Biochem. J. (1973) **136**, 649–658
Printed in Great Britain

The Degradation of L-Histidine in the Rat

THE FORMATION OF IMIDAZOLYLPYRUVATE, IMIDAZOLYL-LACTATE AND IMIDAZOLYLPROPIONATE

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(*Received* 8 *May* 1973)

1. Soluble and mitochondrial forms of histidine–pyruvate aminotransferase were separated from rat liver preparations by chromatography on DEAE-cellulose. 2. These enzymes were characterized with respect to substrate specificity, substrate affinity, pH optimum, stability and molecular weight by chromatography on Sephadex G-200. 3. Each enzyme has a relatively broad specificity showing significant activity towards L-phenylalanine and L-tyrosine and catalysing transamination with a number of monocarboxylic 2-oxo acids. 2-Oxoglutarate is not a substrate for either enzyme. 4. The molecular weights of the two enzymes, by chromatography on Sephadex G-200, are in the range 130000–150000. 5. The formation *in vitro* of imidazolyl-lactate from imidazolylpyruvate and NADH was demonstrated by using liver preparations. 6. From a study of imidazolyl-lactate–NAD^+ oxidoreductase activity after electrophoresis of liver preparations on polyacrylamide gel, and from an examination of the activity of L-lactate–NAD^+ oxidoreductase (EC 1.1.1.27) towards imidazolylpyruvate, it is concluded that this latter enzyme is responsible for the formation of imidazolyl-lactate in the liver. 7. Preparations of bacteria obtained from rat faeces form imidazolylpropionate from L-histidine and urocanate without further subculture. The amount of imidazolylpropionate formed is increased under anaerobic conditions and more so in an atmosphere of H_2. It is suggested that the gut flora of the rat contribute largely, if not exclusively, to the formation of imidazolylpropionate normally found in the urine.

The major pathway for the degradation of L-histidine in the mammal proceeds via urocanate (imidazol-5-ylacrylate), imidazol-4-on-5-ylpropionate and *N*-formimino-L-glutamate to glutamate (Brown & Kies, 1959). However, in addition to intermediates of this pathway a number of other clearly recognizable degradation products of histidine have been identified in mammalian systems. Apart from the methylated derivatives of histidine, probably the most significant are imidazol-5-ylpyruvate, imidazol-5-yl-lactate and imidazol-5-ylpropionate, all of which are normal constituents of mammalian urine, increasing in concentration after dietary histidine loading (Baldridge & Tourtellotte, 1958; Sen *et al.*, 1962). In man, the clinical condition of histidinaemia (Ghadami *et al.*, 1961), where histidase (L-histidine ammonia-lyase, EC 4.3.1.3) is lacking or defective, results in an increased urinary excretion of imidazolylpyruvate and imidazolyl-lactate (Auerbach *et al.*, 1962).

The significance of these compounds with respect to the normal metabolism of histidine has not been fully elucidated. Imidazolylpyruvate is formed by transamination of L-histidine and Spolter & Baldridge (1964) have partially characterized mitochondrial and soluble histidine–pyruvate aminotransferases in crude preparations obtained from rat liver. It has been assumed that imidazolyl-lactate is formed from imidazolylpyruvate and that a reduction of urocanate gives rise to imidazolylpropionate (Sen *et al.*, 1962). However, little appears to be known about the enzymology of these reactions, particularly with respect to the specificities of the systems involved.

The present paper reports the separation and further characterization of the mitochondrial and soluble histidine–pyruvate aminotransferases. The formation of imidazolyl-lactate from imidazolylpyruvate *in vitro* is described, and it is concluded that L-lactate–NAD^+ oxidoreductase (EC 1.1.1.27), rather than a specific enzyme, is responsible for this reaction.

The origin of imidazolylpropionate was also examined and results are given which show that this compound is readily formed from urocanate by preparations of gut bacteria. This observation is of interest, since the report of Van der Heiden *et al.* (1972) that, in man, there is an increased excretion of imidazolylpropionate in urine associated with various intestinal disorders. It is suggested by these authors also that imidazolylpropionate is a bacterial metabolite of histidine.

Materials and Methods

Chemicals

Urocanic acid and imidazolylpropionic acid, including the ^{14}C-labelled forms, were synthesized as described by Coote & Hassall (1973*a*).

Other chemicals were purchased as follows: imidazolylpyruvate from Calbiochem, Los Angeles, Calif., U.S.A.; Tris, L-histidine, imidazolyl-L-lactate, oxo acids, NADH and NADPH from Sigma (London) Chemical Co., London S.W.6, U.K.; [^{14}C]histidine from The Radiochemical Centre, Amersham, Bucks., U.K.; lactate dehydrogenase, malate dehydrogenase, aspartate–oxoglutarate aminotransferase, alanine–oxoglutarate aminotransferase and alcohol dehydrogenase from the Boehringer Corp. (London) Ltd., London W.5, U.K. The remaining reagents were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K., and were AnalaR grade where available.

Methods

Enzyme assays. All assays were carried out at 25°C in a Gilford 2000 multiple-sample absorbance recorder fitted with a Unicam SP.500 monochromator. One unit of enzyme activity is defined as the amount of enzyme transforming 1 μmol of substrate/min under the conditions of the assay.

L-Histidine–pyruvate aminotransferase was assayed by the method of Lin *et al.* (1958) as improved by Spolter & Baldridge (1963). The formation of the enol–borate complex of imidazolylpyruvate was followed spectrophotometrically at 293nm (ϵ12000 litre·mol^{-1}·cm^{-1}) in borate–arsenate buffer. The complete system (3.5ml) consisted of 500μmol of sodium tetraborate (adjusted to pH8.5 with 2M-HCl), 70μmol of sodium arsenate, 80μmol of EDTA (to inhibit histidase), 100μmol of L-histidine, 160μmol of sodium pyruvate and 0.2ml of enzyme preincubated for 10min with 75μg of pyridoxal 5′-phosphate/ml. Pyruvate was omitted from the control reaction.

Other enzymes were assayed as follows: histidase (L-histidine ammonia-lyase, EC4.3.1.3) and urocanase as described by Coote & Hassall (1973*b*); L-aspartate–2-oxoglutarate aminotransferase (EC 2.6.1.1) and L-alanine–2-oxoglutarate aminotransferase (EC 2.6.1.2) by using coupled-assay procedures with malate dehydrogenase (EC 1.1.1.37) and lactate dehydrogenase (EC 1.1.1.27) respectively (Bergmeyer & Bernt, 1963*a*, *b*); lactate dehydrogenase (L-lactate–NAD$^+$ oxidoreductase) as described by Bergmeyer *et al.* (1963) by following NADH oxidation in the presence of pyruvate. For the assay of oxidoreductase activity towards imidazolylpyruvate, the reaction mixture contained 250μmol of KH_2PO_4, 0.4μmol of NADH, 5μmol of imidazolylpyruvate and enzyme in a total volume of 3ml, pH6.8.

Where apparent Michaelis constants are quoted they were determined graphically from plots of rate^{-1} against substrate concn.$^{-1}$ as described by Lineweaver & Burk (1934). Reaction rates were assayed at a minimum of eight different substrate concentrations.

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 (BDH Chemicals Ltd.) as the reference standard. The elution of protein from chromatography columns was monitored by measuring the E_{280} of the eluate.

Preparation of histidine–pyruvate aminotransferase fractions. The livers of adult female albino rats were used. The initial stages of the fractionation scheme followed those of the procedure of Spolter & Baldridge (1964) and, unless otherwise stated, these and subsequent steps were carried out at 0–4°C. The rats were first stunned and killed by bleeding from the neck. The livers from up to 10 animals were homogenized in 10g quantities with 2vol. of ice-cold 0.25M-sucrose in a Potter–Elvehjem homogenizer with a loose-fitting plunger (0.4mm clearance) rotating at 1500rev./min. The homogenate was centrifuged at 1000*g* for 10min and the sedimented material was discarded. The supernatant solution was centrifuged at 78480*g* for 1h. The supernatant from this second centrifugation step is referred to as the 'soluble fraction', and the pellet as the 'particulate fraction'.

The soluble fraction was heated for 15min in a water bath at 60°C with stirring. It was then cooled in ice and centrifuged at 12000*g* for 10min. The precipitated material was discarded and the supernatant solution was fractionated by the addition of solid $(NH_4)_2SO_4$, first to 40% and then to 65% saturation. During this procedure the pH of the preparation was maintained at 7.5–8.0 with aq. 2M-NH_3. The 40–65%-satd. $(NH_4)_2SO_4$ precipitate was dissolved in a minimum volume of 15mM-H_3BO_3 (adjusted to pH8 with 1M-NaOH). This solution (usually 10–15ml) was then freed from $(NH_4)_2SO_4$ by passing it through a column (30cm×1.5cm) of Sephadex G-25 (fine grade) that had been previously equilibrated with the 15mM-H_3BO_3 solution at pH8. The further purification of the enzymes in this fraction is described in the legend to Fig. 1.

The particulate fraction obtained after the first high-speed centrifugation (78480*g*) was washed by suspending it in a volume of 0.25M-sucrose equal to the volume of the removed supernatant solution (soluble fraction), followed by re-centrifugation at 78480*g* for 1h. The supernatant solution was discarded and a similar volume of 5mM-NaOH in 0.14M-KCl was added to the pellet. The suspension was homogenized in a Waring blender. This procedure (Spolter & Baldridge, 1964) disrupts the mitochondria and liberates histidine–pyruvate aminotransferase.

The suspension was then centrifuged again at

78480*g* for 1h and the supernatant solution (the crude mitochondrial preparation) was decanted and fractionated with $(NH_4)_2SO_4$. The protein fraction precipitated with $(NH_4)_2SO_4$ between 30 and 65% saturation contained all the recoverable histidine–pyruvate aminotransferase activity. This precipitate was dissolved in a minimum volume of 15mM-H_3BO_3 (adjusted to pH8 with 1M-NaOH) and desalted on Sephadex G-25 (fine grade). The enzyme was further purified by chromatography on DEAE-cellulose by using conditions identical with those used for the fractionation of the soluble preparation (Fig. 1).

Chromatography of samples on Sephadex G-200. For an approximate determination of the molecular weights of the histidine–pyruvate aminotransferases, fractions containing activity were chromatographed on Sephadex G-200 by the procedure of Andrews (1965). A column (70cm × 1.5cm) of gel was equilibrated with 0.1M-Tris buffer, adjusted to pH8.5 with 2M-HCl. It was then calibrated by measuring the elution volumes of substances of known molecular weights (cytochrome *c*, 12400; bovine serum albumin, 68000; catalase, 240000; Blue Dextran) and finally washed with 500ml of the Tris buffer before use. The sample volume was kept below 2ml for each chromatography run.

Preparation of bacterial suspensions from faeces. Faeces, usually 5g, were collected fresh from several adult albino rats kept in a wire-bottomed cage. They were weighed and homogenized in 2vol. of 0.1M-KH_2PO_4 (adjusted to pH7.2 with 5M-NaOH). The suspension was filtered through lint and the filtrate was centrifuged at 1000*g* to remove debris, mainly fibrous matter. The supernatant solution was re-centrifuged (10000*g* for 10min) and the pellet, containing the bacteria, was suspended in 1ml of 0.1M-KH_2PO_4 buffer and stored frozen until required.

Paper chromatography and high-voltage paper electrophoresis. All paper chromatography was carried out on Whatman no. 1 paper in an ascending direction. The following solvent systems were used: A, the organic phase of 2-methylpropan-1-ol–formic acid–water (19:2:6, by vol.) (Hassall & Greenberg, 1963); B, butan-1-ol–pyridine–water (1:1:1, by vol.); C, butan-1-ol–acetic acid–water (4:1:1, by vol.); D, propan-2-ol–aq. conc. NH_3–water (8:1:1, by vol.) (Sen *et al.*, 1962). High-voltage paper electrophoresis was done on Whatman 3MM paper by using a Pherograph–Original–Frankfurt type 1963–1964 instrument. Two buffers were used: water–pyridine–acetic acid (450:25:1, by vol., pH6.5) and water–pyridine–acetic acid (189:1:10, by vol., pH3.7) (Katz *et al.*, 1959). A potential of 50V/cm was applied to the paper via platinum electrodes for 40–120min. Compounds were detected and identified after paper chromatography and electrophoresis as described by Coote & Hassall (1973*a*).

Measurement of radioactivity. 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 aluminium planchet. These were assayed for radioactivity in a Nuclear–Chicago gas-flow counter.

Electrophoresis of proteins in polyacrylamide gels. Disc electrophoresis of protein samples was carried out in polyacrylamide gel essentially as described by Davis (1964). A small-pore running gel of 7.5% (w/v) polyacrylamide was poured to a height of 7cm in gel tubes of 5mm internal diameter. The sample proteins were applied in 0.2ml of 10% (w/v) sucrose solution placed immediately above the spacer gel. The buffer vessels contained Tris–glycine buffer (pH8.6) and electrophoresis was carried out with a current of 4mA per tube and discontinued when the Bromophenol Blue marker dye, added to the cathode solution, had migrated through the gels. NAD^+ oxidoreductase activity was detected on gels by coupling the reaction to Nitro Blue Tetrazolium with *N*-methylphenazonium methosulphate. Each gel was incubated for 1h in the dark in 5ml of solution containing 10μmol of L-lactate or imidazolyl-L-lactate, 10μmol of NAD^+, 40μmol of Tris buffer (pH9.2), 50μg of Nitro Blue Tetrazolium and 50μg of *N*-methylphenazonium methosulphate. Oxidoreductase acitivity was indicated by the formation of a dark-purple band. Histidine–pyruvate aminotransferase activity was detected by a method similar to that used for histidase (Hassall *et al.*, 1970) and dependent upon the formation of the enol–borate complex of imidazolylpyruvate. The gels were incubated in a reaction mixture consisting of 17.5ml of 0.2M-sodium tetraborate–0.028M-sodium arsenate (pH8.5), 1.5ml of 0.5M-L-histidine, 3ml of 0.2M-EDTA, 7ml of 20% (w/v) sucrose and 50μg of pyridoxal 5′-phosphate. They were then photographed with transmitted u.v. light after 10–15min of incubation to detect histidase and then at 15min intervals after the addition of 2ml of 0.8M-sodium pyruvate. The position of histidine–pyruvate aminotransferase was indicated by the appearance of a white (u.v.-absorbing) band dependent upon the addition of pyruvate.

Results

Separation of histidine–pyruvate aminotransferases

The initial stages of the fractionation procedure have been described in the preceding section. The desalted $(NH_4)_2SO_4$ fractions obtained from the soluble and particulate preparations were chromatographed separately, but under identical conditions, on DEAE-cellulose. Three peaks of activity were obtained from the soluble preparation when the column eluate was assayed for histidine–pyruvate aminotransferase activity (Fig. 1). The third of these

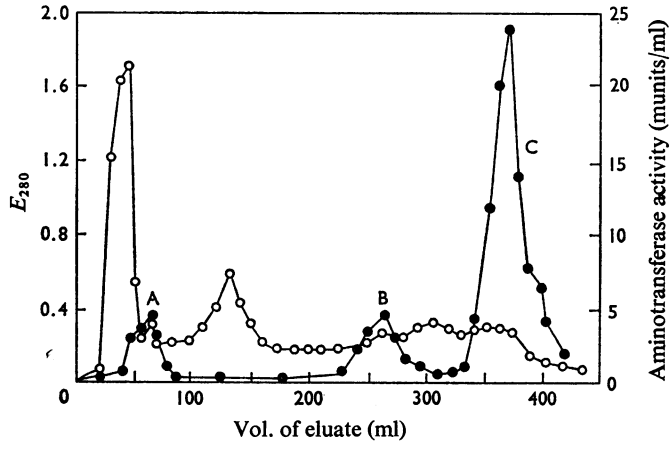

Fig. 1. *Separation of histidine–pyruvate aminotransferase activities by DEAE-cellulose chromatography of a soluble preparation from rat liver*

A desalted $(NH_4)_2SO_4$ fraction (10ml) of homogenate supernatant was chromatographed on a column (20cm×1.5cm) of DEAE-cellulose. The sample was washed into the column with 20ml of 0.015M-H_3BO_3 (buffered at pH8.0 with NaOH) and eluted with a linear gradient of 0–0.2M-NaCl in the same buffer. ○, Protein concentration; ●, histidine–pyruvate aminotransferase activity (peaks A and B) and histidase activity (peak C).

peaks (peak C) proved to be due to histidase that was not completely inhibited by the EDTA added to the borate assay mixture. This enzyme was readily distinguished from the aminotransferases by the fact that, under the assay conditions, there was the same increase in E_{293} whether pyruvate was present or not. The product of the reaction was subsequently shown to be urocanate by paper chromatography and paper electrophoresis.

The fractions corresponding to each peak of aminotransferase activity were pooled to give soluble fractions A and B respectively. Fraction B was always the more active of these two fractions, normally being two to three times as active as fraction A.

When the $(NH_4)_2SO_4$ fraction from the particulate preparation was chromatographed on DEAE-cellulose under the same conditions, only one peak of histidine–pyruvate aminotransferase activity was obtained and this was eluted in the same position as soluble fraction A shown in Fig. 1.

The maximum specific activities of the three aminotransferase preparations were as follows (munits/mg of protein): soluble fraction A, 14; soluble fraction B, 18; mitochondrial (particulate) enzyme, 45. These specific activities compare with a value of 0.9munit/mg obtained for the specific activity of the crude supernatant obtained after the first centrifugation step to remove whole cells, cell debris and nuclei. As might be expected from the elution profile obtained from DEAE-cellulose, all three preparations showed considerable heterogeneity with respect to protein content when subjected to polyacrylamide-gel electrophoresis. However, all three enzyme fractions were free from histidase and urocanase and showed negligible L-aspartate–2-oxoglutarate aminotransferase and L-alanine–2-oxoglutarate aminotransferase activities.

Properties of histidine–pyruvate aminotransferases

Because of its elution position during DEAE-cellulose chromatography, it seemed possible that the minor component of the soluble fraction (fraction A) was enzyme that had been released from damaged mitochondria during the fractionation procedure. Further studies confirmed that this was indeed highly probable in that, with respect to the properties described below, no significant differences could be observed between the particulate (mitochondrial) enzyme and soluble fraction A.

Apparent Michaelis–Menten constants

The various K_m values were determined by the normal assay procedure for histidine–pyruvate aminotransferase, but first the concentration of pyruvate (2.8–46mM) and then the concentration of L-histidine (0.28–28mM) was varied while keeping the concentration of the other substrate constant. In both cases, the particulate (mitochondrial) enzyme and soluble fraction A enzyme behaved similarly. The K_m values for pyruvate are also very similar for the two major preparations (8.5mM and 9mM for the

mitochondrial and soluble B forms respectively) whereas those for histidine are significantly different. The soluble enzyme has a much lower K_m value towards histidine (1.2mM) than has the mitochondrial enzyme (8.3mM) and is also subject to inhibition at high concentrations of substrate (Fig. 2).

Substrate specificity

The specificities of the enzymes with respect to certain amino and oxo acids were determined by the standard enol–borate assay procedure but with each substrate replaced accordingly. The range of oxo acids that can be used is limited to those that do not interfere with the assay by absorbing at 293nm either directly or after complexing with borate. For similar reasons, only amino acids with oxo acid analogues that form complexes that absorb in the u.v. can be used in place of L-histidine. The assay is suitable for monitoring transamination of phenylalanine or tyrosine since they give rise to the oxo acids, phenylpyruvic acid and *p*-hydroxyphenylpyruvic acid, which form enol–borate complexes with characteristic absorption spectra (ϵ_{310} 12400 and ϵ_{300} 9150 litre·mol^{-1}·cm^{-1} respectively). Data for the rates with different oxo acids and aromatic amino acids are shown in Table 1.

Samples (0.2ml) of the soluble enzyme (fraction B) were subjected to disc polyacrylamide-gel electrophoresis. The gels were then examined for L-histidine aminotransferase activity with, respectively, pyruvate, glyoxylate, 2-oxobutyrate, 2-oxohexanoate and 4-methyl-2-oxopentanoate as the amino acceptor. In each assay system, and when using mixed substrates, only one band of activity was detected, showing that probably only one enzyme was involved in the transamination of the compounds tested.

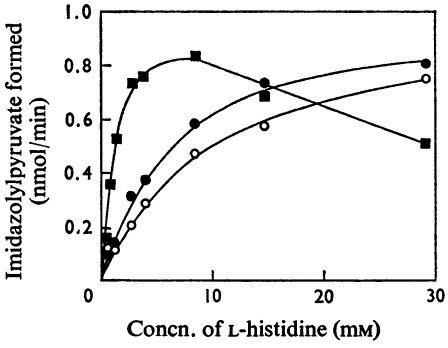

Fig. 2. *Effect of* L-*histidine concentration on the activities of histidine–pyruvate aminotransferases fractionated from rat liver*

The preparations used were the pooled fractions corresponding to the peaks of activity separated by DEAE-cellulose chromatography. Each reaction mixture (3.5ml) contained 160μmol of sodium pyruvate and 0.1ml of enzyme preparation. ○, Peak A from the soluble fraction; ■, peak B from the soluble fraction; ●, mitochondrial enzyme.

pH optima

For the determination of the pH optima of the soluble and mitochondrial enzymes, buffer solutions were prepared containing final concentrations of 0.1M-sodium tetraborate and 0.014M-sodium arsenate with the pH adjusted to the required value with 2M-HCl. EDTA, histidine and pyruvate concentrations were as given under 'Methods'.

A pH optimum of 8.9–9.1 was obtained for the mitochondrial enzyme and 9.2–9.4 for the soluble enzyme (soluble fraction B).

Stability

Crude preparations of the enzymes were stable to repeated freezing and thawing from storage at −18°C, with little loss in activity over several weeks. Similarly, the partially purified enzymes could be stored frozen although these preparations were not subjected to repeated freezing and thawing.

At 4°C and pH6.0, the soluble enzyme (fraction B) was completely inactive after 15 days of storage but, at pH7.2 and 8.0, it retained 100 and 75% of its activity respectively. The mitochondrial enzyme lost all its activity under these storage conditions.

A similar pattern was observed with respect to heat stability. Samples of the enzymes in 15mM-sodium borate buffer (pH8.0) were heated in a water bath at 50° and 70°C for 15min. After cooling them in ice and removing the denatured protein by centrifugation, the aminotransferase activities were assayed. The soluble fraction (fraction B) retained all its activity at 50°C and 54% at 70°C. The mitochondrial enzyme was again less stable and retained only 66 and 17% of its activity respectively when heated at these temperatures.

Requirement for pyridoxal 5′-phosphate

An unequivocal demonstration of an absolute requirement for pyridoxal phosphate was not obtained, although some degree of dependence of histidine–pyruvate aminotransferase activity upon added cofactor was observed. If pyridoxal phosphate was omitted from reaction mixtures, then the rate of reaction was decreased by an amount that varied from 25 to 60%, depending on the preparation. No correlation was obtained between the extent of purification of the enzymes and pyridoxal phosphate activation, since crude preparations frequently

Table 1. *Substrate specificities of* L-*histidine–pyruvate aminotransferases obtained from the soluble and mitochondrial fractions of rat liver*

Preparations were used after chromatography on DEAE-cellulose (Fig. 1). Aminotransferase activity towards aromatic amino acids was measured spectrophotometrically by following the appearance of the enol–borate complex of the particular oxo acid formed. For the determination of rates with different oxo acids, 100μmol of L-histidine and 16μmol of oxo acid were present in 3.5ml of reaction mixture. Other reaction mixtures contained 150μmol of pyruvate and 20μmol of amino acid. The activity obtained with L-histidine and pyruvate as substrates is in every case set at 100.

Substrates present in the reaction mixture	Relative reaction rate: Soluble enzyme (fraction B)	Relative reaction rate: Mitochondrial enzyme
Pyruvate + L-histidine	100	100
Pyruvate + D-histidine	0	0
Pyruvate + DL-phenylalanine	38	40
Pyruvate + DL-tyrosine	85	18
L-Histidine + pyruvate	100	100
L-Histidine + 2-oxobutyrate	160	21
L-Histidine + 2-oxohexanoate	730	69
L-Histidine + 4-methyl-2-oxopentanoate	146	32
L-Histidine + glyoxylate	146	160
L-Histidine + 2-oxoglutarate	0	9

showed as great a dependence upon the cofactor as did the partially purified enzymes.

Chromatography of histidine–pyruvate aminotransferase on Sephadex G-200

Both the mitochondrial enzyme and the soluble enzyme were eluted from a column of Sephadex G-200 when the ratio of elution volume to exclusion volume was 1.6–1.7. By comparison with the elution pattern obtained with proteins of known molecular weight, this range corresponds to a molecular weight of 130000–150000.

Formation of imidazolyl-lactate

For studies of the formation of imidazolyl-lactate, rat liver was homogenized in 2vol. of 0.1M-KH_2PO_4 (adjusted to pH7.2 with 5M-NaOH). The supernatant solution obtained from this preparation after centrifuging at 78480*g* for 1h was fractionated with solid $(NH_4)_2SO_4$ and the precipitate at 35–60% satn. at 0°C was collected. This was dissolved in 0.1M-KH_2PO_4 (adjusted to pH6.8 with 5M-NaOH) to give a solution equal in volume to one-third that of the original homogenate.

This preparation was then assayed for its ability to catalyse the oxidation of NADH in the presence of imidazolylpyruvate. The pH optimum for this was pH7.0–7.1, and was fairly sharp. Under these optimal conditions, the rate of oxidation of NADH was 44nmol·min^{-1}·ml^{-1} for the enzyme preparation; the rate of oxidation of NADPH under similar conditions was 45% that of NADH. The apparent K_m values for imidazolylpyruvate and NADH were 1.75×10^{-3}M and 1×10^{-5}M, when determined at fixed concentrations of NADH (0.13mM) and imidazolylpyruvate (1.7mM) respectively.

Identification of imidazolyl-lactate as the product of imidazolylpyruvate metabolism

Imidazolylpyruvate (245μmol) was incubated with 5mg of yeast alcohol dehydrogenase (alcohol–NAD^+ oxidoreductase, EC 1.1.1.1), 13μmol of NAD^+, 500μmol of ethanol and 3.5ml of a dialysed 35–60%-satd. $(NH_4)_2SO_4$ fraction from rat liver. The final volume was made up to 6ml with 0.1M-KH_2PO_4 (pH7.0). After incubation of this reaction mixture at 35°C for 12h, paper chromatography of a 5μl sample showed that no imidazolylpyruvate remained. A small-scale control reaction mixture, from which the liver preparation had been omitted, showed no metabolism of imidazolylpyruvate.

The protein in the main reaction mixture was precipitated by the addition of 1ml of 2M-HCl and then heating the suspension at 100°C for 2min. The supernatant solution was chromatographed on a column (20cm×1cm) of Dowex 50 (H^+ form) by using 250ml of 0.3M-HCl, then 0.5M-HCl, as the eluent. The fractions collected after passage of 50–150ml of 0.5M-HCl through the column were imidazole-positive. They were pooled and evaporated

to dryness under reduced pressure. The residue (31 mg) was dissolved in the minimum amount of ethanol and precipitated with an excess of diethyl ether. The identity of this as imidazolyl-lactic acid hydrochloride was established by paper chromatography and high-voltage paper electrophoresis and by elementary analysis (Found: C, 37.5; H, 4.6; N, 14.8; halogen, 18.7. Calc. for $C_6H_8O_3N_2$,HCl: C, 37.4; H, 4.7; N, 14.6; Cl, 18.4%).

Activity of lactate dehydrogenase towards imidazolylpyruvate

Because of the high activity of lactate dehydrogenase in the $(NH_4)_2SO_4$ fraction obtained from rat liver ($280\mu mol \cdot min^{-1} \cdot ml^{-1}$), it was considered probable that this enzyme was responsible for the formation of imidazolyl-lactate. This was investigated in two ways: first, by locating oxidoreductase activity after electrophoresis of the sample on polyacrylamide gels, with both lactate and imidazolyl-lactate as substrates in the presence of NAD^+, *N*-methylphenazonium methosulphate and Nitro Blue Tetrazolium; secondly, by assaying the activity of purified lactate dehydrogenase towards imidazolylpyruvate.

After disc polyacrylamide-gel electrophoresis of the rat liver preparation, one main band of activity was observed when the gel was stained for NAD^+ oxidoreductase activity in the presence of lactate; a second fainter band, migrating nearer the anode, was also detected. The pattern obtained with imidazolyl-lactate as the substrate was the same as that given by lactate. Different amounts of the preparation were subjected to electrophoresis to allow for the previously observed differences in activities towards the two substrates.

Commercially obtained lactate dehydrogenase (from pig heart) with an activity towards pyruvate of $330\mu mol \cdot min^{-1} \cdot mg^{-1}$ showed a low, but definite, activity when imidazolylpyruvate was used as the substrate ($42 nmol \cdot min^{-1} \cdot mg^{-1}$) and was readily detected after gel electrophoresis when NAD^+ and imidazolyl-lactate were used as substrates. The apparent K_m value of this enzyme towards imidazolylpyruvate was 6.7×10^{-4} M.

Relative activities of histidine-degrading enzymes in rat liver

The activities of histidase, urocanase, histidine–pyruvate aminotransferase and 'imidazolyl-lactate–NAD^+ oxidoreductase' were compared. A 30–65%-satd. $(NH_4)_2SO_4$ fraction was used to facilitate the assay of the oxidoreductase activity by avoiding the high endogenous oxidation of NADH found with crude homogenate supernatant solutions. The 30–65%-satd. $(NH_4)_2SO_4$ fraction contained all the recoverable activities of the other enzymes assayed. The observed activities were as follows ($nmol \cdot min^{-1} \cdot ml^{-1}$): histidase, 66.7; urocanase, 30.3; histidine–pyruvate aminotransferase, 47.5; imidazolyl-lactate–NAD^+ oxidoreductase 26.6.

Formation of imidazolylpropionate

With homogenates of rat liver and rat kidney we were unable to detect the formation of [^{14}C]imidazolylpropionate from either [^{14}C]histidine or [^{14}C]urocanate. The ability of intestinal bacteria to form imidazolylpropionate from these substrates was then investigated.

Reaction mixtures were deproteinized by heating them at 100°C for 2min. The insoluble material was removed by centrifugation and samples of the supernatant solution were analysed by two-dimensional paper chromatography in solvents C and B, followed by radioautography; 5μg of unlabelled urocanate was run on each chromatogram as an internal marker and located after chromatography as a dark spot by u.v. light; it had R_F values of 0.55 and 0.65 in solvents C and B respectively.

Incubation of suspensions of faecal bacteria with [*ring*-2-^{14}C]urocanate gave several radioactive products of which only imidazolylpropionate, formylisoglutamine and formiminoglutamate were identified. Imidazolylpropionate and formylisoglutamine were poorly resolved by the two solvents used. The combined radioactive area was eluted from the paper and subjected to paper electrophoresis at pH6.5. The two compounds were again treated by radioautography and their relative proportions determined.

When L-[*ring*-2-^{14}C]histidine was used as the substrate, in place of [^{14}C]urocanate, a similar pattern of metabolites was obtained; [*ring*-2-^{14}C]imidazolylpropionate was not degraded. Table 2 shows the amounts of the principal ^{14}C-labelled products that are formed from the various substrates. The recovery of total radioactivity from [^{14}C]histidine and [^{14}C]urocanate was usually 15–20%, showing that considerable radioactivity was lost as CO_2 or other volatile products.

The amount of imidazolylpropionate that was produced under normal conditions of aerobic incubation varied considerably with each preparation of faecal bacteria. In all cases, however, the amount formed could be increased by carrying out the incubation anaerobically, and it could be increased even further if a reducing atmosphere of H_2 was used. These stimulatory effects of an anaerobic or reducing atmosphere were particularly pronounced where the aerobic formation of imidazolylpyruvate was relatively slight. This is shown in Table 3. Heat-treated suspensions of active cells did not metabolize urocanate or histidine either aerobically, anaerobically or in an atmosphere of H_2.

Table 2. *Radioactive products formed from [^{14}C]histidine, [^{14}C]urocanate and [^{14}C]imidazolylpropionate by suspensions of rat faecal bacteria*

Bacterial suspension (0.2ml) was incubated aerobically at 37°C for 2h with 0.2ml of *ring*-2-^{14}C-labelled substrate (5μCi/ml; 35μCi/μmol). After heat precipitation of the cells, a 50μl sample of each reaction mixture was chromatographed in two dimensions on paper in solvents C and B. The compounds were located by radioautography and assayed for radioactivity. Chromatographic mobilities are expressed relative to urocanate (R_U).

Compound formed or remaining	Mobility (R_U)		Percentage distribution of radioactivity recovered by paper chromatography from *ring*-2-^{14}C-labelled substrate:		
	Solvent C	Solvent B	L-Histidine	Urocanate	Imidazolyl-propionate
Urocanate	1.0	1.0	4.8	0.1	0
Imidazolylpropionate	0.93	0.55	8.6	16.0	100
Formiminoglutamate	0.40	0.32	37.0	33.0	0
Formylisoglutamine	0.85	0.55	23.0	31.0	0
Unknown 1	0.27	0.61	9.4	5.6	0
Unknown 2	0.73	0.93	6.1	4.3	0

Table 3. *Radioactive products formed from [ring-2-^{14}C]urocanate by suspensions of rat faecal bacteria incubated aerobically, anaerobically and in a reducing atmosphere*

Reaction conditions were as described in Table 2 with the exception that the gas phase was varied as shown.

Compound formed or remaining	Percentage distribution of radioactivity recovered by paper chromatography in an atmosphere of:		
	Air	N_2	H_2
Urocanate	5.3	4.0	4.9
Imidazolylpropionate	0.2	1.7	21.0
Formiminoglutamate	22.0	12.0	24.0
Formylisoglutamine	45.0	62.0	41.0
Unknown 1	5.0	1.7	2.0
Unknown 2	16.0	3.9	1.9

Discussion

The results obtained from our studies of the transamination of histidine are in broad agreement with those of Spolter & Baldridge (1964), who presented evidence for the existence of two histidine–pyruvate aminotransferases in preparations of rat liver. Although these authors did not completely separate the two enzymes, they were able to show that one was mitochondrial and the other was from the cytosol. By using relatively simple fractionation techniques, consisting essentially of $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography, we have been able to separate and characterize further the two aminotransferases.

The pH optima of the two enzymes are similar in that a value of pH8.9–9.1 was obtained for that from the mitochondria and pH9.2–9.4 for the pH optimum of the soluble one. For the unfractionated enzymes, Spolter & Baldridge (1964) reported optima of pH8.4–8.6 and pH8.8–9.4 respectively.

The soluble enzyme is readily differentiated from the mitochondrial one by its relative stability, its lower K_m value for L-histidine and its inhibition at high substrate concentrations of L-histidine. Both enzymes have broad specificities in that phenylalanine and tyrosine can replace histidine as the substrate and pyruvate can be replaced by a number of monocarboxylic 2-oxo-acids. Thus one cannot with certainty identify the substrates for these enzymes *in vivo*. Neither of them, however, showed any activity towards histidine, tyrosine or phenylalanine in the presence of 2-oxoglutarate and they were presumed,

therefore, to be free from tyrosine–2-oxoglutarate aminotransferase (EC 2.6.1.5). Similarly, our preparations of histidine–pyruvate aminotransferase contained negligible amounts of glutamate–pyruvate aminotransferase and glutamate–oxaloacetate aminotransferase whereas both these enzymes were extremely active in crude homogenates of rat liver.

Although the activity of histidine–pyruvate aminotransferases in rat liver is comparable with the activities of histidase and urocanase, enzymes concerned exclusively with histidine degradation, it seems unlikely that transamination is of any significance in normal histidine catabolism. As intra- and extra-mitochondrial enzymes are present, transamination of histidine may be important as part of a mechanism for the transport of histidine (as imidazolylpyruvate) from the cytosol into the mitochondrion. In postulating a transportation role for the histidine–pyruvate aminotransferase system, it is perhaps of significance that, whereas the main degradative enzyme for histidine (histidase) is negligible in rat liver at birth, and remains low for 2–3 weeks, aminotransferase activity towards histidine is present in the embryo 15 days after gestation and is at a maximum within 24h of birth (Makoff & Baldridge, 1964).

The formation of imidazolyl-lactate and imidazolylpropionate was investigated because of the occurrence of these compounds in mammalian urine. Although we have been able to demonstrate the formation of imidazolyl-lactate from imidazolylpyruvate by the liver preparations, there is no evidence to suggest the existence of a separate specific enzyme. In fact the converse is true in that L-lactate–NAD^+ oxidoreductase is able to catalyse the formation of imidazolyl-lactate.

The rate of reduction of imidazolylpyruvate, relative to that of pyruvate, is extremely low, but, nevertheless, because of the large amounts of lactate dehydrogenase present in liver, the absolute activity towards imidazolylpyruvate is of the same order as the activities of other enzymes concerned with the metabolism of histidine and its derivatives. Disc polyacrylamide-gel electrophoresis and the assay of commercially obtained lactate dehydrogenase showed that both heart and liver forms of the enzyme had imidazolyl-lactate–NAD^+ oxidoreductase activity.

The apparent K_m values of rat liver lactate dehydrogenase towards imidazolylpyruvate and NADH (1.75×10^{-3}M and 1×10^{-5}M respectively) do not preclude the functioning of this enzyme *in vivo* in the formation of imidazolyl-lactate. The formation of imidazolyl-lactate in this way could be expected to be particularly pronounced when there is either dietary histidine loading or in cases of histidinaemia where the lack of histidase activity leads to an increased rate of imidazolylpyruvate formation.

The formation of imidazolylpropionate from urocanate by preparations of faecal bacteria has been conclusively demonstrated. Whether or not the intestinal flora are capable of accounting for all the imidazolylpropionate found in urine is uncertain. Since only relatively small amounts of the compound are excreted normally (Sen *et al.*, 1962), and because it is not further metabolized in mammals (Kraml & Bouthillier, 1955) and hence is not an intermediate in a pathway of histidine degradation, it is probably formed by reduction of urocanate in the gut and then absorbed into the blood and subsequently excreted in the urine. This is in agreement with the suggestion of Van der Heiden *et al.* (1972), who have shown greatly increased amounts of imidazolylpropionate in urine in patients with malabsorptive intestinal disorders. The formation of imidazolylpropionate from urocanate had previously been shown to occur in suspensions of washed micro-organisms from the rumen of the cow (Van den Hende *et al.*, 1963).

The reduction of urocanate to imidazolylpropionate is in many ways analogous to other reductions that can occur in the digestive tract. For example, linolenic acid, containing three unsaturated C=C bonds, is hydrogenated in stages to give the fully saturated acid, stearic acid (Wilde & Dawson, 1966).

The increased formation of imidazolylpropionate from urocanate occurring in an atmosphere of H_2 and to a lesser extent in one of N_2 is consistent with the findings of Polan *et al.* (1964), which were that biohydrogenation of unsaturated fatty acids by rumen bacteria requires complete anaerobiosis and that greatest activity is obtained when H_2 is present. Similarly, suspensions of *Vibrio succinogenes* readily catalyse the formation of succinate from fumarate in the presence of molecular H_2 (Wolin *et al.*, 1961).

The stimulation of imidazolylpropionate formation by H_2 may be caused by the reduction of systems oxidized during the preparation of the bacterial suspensions rather than by the direct reaction of H_2 with urocanate. This is to some extent suggested by the fact that the capacity of faecal bacteria to form imidazolylpropionate aerobically varied from preparation to preparation. However, fumarate can be reduced with molecular H_2 to succinate by the action of hydrogenases from *Escherichia coli* and *Micrococcus lactilyticus* (Peck *et al.*, 1957) so that this reaction also might be a property of the hydrogenase system.

We are grateful to the M.R.C. for financial support to A. V. E., and to Mr. F. R. Daubney of the Department of Organic Chemistry, University of Leeds for doing the elementary analysis.

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