the passage of sterile air. Cells in the exponential phase of growth were harvested either in a De Laval continuous-flow centrifuge or by centrifuging at 15000g for 10 min in a MSE High-speed 18 centrifuge. They were washed once by suspension in 0.1M- KH_2PO_4 buffer (adjusted to pH 7.0 with 5M-NaOH) and were then re-centrifuged.

Isolation of mutants. Cells growing exponentially in 10ml of medium with urocanate as the carbon source were collected on a sterile Oxoid cellulose acetate filter (0.5–1 μ m pore size) and resuspended at approx. 0.25mg dry wt./ml in 10ml of Tris–maleate medium containing 0.3mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine/ml. The Tris–maleate medium consisted of basal salts medium with KH_2PO_4 replaced by 0.05M-Tris and 0.05M-maleic acid (final concentrations), adjusted to pH 6.0 with 5M-NaOH. The suspension was incubated at 30°C for 30min and a 1–2ml sample was then withdrawn and washed free of mutagen on a second sterile filter. The cells were grown overnight in nutrient broth medium and the culture was then diluted with sterile water to approx. 5×10^3 cells/ml. Samples of this suspension were then spread on 1.5% (w/v) agar plates containing L-glutamate (0.02g/litre) and imidazolyl compound (L-histidine, urocanate, imidazolylpropionate or imidazolyl-L-lactate) at 1g/litre. The plates were incubated for 3–4 days and the pin-point colonies, easily distinguished from the large wild-type colonies, were removed and placed on nutrient agar plates. When grown, they were replated on to plates containing either L-histidine, urocanate, imidazolylpropionate, imidazolyl-L-lactate or L-glutamate as the carbon source (1g/litre) and $(\text{NH}_4)_2\text{SO}_4$ (1g/litre) as the nitrogen source. Mutants were initially classified according to the growth pattern observed. For mutants with impaired histidase or urocanase activity, the deficiency was confirmed by assay of the enzyme after growth in liquid media with either urocanate as the carbon source (for histidase[−] mutants) or with succinate as the carbon source and histidine as the nitrogen source (for urocanase[−] mutants). In the latter medium, urocanase[−] mutants were characterized by an extracellular accumulation of urocanate.

The mutants that were used were: A26, urocanase[−]; 11/13, possessing less than 10% of the inducible histidase of the wild-type organism; 13/18, unable to use imidazolylpropionate as a carbon source for growth.

Disruption of cells. For most of the experiments described, cells were disrupted with a Hughes press (Hughes, 1951). Occasionally, as indicated in the text, they were disintegrated by exposure of 3ml of suspension for 1.5min to the output of a Dawe Soni-probe type 1130A ultrasonic disintegrator used at a setting of 1A, by grinding with an equal weight of alumina, or by passage through a French pressure cell (Milner *et al.*, 1950) with a pressure difference of 1.38×10^7 N/m² (20000 lbf/in²) at the outlet.

Preparation of cell extracts. The crushed material from the Hughes press was dispersed in 3vol. of phosphate buffer (0.1M) and the suspension clarified by centrifugation at 30000g for 30min at 2°C. Other broken-cell preparations were centrifuged under the same conditions after being diluted with buffer where appropriate. The protein concentration of cell ex-

tracts was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin (BDH Chemicals Ltd., Poole, Dorset, U.K.) as the reference standard.

Particulate fractions were prepared by suspending cells in 3vol. of 0.1M-phosphate buffer and disintegrating them ultrasonically. The supernatant obtained after centrifuging this preparation at 10000g for 10min was then recentrifuged at 45000g for 1h and the pellet obtained was resuspended in phosphate buffer to one-quarter the volume of the original suspension.

Manometric experiments. O₂ uptake was measured in conventional Warburg manometers at 30°C. The centre well contained 20% (w/v) KOH and substrate was tipped from the side arm. The total volume of the reaction mixture was 2.8ml.

In some experiments, O₂ consumption was measured with an O₂ electrode fitted with a linear chart recorder. The reaction volume was 2.8ml and the concentration of O₂ in the reaction mixture equilibrated with air at 30°C was taken as 0.24mM.

Buffers. Phosphate buffer consisted of KH_2PO_4 with the pH of the solution adjusted to 7.0 with 5M-NaOH. Aminomethylpropanediol and Tris buffers consisted of 2-amino-2-methylpropane-1,3-diol or Tris, respectively, of the required concentration and with the pH adjusted with 2M-HCl. Buffer concentration is given as the concentration of phosphate, aminomethylpropanediol or Tris, respectively. The borate–arsenate buffer that was used in reaction mixtures where the accumulation of imidazolyl-pyruvate was measured consisted of 0.2M-sodium tetraborate and 0.028M-sodium arsenate with the pH brought to 8.0 with 2M-HCl.

Paper chromatography and high-voltage paper electrophoresis. The reaction products of [2-¹⁴C]-urocanate ([2-¹⁴C]imidazolylacrylate) and [2-¹⁴C]-imidazolyl-L-lactate were chromatographed on Whatman no. 1 paper in solvent A, consisting of the organic phase of 2-methylpropan-1-ol–formic acid–water (19:2:6, by vol.) (Hassall & Greenberg, 1963). Other solvents used for the further characterization of intermediates were: B, butan-1-ol–pyridine–water (1:1:1, by vol.) and C, butan-1-ol–acetic acid–water (4:1:1, by vol.) (Sen *et al.*, 1962). High-voltage paper electrophoresis was carried out on Whatman no. 3 paper by using a Pherograph-Original-Frankfurt type 1963–64 instrument. Two buffers were used: water–pyridine–acetic acid (450:25:1, by vol., pH 6.5) and water–pyridine–acetic acid (189:1:10, by vol., pH 3.7) (Katz *et al.*, 1959). A potential of 50V/cm was applied to the paper via platinum electrodes for 40–120min.

Detection of compounds after chromatography or electrophoresis. Imidazolyl derivatives were detected by spraying the papers with diazotized *p*-chloroaniline solution and then exposing them to NH₃ vapour. The

reagent was prepared immediately before use by mixing equal volumes of ice-cold 5% (w/v) NaNO_2 and 1% (w/v) *p*-chloroaniline in 10% (v/v) HCl. Imidazolyl compounds, with some exceptions, give red, orange or brown spots on a yellow background when treated this way (Hall, 1952). Urocanate was also detected as a dark-purple spot when papers were viewed in u.v. light. Other compounds were detected as described previously (Hassall, 1971).

Identification of ^{14}C -labelled products. Small amounts of ^{14}C -labelled products were identified (after elution from unsprayed chromatograms) by co-chromatography and co-electrophoresis. The radioactive material was mixed with authentic sample and the mixture chromatographed in two-dimensions on paper or subjected to paper electrophoresis. The radioactive areas were located by radioautography on Kodirex (Kodak Ltd., London W.C.2, U.K.) X-ray film and the papers subsequently treated with the appropriate reagent for the detection of the unlabelled carrier compound. When the radioautogram and paper were superimposed, the coincidence of radioactive area and chemically detected spot was obtained in all systems.

Identification of urocanate as a product of imidazolylpropionate metabolism. A reaction mixture (200 ml) containing 0.8 g of imidazolylpropionate, 6 mmol of KH_2PO_4 and 300 mg dry wt. of cells previously grown with imidazolylpropionate as the carbon source was incubated at 30°C for 6 h. The cells were removed by filtering the suspension through an Oxoid cellulose acetate filter and the filtrate, with E_{277} 26.7, was evaporated to dryness on a rotary evaporator. The dried material was dissolved in 10 ml of hot water and the pH adjusted to approx. pH 4.5 with acetic acid. The crystals, which appeared as the solution cooled, were collected by filtration and recrystallized from 5 ml of hot water. The final product consisted of 40 mg of fine white needles which melted at 223°C (uncorr.) (urocanic acid dihydrate; m.p. 225°C; Mehler & Tabor, 1953). The u.v.-absorption spectra in acidic, neutral and alkaline solutions and the i.r. spectrum were identical with those obtained for authentic urocanic acid dihydrate (Found: C, 41.6; H, 5.7; N, 16.0. Calc. for $\text{C}_6\text{H}_6\text{N}_2\text{O}_2 \cdot 2\text{H}_2\text{O}$: C, 41.5; H, 5.7; N, 16.1%).

Measurement of radioactivity. Material was assayed on aluminium planchets in a Nuclear-Chicago gas-flow counter. The radioactivity of compounds on unsprayed chromatograms was determined by cutting out the appropriate area of paper and placing it directly on to a lightly gummed planchet. The radioactivity of whole cells collected by filtration on Oxoid discs was determined in the same way.

Spectrophotometry. Continuous changes in extinction were monitored automatically in a Gilford 2000 multiple-sample absorbance indicator fitted with a Unicam SP.500 monochromator. U.v. and i.r.

spectra were determined with Unicam SP.800 and Unicam SP.200 spectrophotometers respectively.

Assay of histidine aminotransferase activity. The assay used was based on the method described by Spolter & Balldridge (1963) for the assay of histidine-pyruvate aminotransferase. The formation of the enol-borate complex of imidazolylpyruvate was followed spectrophotometrically at 293 nm (ϵ 12000) in borate-arsenate buffer. The complete system (3.5 ml) consisted of 2 mmol of sodium borate (pH 8.0), 20 μmol of sodium arsenate, 80 μmol of EDTA, 100 μmol of L-histidine, 100 μmol of oxo acid and 0.1 ml of enzyme preincubated for 10 min with 75 μg of pyridoxal 5'-phosphate/ml. Oxo acid was omitted from the control reaction mixture. One unit of enzyme activity was defined as the formation of 1 μmol of product/min at 30°C. When activity towards phenylalanine and tyrosine was assayed, extinction changes were followed at the appropriate wavelength for the enol-borate complex formed (phenylpyruvate, ϵ_{310} 12400; *p*-hydroxyphenylpyruvate, ϵ_{300} 9150).

Assay of imidazolyl-lactate oxidase activity. The ability of preparations to form imidazolylpyruvate from imidazolyl-L-lactate was measured spectrophotometrically by following the formation of the enol-borate complex of imidazolylpyruvate at 293 nm. The reaction mixture contained 10 μmol of imidazolyl-L-lactate, 2 mmol of sodium borate, 20 μmol of sodium arsenate (pH 8.0) and sample to a total volume of 3.5 ml. A control reaction mixture from which imidazolyl-lactate was omitted showed no change in E_{293} .

Removal of cells from samples of suspension. For two types of experiment it was necessary to remove cells quantitatively from samples of suspension: first, to determine the extracellular accumulation of urocanate by measurement of the E_{277} of the filtrate, and secondly, to measure the uptake of ^{14}C -labelled substrates by whole cells. In each case, the cells were removed by filtering the suspension, with suction, through 2.4 cm diam. Oxoid filters held in a stainless-steel filter-tower assembly. When the cells were needed for the measurement of radioactivity, the filter was washed twice with 3 ml of water and allowed to dry at 35°C.

Chemicals

Urocanic acid was synthesized enzymically by the method of Mehler *et al.* (1955). [$2\text{-}^{14}\text{C}$]Urocanic acid was prepared from [$\text{ring-2-}^{14}\text{C}$]histidine by a similar procedure except that the product was isolated by elution from Amberlite IRC-50 (Na^+ form) with a gradient of 0–0.1 M-acetic acid (Rao & Greenberg, 1960). Imidazolyl-L-lactic acid hydrochloride was prepared by the method of Fränkel (1903). It was purified by passage through a Dowex 50(H^+ form)

column and eluted with 0.4M-HCl. The fractions containing imidazolyl-lactic acid were pooled and evaporated to dryness in a rotary evaporator. The product was then dissolved in water and precipitated with acetone; final purification was obtained by precipitation from ethanol with diethyl ether. [2-¹⁴C]-Imidazolyl-L-lactic acid was prepared by incubating 0.2ml of 0.5mM-[ring-2-¹⁴C]histidine (5 μ Ci) with 0.1ml of 50%-saturated aq. NaNO₂ at 30°C for 30min. The product was purified by paper chromatography in solvent C and high-voltage paper electrophoresis at pH3.6. Imidazolylpropionic acid was prepared by the catalytic hydrogenation of urocanic acid (Mehler & Tabor, 1953; Kraml & Bouthillier, 1955). The conversion into imidazolylpropionic acid was virtually complete and the crude product was purified by precipitation from ethanol with diethyl ether. [2-¹⁴C]Imidazolylpropionic acid was prepared from [2-¹⁴C]urocanic acid by the same method except that the product was purified by paper chromatography and paper electrophoresis by using the systems used for the purification of [¹⁴C]imidazolyl-lactic acid. *N*-Formyl-L-glutamic acid was synthesized as described by Tabor & Mehler (1954). *N*-Formimino-L-glutamate was obtained as the barium salt (BDH Chemicals Ltd.) and was converted into the sodium salt by the addition of an equivalent amount of Na₂SO₄; BaSO₄ was removed by centrifugation.

Other chemicals were purchased as follows: imidazolylpyruvate from Calbiochem, Los Angeles, Calif., U.S.A.; Tris, L-histidine, 2-oxoglutarate, NADH and NADPH from Sigma (London) Chemical Co., London S.W.6, U.K.; 2-amino-2-methylpropane-1,3-diol from BDH Chemicals Ltd.; chloramphenicol from Parke, Davis and Co. Ltd., Hounslow, Middx., U.K.; *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine from Aldrich Chemical Co., Milwaukee, Wis., U.S.A.; [¹⁴C]histidine from The Radiochemical Centre, Amersham, Bucks., U.K. The remaining reagents were supplied by BDH Chemicals Ltd. and were AnalaR grade where available.

Results

Oxidation of substrates by whole cells

The existence of a similar respiratory pattern for cells grown with L-imidazolyl-lactate, L-histidine or urocanate was established. Suspensions of washed cells grown on any of these substrates were able to oxidize L-imidazolyl-lactate, imidazolylpyruvate, *N*-formimino-L-glutamate, *N*-formyl-L-glutamate and L-glutamate, rapidly and without a lag period. L-Histidine was in all cases oxidized at a lower rate, whereas imidazolylpropionate was only oxidized when it had served as the source of carbon for growth (Table 1). If cells were grown with L-glutamate, then

Table 1. *Growth rates of P. testosteronei with different carbon sources and rates of oxidation of substrates by washed cells*

Cells were grown in media containing (NH₄)₂SO₄ as the nitrogen source and with the carbon sources shown. Growth rates were measured spectrophotometrically at 580nm. Cells were harvested in the exponential phase and washed with 0.1M-KH₂PO₄ buffer (pH7.0). Rates of oxidation were measured manometrically in reaction mixtures containing 3–5mg dry wt. of cells, 100 μ mol of KH₂PO₄ buffer (pH7.0) and 5 μ mol of substrate in 2.8ml. All rates are corrected for endogenous respiration.

Carbon source during growth	Doubling time (h)	Substrates added to manometer flasks and rate of oxidation (μ l of O ₂ /h per mg dry wt.)							
		L-Histidine	Urocanate	Imidazolyl-L-lactate	Imidazolyl-propionate	Imidazolyl-pyruvate	<i>N</i> -Formimino-L-glutamate	<i>N</i> -Formyl-L-glutamate	L-Glutamate
L-Histidine	4.7	45	88	65	2	—	65	55	70
Urocanate	2.0	38	81	62	2	66	60	57	68
Imidazolyl-L-lactate	5.0	40	88	70	3	67	62	62	65
Imidazolyl-propionate	2.3	34	78	62	74	68	60	63	70
L-Glutamate	1.6	2	4	3	3	3	4	5	74

only L-glutamate of the substrates listed above was oxidized without a pronounced lag period.

These results show that imidazolylpropionate and imidazolyl-lactate are oxidized by inducible enzyme systems and that in each case the ability of cells to accomplish the oxidation is associated with the induction of the histidine catabolic pathway. With respect to the ability to metabolize imidazolyl-lactate, a certain amount of non-specific induction is apparent since this compound is oxidized by cells grown with histidine, urocanate or imidazolylpropionate as well as by those for which it has been the growth substrate.

The results are consistent with the formation of urocanate from imidazolylpropionate and suggest that imidazolyl-lactate might also be degraded to urocanate via imidazolylpyruvate and histidine. The oxidation of histidine by cells grown with urocanate is in agreement with the finding that histidase (L-histidine ammonia-lyase, EC 4.3.1.3) and urocanase are co-ordinately induced in this organism (Coote & Hassall, 1973).

The mean generation times of the organism with different substrates as the sole source of carbon and energy for growth are also shown in Table 1. The fastest growth rate is observed when glutamate is the source of carbon but growth on urocanate or imidazolylpropionate is also rapid. Growth with histidine or imidazolyl-lactate is relatively slow and, at least for histidine, this could be due to the poor rate of entry of substrate into the cell. This is also probably the reason for the slow manometric oxidation of histidine obtained with whole cells since results described later show that sonically disrupted cells are able to convert histidine into urocanate at a faster rate than can the original whole cells. If the organism is grown in a medium containing succinate as the carbon source and histidine as the sole source of nitrogen, instead of in the medium where histidine is the sole source of carbon, then the mean generation time is decreased to 1.9 h from 4.7 h.

To determine whether the pathway of urocanate catabolism in *P. testosteronei* was the same as that used by the fluorescent species of *Pseudomonas*, the degradation products of urocanate were identified. The successive formation of formiminoglutamate and formylglutamate was demonstrated by incubating [2-¹⁴C]urocanate with extracts of cells grown with histidine, imidazolylpropionate or imidazolyl-lactate. Samples of each reaction mixture were analysed by paper chromatography and radioautography for non-volatile, radioactive intermediates. The results were quantitatively similar for the three different extracts; a typical reaction sequence is shown in Fig. 1. Imidazolylpropionate is not detected as an intermediate in an experiment of this kind, since it is oxidized non-enzymically to 4-oxoglutaramate, NH₃ and formate by the conditions used to stop the

reaction (Hassall & Greenberg, 1963). The formic acid, which in this case contains the ¹⁴C, is volatile and is consequently lost during the chromatography step. Similarly, since the enzymic hydrolysis of [1-¹⁴C]formylglutamate yields [1-¹⁴C]formate, no intermediates beyond formylglutamate are detected by the procedure used.

Fig. 1. Kinetics of [2-¹⁴C]urocanic acid metabolism by a cell-free extract of *P. testosteronei*

The reaction mixture (0.9 ml) incubated at 30°C contained 0.2 μ Ci of [2-¹⁴C]urocanic acid (58 μ Ci/ μ mol), 50 μ mol of Tris buffer (pH 7.0) and cell-free extract (1.2 mg dry wt. of protein) prepared from cells grown with imidazolyl-lactate as the source of carbon. Samples (0.1 ml) were taken at the times shown and deproteinized by heating at 100°C for 1 min followed by the addition of 50 μ l of 2 M-HCl. A portion (50 μ l) of each supernatant was chromatographed by upward flow in solvent A, the solvent front travelling 25 cm from the origin. After radioautography, each radioactive area was cut from the paper chromatogram for the determination of radioactivity. The values refer to the activities present in the sample chromatographed. ●, Urocanate; ○, N-formiminoglutamate; □, N-formylglutamate.

Extracts prepared from cells grown previously with either imidazolypropionate or imidazoly-lactate as the source of carbon were, in each case, inactive towards the growth substrate. Incubation of [^{14}C]imidazolypropionate or [^{14}C]imidazoly-lactate with the appropriate extract gave no detectable metabolites after chromatography and radioautography of deproteinized samples of the reaction mixtures. Attempts to stimulate activity by the addition of such cofactors as NAD^+ , NADP^+ , FAD , ATP , Mg^{2+} , CoA , GSH and Fe^{2+} were unsuccessful in that no degradation products of the substrate could be detected. Also, the preparation of cell extracts by a variety of means, including treatment with lysozyme and EDTA , followed by osmotic shock (Flessel *et al.*, 1967) gave no improvement in the recovery of activity. There are, however, two major difficulties with this type of experiment. First, if the initial step in the metabolism of the substrate is very slow compared with the following steps, then no intermediates will accumulate. Secondly, where a slow disappearance of the substrate is observed there is always the possibility that this might be brought about by residual whole cells.

Extracellular accumulation of urocanate by whole cells incubated with imidazolypropionate

When washed cells, grown with imidazolypropionate as the source of carbon, were resuspended in fresh medium containing imidazolypropionate there was an immediate extracellular accumulation of urocanate (Hassall & Rabie, 1966); this accumulation was not affected by the presence of chloramphenicol in the resuspension medium (Fig. 2). Cells grown previously on urocanate, when incubated with imidazolypropionate, only accumulated urocanate after a lag period of some 2–2½ h. Further, the accumulation was prevented by chloramphenicol in the reaction mixture. These results indicate that cells grown on urocanate require the induction of a permease or an enzyme system (or both) before they can metabolize imidazolypropionate. This is in complete agreement with the manometric results shown in Table 1.

Uptake of [^{14}C]imidazolypropionate by whole cells

To determine whether the non-metabolism of imidazolypropionate by cells grown on urocanate was due to non-permeability of the cells to the substrate or to the lack of an enzyme system, the ability of cells to accumulate [^{14}C]imidazolypropionate was investigated.

Cells grown respectively on urocanate, imidazolypropionate and L-glutamate were harvested, washed and resuspended in media containing [$2\text{-}^{14}\text{C}$]imidazolypropionate. The radioactivity of the cells in each suspension was determined at specific time-

intervals (Fig. 3). There was a rapid uptake of radioactivity by cells that had been grown on urocanate or imidazolypropionate but not by those grown on glutamate. The radioactivity of the cells grown on imidazolypropionate decreased markedly after the initial rise. This was assumed to be due to the metabolism of imidazolypropionate, rather than to its release into the suspension medium. The assumption would appear to be valid, since the total radioactivity of a dried sample of this reaction mixture decreased to some 10% of the initial value over a 15 min reaction period. The total radioactivity in each of the other two reaction mixtures remained constant.

Fig. 2. Accumulation of urocanate from imidazolypropionate by resuspended cells of *P. testosteroni*

Exponentially growing cells were harvested from media containing either imidazolypropionate or urocanate as the carbon source. They were washed and resuspended to 1.2 mg dry wt./ml in 10 ml of reaction mixture, which contained 150 μmol of imidazolypropionate and 200 μmol of KH_2PO_4 buffer adjusted to pH 7.0 with 5 M- NaOH . Chloramphenicol, where present, was added at 100 $\mu\text{g}/\text{ml}$. Samples (1 ml) were taken at the times indicated and filtered through Oxoid discs. Urocanate was estimated spectrophotometrically at 277 nm ($\epsilon 18800$) after tenfold dilution of samples of filtrate with water. Urocanate-grown cells, with (■) and without (□) chloramphenicol. Imidazolypropionate-grown cells, with (●) and without (○) chloramphenicol.

L-Histidine-2-oxoglutarate aminotransferase: the formation of histidine from imidazolylpyruvate

Cell extracts were examined for their ability to catalyse the transamination of imidazolylpyruvate and L-histidine. Aminotransferase activity was first assayed in extracts of cells grown on imidazolyl-lactate with imidazolylpyruvate as the substrate. The change in E_{282} was followed when various amino acids were added to the reaction mixture. In the absence of L-glutamate no decrease in extinction occurred, and no activity was observed if L-alanine or L-aspartate replaced L-glutamate.

An assay system some five times more sensitive was obtained by following the reaction in the reverse direction, borate being used to complex the imidazolylpyruvate formed and the increase in E_{293} due to this complex being measured. Table 2 shows the specificity of histidine aminotransferase activity towards a number of oxo acids. Of the oxo acids used 2-oxoglutarate gave the highest activity.

The specificity of the enzyme with respect to the amino acid could only be tested within the limits of the spectrophotometric assay procedure, which is dependent on the formation of an enol-borate complex of the oxo acid produced. The assay is suitable for monitoring transamination of phenylalanine or tyrosine, since they give rise to oxo acids, phenylpyruvate and *p*-hydroxyphenylpyruvate, which form enol-borate complexes with characteristic absorption spectra (ϵ_{310} 12400 and ϵ_{300} 9150 respectively). However, a lower concentration of substrate is necessary when tyrosine is used, because of its poor solubility.

Fig. 3. Uptake of [^{14}C]imidazolylpropionate by whole cells of *P. testosteroni*

Whole cells were used which had been grown with urocanate (○), imidazolylpropionate (●) or L-glutamate (□) as the carbon source. Each reaction mixture, at 30°C, contained 0.02 μmol of [$2\text{-}^{14}\text{C}$]imidazolylpropionate (0.01 μCi), 0.5 mg of chloramphenicol and 100 μmol of KH_2PO_4 buffer (pH 7.0) in 5 ml. The reaction was started by the addition of 5 ml of the appropriate cell suspension (1 mg dry wt./ml) and 1 ml samples were withdrawn at the times shown for the determination of radioactivity in the cells.

Table 2. Specificity of L-histidine-2-oxoglutarate aminotransferase activity in crude cell-free extracts of *P. testosteroni*

Extracts were prepared from cells grown in media containing either imidazolyl-L-lactate or succinate as the sole source of carbon. Aminotransferase activity towards aromatic amino acids was measured spectrophotometrically by following the rate of appearance of the enol-borate complex of the oxo acid formed. For the determination of rates with different oxo acids 100 μmol of L-histidine and 100 μmol of oxo acid were present. Other reaction mixtures contained 100 μmol of 2-oxoglutarate and 20 μmol of amino acid.

Substrates present in the reaction mixture		Activity (munits/mg of protein)	
		Cells grown with imidazolyl-L-lactate	Cells grown with succinate
2-Oxoglutarate + L-Histidine		260	13
	+D-Histidine	0	0
	+DL-Phenylalanine	151	126
	+L-Tyrosine	216	—
L-Histidine	+2-Oxoglutarate	300	—
	+Pyruvate	18	—
	+Glyoxylate	3	—
	+2-Oxobutyrate	24	—
	+4-Methyl-2-oxo-pentanoate	18	—

Table 2 also shows that an extract prepared from cells grown on imidazoyl-lactate had a general aminotransferase activity towards the aromatic amino acids. The activity towards phenylalanine and tyrosine was again specific for 2-oxoglutarate as the oxo acid, since only minimal activity was obtained with glyoxylate, pyruvate, 2-oxobutyrate or 4-methyl-2-oxo-pentanoate. A low, but distinct, basal activity of the enzyme towards L-histidine was detected in an extract of cells grown on succinate as the carbon source. Surprisingly, the activity of this extract towards phenylalanine was comparable with that of the extract of cells grown on imidazoyl-lactate. This activity towards phenylalanine in uninduced cells strongly suggests that the enzyme catalysing the transamination of histidine is not responsible for all the activity towards phenylalanine and that this, in part, could be due to an aminotransferase normally concerned with the biosynthesis of phenylalanine.

Formation of imidazoylpyruvate from imidazoyl-lactate

Three distinct enzyme systems were recognized that could catalyse the formation of imidazoylpyruvate from imidazoyl-lactate. These were imidazoyl-lactate-NADP⁺ oxidoreductase, and particulate and non-particulate imidazoyl-lactate oxidases.

The equilibrium position of the reaction catalysed by imidazoyl-lactate-NADP⁺ oxidoreductase is strongly in favour of imidazoyl-lactate formation, since the reaction could only be demonstrated in this direction. The relative rates of oxidation of NADH and NADPH were determined with imidazoylpyruvate, phenylpyruvate and *p*-hydroxyphenyl-

pyruvate as substrates by using an extract of cells grown on imidazoyl-lactate (Table 3).

The formation of imidazoylpyruvate from imidazoyl-lactate, in the absence of added cofactors, was followed in borate buffer by measuring the increase in E_{293} caused by the formation of the enol-borate complex of the oxo acid. Table 4 shows that the activity towards imidazoyl-lactate, with respect to the formation of imidazoylpyruvate, was confined mainly to the particulate fraction of the cell. Results in Table 4 also show that this particulate oxidase activity is constitutive in that preparations obtained from cells grown on glutamate as the carbon source are as active as those obtained from cells grown on

Table 3. *Rates of oxidation of NADH and NADPH by cell-free extract in the presence of aromatic oxo acids*

Each reaction mixture (3ml), incubated at 30°C, contained 5 μ mol of oxo acid, 0.5 μ mol of NADH or NADPH, 100 μ mol of KH₂PO₄ buffer (pH 7.0) and extract from *P. testosteroni* grown with imidazoyl-L-lactate as the carbon source. Rates of oxidation were measured at 340nm and corrected for the rate observed in the absence of oxo acid.

Oxo acid added	Oxidation rate (nmol/min per mg)	
	NADH	NADPH
Imidazoylpyruvate	11.6	20.0
Phenylpyruvate	4.6	22.0
<i>p</i> -Hydroxyphenylpyruvate	4.8	24.8

Table 4. *Intracellular distribution of imidazoyl-lactate oxidase activity in P. testosteroni*

Cells were grown with either imidazoyl-L-lactate or L-glutamate as the carbon source and were collected by centrifugation and resuspended to a density of 5mg dry wt./ml. Samples of suspension (5ml) were subjected to sonic treatment and centrifuged at 10000g for 15min. The supernatant solutions were re-centrifuged at 45000g for 60min. The pellets from the final centrifugation were suspended in 0.1 M-KH₂PO₄ buffer (pH 7.0) to the original volume allowing for the removal of a sample for assay at each stage. Each fraction was assayed for its ability to form imidazoylpyruvate from imidazoyl-L-lactate by using the enol-borate system as described in the Materials and Methods section.

Fraction	Rate of imidazoylpyruvate formation (increase in E_{293} /min per ml)	
	Imidazoyl-lactate as carbon source	L-Glutamate as carbon source
Whole cells	0.043	0.035
Total sonicate	0.336	0.360
10000g supernatant	0.210	0.232
45000g supernatant	0.027	0.040
Resuspended pellet from 45000g centrifugation	0.170	0.161

imidazolyl-lactate. The stoicheiometry of the reaction is shown in Table 5; 2 μ mol of imidazolylpyruvate are formed/ μ mol of O₂ consumed.

When soluble extracts of cells able to oxidize imidazolyl-lactate were subjected to polyacrylamide-gel electrophoresis and the resulting gels incubated in a reaction mixture consisting of imidazolyl-lactate, phenazine methosulphate and Nitro Blue Tetrazolium, an intense, purple band was obtained (Plate 1). Phenyl-lactate or *p*-hydroxyphenyl-lactate would also act as substrates for this reaction, indicating a certain degree of non-specificity of action. However, no activity was detected in extracts of cells grown with either succinate or glutamate as the carbon source. The possibility of there being a similar oxidase concerned with the metabolism of imidazolylpropionate was investigated by using the same phenazine methosulphate–Nitro Blue Tetrazolium assay system but with imidazolylpropionate as substrate. The results were negative in that no band of activity was

observed, even when extracts were prepared from cells previously grown with imidazolylpropionate as the carbon source.

Studies with mutants of P. testosteroni

It was found during the screening of potential mutants that those clones showing weak growth on L-histidine plates also showed weak growth on imidazolyl-lactate plates. Similarly, mutants selected because of an inability to grow on imidazolyl-lactate were frequently found to grow poorly on L-histidine. Thus mutant 11/13, a 'leaky' histidase⁻ mutant, grew readily on urocanate or imidazolylpropionate but showed only poor growth on histidine or imidazolyl-lactate plates even after 4 or 5 days. Revertants of this mutant, selected after heavy seeding of either histidine or imidazolyl-lactate plates, grew readily on both histidine and imidazolyl-lactate. Mutant A26, a urocanase⁻ mutant, showed no growth on histidine,

Table 5. *Stoicheiometry of O₂ consumption during the formation of imidazolylpyruvate from imidazolyl-L-lactate by a particulate preparation of P. testosteroni*

The reaction vessel of the O₂ electrode contained 1.6 ml of borate–arsenate buffer (pH 8.0) and 1 ml of particulate preparation from cells grown with imidazolyl-L-lactate as the carbon source; 0.2 ml (20 μ mol) of imidazolyl-L-lactate was added to start the reaction. O₂ consumption was recorded and separate reactions were stopped by the addition of 0.2 ml of 35 % (w/v) HClO₄. The mixtures were centrifuged and 2 ml of each supernatant solution was adjusted to pH 8.0 by the addition of 0.75 M-NaOH in borate buffer. Imidazolylpyruvate was estimated spectrophotometrically at 293 nm. A control reaction was used to correct for endogenous O₂ consumption and as a blank for the determination of imidazolylpyruvate.

Reaction time (min)	Imidazolylpyruvate formed (μ mol)	O ₂ consumed (μ mol)	Imidazolylpyruvate formed O ₂ consumed
15	0.546	0.294	1.86
30	1.044	0.540	1.92

Table 6. *Formation of urocanate from various precursors by a urocanase⁻ mutant (A26) of P. testosteroni*

Cells were grown in the presence of imidazolyl-L-lactate with succinate as the carbon source and (NH₄)₂SO₄ as the nitrogen source. They were harvested, washed and resuspended to approx. 7 mg dry wt./ml. Samples were disrupted by ultrasonic treatment. Whole cells and disintegrated preparations were assayed in 3 ml of reaction mixture containing 1 μ mol of substrate, 100 μ mol of Tris buffer (pH 8.0) and 0.1 ml of preparation at 30°C. The amount of urocanate formed was calculated from the change in *E*₂₇₇. The decrease in extinction caused by the metabolism of imidazolylpyruvate was allowed for in the appropriate calculations.

Substrates	Rate of urocanate formation (nmol/min per mg dry wt.)	
	Whole cells	Disintegrated cells
L-Histidine	2.0	19.5
Imidazolylpyruvate	6.0	2.8
Imidazolylpyruvate + L-glutamate	—	5.6
Imidazolyl-L-lactate	6.5	3.1
Imidazolyl-L-lactate + L-glutamate	—	4.8

EXPLANATION OF PLATE I

Photographs of polyacrylamide gels stained for imidazolyl-lactate oxidase activity

Samples of crude cell-free extract of *P. testosteroni* were subjected to electrophoresis until the Bromophenol Blue marker bands had emerged from the gels. Each gel was incubated for 1 h in the dark in 5 ml of solution containing 10 μ mol of imidazolyl-L-lactate, 40 μ mol of Tris buffer (pH 9.2), 50 μ g of Nitro Blue Tetrazolium and 50 μ g of *N*-methylphenazonium methosulphate. The gels were finally washed and stored in 7% (v/v) acetic acid. The carbon sources for the growth of the cells from which the extracts were prepared and the amounts of protein (mg) subjected to electrophoresis, were as follows: (a) imidazolyl-L-lactate (0.29); (b) imidazolylpropionate (0.23); (c) L-histidine (0.29); (d) urocanate (0.35); (e) succinate (0.25).

Fig. 4. Glutamate-dependent accumulation of urocanate from imidazolylpyruvate by a cell-free extract of mutant A26 (urocanase⁻)

The complete reaction mixture contained 1 μ mol of imidazolylpyruvate, 10 μ mol of L-glutamate, 200 μ mol of Tris buffer (pH 7.0), and crude cell-free extract (0.3mg of protein). Spectra were recorded automatically; trace 1, before addition of glutamate; traces 2 and 3, at 20 min and 30 min after the addition of glutamate. When glutamate was omitted, the spectrum after 30 min was superimposable on trace 1.

imidazolyl-lactate, urocanate or imidazolylpropionate. Revertants, obtained from any one of the four plates, were indistinguishable from the wild-type organism and could use all these compounds as growth substrates. Mutant 18/13 could not grow with imidazolylpropionate as the sole source of carbon but it grew normally on imidazolyl-lactate, histidine or urocanate.

Overnight growth of mutant A26 (urocanase⁻) in liquid culture in L-histidine-succinate medium or in succinate-NH₄⁺ medium in the presence of imidazolyl-lactate or imidazolylpropionate gave an accumulation of urocanate in the growth medium.

The activity of various cell preparations of mutant A26, with respect to their ability to form urocanate from a precursor, was assayed spectrophotometrically by following the increase in E_{277} of reaction mixtures. Provided that the cell density was not too high, this activity could be assayed by using whole cells or sonicated preparations. Whole cells of this mutant,

Fig. 5. Formation of [¹⁴C]urocanate from [2-¹⁴C]-imidazolyl-L-lactate by a broken-cell suspension of mutant A26 (urocanase⁻)

The reaction mixture (0.7 ml) contained 0.02 μ Ci of imidazolyl-L-lactate (58 μ Ci/ μ mol), 50 μ mol of Tris buffer (pH 8.0), 1 μ mol of L-glutamate and 6 mg dry wt. of sonically disrupted cells of mutant A26 grown with succinate as the carbon source in the presence of imidazolyl-L-lactate. The analytical procedures are as described in the legend to Fig. 1. ●, Imidazolyl-L-lactate; ○, urocanate.

after previous growth in succinate-NH₄⁺ medium containing L-imidazolyl-lactate, accumulated urocanate from imidazolyl-lactate, imidazolylpyruvate and histidine, with the accumulation from histidine being the slowest (Table 6). Sonic disruption of the cell suspension caused a significant decrease in the rate of urocanate accumulation from imidazolyl-lactate and imidazolylpyruvate but greatly stimulated that from histidine, indicating that there is restricted entry of histidine into whole cells. The formation of urocanate from imidazolyl-lactate or imidazolylpyruvate, by sonicated cells, was markedly stimulated by the addition of L-glutamate, this presumably being required in the aminotransferase reaction for the formation of histidine. In keeping with this,

cell-free extracts of mutant A26 grown in the presence of imidazolyl-lactate showed no change in E_{277} on incubation with imidazolylpyruvate unless L-glutamate was added to the reaction mixture (Fig. 4). A steady increase in extinction was then observed, and concomitant with this there was a change in λ_{max} from 282 nm to 277 nm, corresponding to the conversion of imidazolylpyruvate into urocanate. Soluble, cell-free extracts of mutant A26 did not accumulate urocanate from imidazolyl-lactate although, as shown in Table 6, sonicated preparations of broken cells would do so. This conversion was examined quantitatively by using [2- ^{14}C]imidazolyl-lactate in a reaction mixture containing sonically disrupted cells previously grown in the presence of imidazolyl-lactate. The results (Fig. 5) show that at any particular time during the reaction, all the radioactivity lost from imidazolyl-lactate was accounted for as urocanate.

Discussion

The pathways of degradation of imidazolylpropionate and imidazolyl-lactate, and their relationship to the pathway for histidine degradation, are shown in Scheme 2. That the degradation of histidine proceeds via formylglutamate, as in *P. fluorescens*, *Pseudomonas putida* and *P. aeruginosa*, is worthy of emphasis since in many respects the Pseudomonads of the fluorescent group differ substantially from those of the acidovorans group, of which *P. testosteroni* is a member. Good examples of these differences are found in the pathways by which representatives of the two groups of organism degrade tryptophan and in the way in which they accomplish the cleavage of the aromatic ring when oxidizing compounds such as protocatechuate (Stanier *et al.*, 1966).

Although our initial experiments showed that extracts of cells grown on imidazolylpropionate were able to degrade urocanate, this alone was not sufficient to establish that urocanate was an intermediate of imidazolylpropionate catabolism. Since imidazolylpropionate has been shown to be a gratuitous inducer for the enzymes of the histidine pathway in a number of organisms (Magasanik, 1963; Schlesinger & Magasanik, 1965; Lessie & Neidhardt, 1967) it was possible that it behaved similarly in *P. testosteroni* without actually being degraded via urocanate. Even with the assumption that some enzymes of the histidine pathway were utilized for the degradation of imidazolylpropionate it was still uncertain which was the point of entry into this pathway. If imidazolylpropionate were first metabolized to urocanate then the co-ordinate induction of histidase and urocanase in this organism would explain the presence of histidase. But similarly, if, as is possible, imidazolonylpropionate hydrolase (EC 3.5.2.7) were co-ordinately induced with histidase and urocanase, then imidazolylpropionate might be hydroxylated to imidazolonyl-

propionate as the first intermediate and thus provide an explanation for the presence of urocanase without implicating urocanate as an intermediate in the pathway.

The hydroxylation of imidazolylpropionate to imidazolonylpropionate was at first considered to be the most-probable initial step, particularly since an analogous reaction has been shown to occur for the degradation of imidazolylacetate in *P. fluorescens* (Hayaishi *et al.*, 1957). This hydroxylation is catalysed by a mixed-function oxygenase (EC 1.14.1.5) that utilizes NADH as the reductant. However, no imidazolylpropionate hydroxylase activity could be detected in extracts of *P. testosteroni* grown on imidazolylpropionate. It is perhaps also of significance that *P. testosteroni* cannot utilize imidazolylacetate as the sole source of carbon for growth.

The accumulation of urocanate from imidazolylpropionate by cells grown with imidazolylpropionate as the carbon source indicates clearly that urocanate is a degradation product. The extent of this accumulation, caused by the inhibition of urocanase (Hassall & Rabie, 1966), and the fact that the equilibrium position of the urocanase reaction is greatly in favour of imidazolonylpropionate formation, does not allow the accumulation of urocanate to be explained in terms of the initial conversion of imidazolylpropionate into imidazolonylpropionate followed by a reversal of the urocanase reaction.

The independent nature of the system catalysing the formation of urocanate from imidazolylpropionate is shown by a number of experiments. First, there is some evidence from the fact that cells grown on histidine, urocanate or imidazolyl-lactate cannot oxidize imidazolylpropionate as determined manometrically. Secondly, cells grown on urocanate are unable to accumulate urocanate from imidazolylpropionate. Both these findings contrast with the observation of Hug *et al.* (1966) that cells of *P. fluorescens* were able to oxidize imidazolylpropionate without a lag period after preincubation with urocanate. The results of the experiments following the uptake of [^{14}C]imidazolylpropionate show that although there is an inducible system for the transport of imidazolylpropionate into the cell, this system is present after growth of the organism on urocanate. Thus non-metabolism of imidazolylpropionate under these conditions is due to the non-induction of the enzyme system necessary for its conversion into urocanate rather than to the non-entry of the substrate into the cell. Results presented in the following paper (Coote & Hassall, 1973) show that, as in *K. aerogenes* (Schlesinger & Magasanik, 1965) imidazolylpropionate enters the cell via a transport system normally responsible for the uptake of urocanate.

It has not been possible to detect any activity towards imidazolylpropionate in either soluble extracts or particulate fractions prepared from cells able to

Scheme 2. Degradation pathways of imidazolyllactate, imidazolypropionate and L-histidine in *Pseudomonas testosteroni*

oxidize imidazolypropionate. The enzyme system responsible for the initial transformation of this compound could be membrane-bound, since it appears to be readily inactivated once the structural integrity of the cell is destroyed. Alternatively, it is possible that we have not succeeded in obtaining the necessary reaction conditions when using preparations from disrupted cells.

The pathway of imidazolyllactate degradation proceeds via imidazolypyruvate and histidine to urocanate. However, the enzymology of the first step, the formation of imidazolypyruvate from imidazolyllactate, is somewhat obscure and, as in imidazolypropionate metabolism, there may be considerable loss of enzymic activity on disruption of the cells. It is probable that the imidazolyllactate-NADP oxidoreductase has insufficient activity in the direction of imidazolypyruvate formation for it to be of significance in the oxidation of imidazolyllactate as originally suggested (Coote & Hassall, 1969). The particulate imidazolyllactate oxidase, although apparently constitutive and of low specificity, seems likely to be the enzyme catalysing the

formation of imidazolypyruvate, since it has higher measurable activity than the oxidoreductase. The role of the enzyme detected on polyacrylamide gels by coupling the oxidation of imidazolyllactate to the reduction of Nitro Blue Tetrazolium is not clear, since its activity has not been shown by any other method. This could simply reflect the low activity of this enzyme, since the gel assay procedure is a very sensitive one. The enzyme remains of interest, and possibly of relevance to the degradation of imidazolyllactate, because, unlike the particulate oxidase, it is only present in extracts prepared from cells that are able to oxidize imidazolyllactate.

The second reaction in the pathway of imidazolyllactate metabolism, that catalysed by the aminotransferase, is freely reversible. The enzyme is specific for L-glutamate and has a high activity in cells that are induced with respect to imidazolyllactate degradation. Similar transamination reactions have been reported by other workers. Feldman & Gunsalus (1950) detected the formation of L-glutamate from L-histidine and 2-oxoglutarate by using a strain of *P. fluorescens* unadapted to L-histidine. In addition, an enzyme for

the reversible transamination of L-histidine, found in *E. coli* B (Feldman & Gunsalus, 1950; Wickramasinghe *et al.*, 1967) and *E. coli* W (Westley & Ceithaml, 1956) has also been shown to occur in other organisms, including *K. aerogenes* and *Proteus vulgaris* (Brevet *et al.*, 1968). There is no evidence, however, that this is an inducible enzyme or that it plays a major role in any catabolic pathway.

Further support is given to the validity of the pathways shown in Scheme 2 by the results of the experiments with the various mutant strains of the organism. The urocanase⁻ mutant is unable to grow on histidine, imidazolypropionate or imidazoly-lactate and accumulates urocanate in the medium when presented with these compounds. A mutant lacking histidase cannot grow on histidine or on imidazoly-lactate but it is still able to grow on either imidazolypropionate or urocanate. A third mutant, unable to grow on imidazolypropionate, behaves normally with respect to growth on urocanate, histidine or imidazoly-lactate.

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References

- Baldrige, R. C. & Tourtellotte, C. D. (1958) *J. Biol. Chem.* **233**, 125–127
- Brevet, J., Hoffmeyer, J., Roche, J. & Hedegaard, J. (1968) *C.R. Soc. Biol.* **162**, 1054–1058
- Coote, J. G. & Hassall, H. (1969) *Biochem. J.* **111**, 237–239
- Coote, J. G. & Hassall, H. (1973) *Biochem. J.* **132**, 423–433
- Feldman, I. L. & Gunsalus, I. C. (1950) *J. Biol. Chem.* **187**, 821–830
- Flessel, C. R., Ralph, P. & Rich, P. (1967) *Science* **158**, 658–660
- Fränkel, S. (1903) *Sitzungsber. Akad. Wiss. Wien, Math.-Naturwiss. Kl., Abt. 2B* **112**, 78–98
- Hall, D. A. (1952) *Biochem. J.* **51**, 499–504
- Hassall, H. (1966) *Biochem. J.* **101**, 22p
- Hassall, H. (1971) *Methods Enzymol.* **17B**, 99–102.
- Hassall, H. & Greenberg, D. M. (1963) *J. Biol. Chem.* **238**, 1423–1431
- Hassall, H. & Rabie, E. F. (1966) *Biochim. Biophys. Acta* **115**, 521–523
- Hayaishi, O., Tabor, H. & Hayaishi, T. (1957) *J. Biol. Chem.* **227**, 161–180
- Hedegaard, J., Brevet, J. & Roche, J. (1966) *Biochem. Biophys. Res. Commun.* **25**, 335–339
- Hug, D. H., Wyborny, L. & Roth, D. (1966) *Bacteriol. Proc.* **66**, 103
- Hughes, D. E. (1951) *Brit. J. Exp. Pathol.* **32**, 97–109
- Katz, A. M., Dreyer, W. J. & Anfinsen, C. B. (1959) *J. Biol. Chem.* **234**, 2897–2900
- Kraml, M. & Bouthillier, L. P. (1955) *Can. J. Biochem. Physiol.* **33**, 590–598
- Lessie, T. G. & Neidhardt, F. C. (1967) *J. Bacteriol.* **93**, 1800–1810
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Magasanik, B. (1963) in *Informational Macromolecules* (Vogel, H. J., Bryson, V. & Lampen, J. O., eds.), pp. 271–286, Academic Press, New York and London
- Mehler, A. H. & Tabor, H. (1953) *J. Biol. Chem.* **201**, 775–784
- Mehler, A. H., Tabor, H. & Hayaishi, O. (1955) *Biochem. Prep.* **4**, 50–53
- Milner, H. W., Lawrence, N. S. & French, C. S. (1950) *Science* **111**, 633–634
- Rao, D. R. & Greenberg, D. M. (1960) *Biochim. Biophys. Acta* **43**, 404–418
- Schlesinger, S. & Magasanik, B. (1965) *J. Biol. Chem.* **240**, 4325–4330
- Sen, N. P., McGeer, P. L. & Paul, R. M. (1962) *Biochem. Biophys. Res. Commun.* **9**, 257–261
- Spolter, P. D. & Baldrige, R. C. (1963) *J. Biol. Chem.* **238**, 2071–2074
- Stanier, R. Y., Palleroni, N. J. & Doudoroff, M. (1966) *J. Gen. Microbiol.* **43**, 159–271
- Tabor, H. & Mehler, A. H. (1954) *J. Biol. Chem.* **210**, 559–568
- Westley, J. & Ceithaml, J. (1956) *Arch. Biochem. Biophys.* **60**, 215–225
- Wickramasinghe, R., Hedegaard, J. & Roche, J. (1967) *C.R. Soc. Biol.* **161**, 1891–1896