New Derivatives of Argininosuccinic Acid in the Urine of a Patient with Argininosuccinaciduria

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The urine of a patient with argininosuccinaciduria contains several previously undescribed compounds that can be derived chemically from argininosuccinic anhydride (I). One of the major compounds is 8-carboxy-2-carboxymethyl-3-oxo-2,3,5,6,7,8-hexahydro-1*H*-imidazo[1,2-*a*][1,3]diazepine, which exists as two diastereoisomers that are readily interconvertible at pH 5. The corresponding ring-opened monocyclic diazepines are also present, as well as traces of the isomeric hexahydroimidazodiazepines that are formally related to argininosuccinic anhydride (II). The formation and stereochemistry of these compounds are discussed.

Argininosuccinic acid is an intermediate in the Krebs–Henseleit urea cycle. In normal humans it is cleaved by argininosuccinate lyase to give arginine and fumarate. In argininosuccinaciduria, this enzyme appears to be largely absent and quantities of argininosuccinic acid and its derivatives are excreted in the urine. Prominent among these derivatives are two ninhydrin-positive lactams (I) and (II) formed between the guanidino residue and the original α -carboxyl group of the aspartate moiety (Kowalsky & Ratner, 1969; Lee & Pollitt, 1970). These anhydrides were first recognized in argininosuccinaciduria by Westall (1960), though anhydride (I) had been previously prepared by Ratner *et al.* (1953). In this paper the anhydride notation of Ratner and co-workers is used. Anhydrides (I) and (II) correspond to Westall's (1960) compounds C and B respectively. The skeleton notation of Kowalsky & Ratner (1969) is followed for the anhydrides and for argininosuccinic acid (III).

Westall (1960) found indications of a ninhydrin-negative derivative of argininosuccinic acid in the urine from his patient. By using radioactive tracers, Ratner & Kunkemueller (1966) showed that a number of unidentified ninhydrin-negative derivatives of argininosuccinic acid and anhydride (I) were formed by heating at neutral pH. In the present paper, the derivatives found in the urine of a patient with argininosuccinaciduria and those formed chemically from argininosuccinate and its anhydrides are compared, and structures are suggested for some of these compounds.

Experimental

Spectra

N.m.r. spectra were taken on Varian Associates A-60 and HA-100 instruments, with internal tetramethylsilane standards for trifluoroacetic acid

solutions and external tetramethylsilane standards for aqueous solutions. The probe temperature was 30°C. U.v. spectra were recorded on a Beckman DB spectrophotometer and i.r. spectra on a Perkin–Elmer 137 Infracord instrument. Optical rotations of aqueous 0.5–2.0% (w/v) solutions were measured with a Bendix–Ericsson type 143 A automatic polarimeter in a 20 mm cell.

Potentiometric titrations

The compounds were present initially as 0.01 M-hydrochlorides, obtained by addition of HCl to the zwitterionic forms in aqueous solution. These solutions were supersaturated during parts of the titrations. The titrations were done under N₂, the pH being recorded by means of an Electronic Instruments Ltd. model 34 A pH-meter with output to a chart recorder.

Electrophoresis

The pH 2.0 buffer contained formic acid (19 ml/l) and acetic acid (15 ml/l); the pH 5.3 buffer contained pyridine (10 ml/l) and acetic acid (4 ml/l). Electrophoresis was done on Whatmann 3MM paper in either a cooled-plate or white-spirit-cooled apparatus at approx. 5 V/mm. For detection the nitroprusside–ferricyanide reagent (Smith, 1968) or ninhydrin–collidine–acetic acid reagent (von Arx & Neher, 1963) was used.

Sources of compounds

The urine was obtained from a patient with argininosuccinaciduria and was frozen immediately at –14°C without adjusting the pH. This patient was one of the original pair described by Allen *et al.* (1958). *N*²,3-Dimethylglycocyamidinium hydrochloride

(I)

(II)

(III)

had been prepared by the method of Matsumoto & Rapoport (1968). Other compounds were prepared as described by Lee & Pollitt (1970).

Column chromatography

The sulphonic acid (Zeo-Karb 225, 4.5% cross-linkage, passing 200 mesh, SRC 12, and Zeo-Karb 225, 4.5% cross-linkage, 59–100 mesh, SRC 10) resins were prepared and packed by standard methods (Morris & Morris, 1964). Buffers were pumped at constant rate and the effluents were divided, part being collected in a fraction collector and the other portion being used for detection of the compounds. For this a continuous monitoring system, using the Jaffé reaction (Bonsnes & Taussky, 1945), was developed by using standard Technicon autoanalyser parts. The solutions were adjusted to allow for the acidity of the column effluents.

Isolation of (8*S*,2*RS*)-8-carboxy-2-carboxymethyl-3-oxo-2,3,5,6,7,8-hexahydro-1*H*-imidazo[1,2-*a*][1,3]-diazepine

Crude barium argininosuccinate (3 g, was dissolved in water and adjusted with 1*M*-H₂SO₄ to pH 3. The precipitate of BaSO₄ was removed, the supernatant adjusted to pH 5 by the addition of acetic acid (1 ml) followed by 4*M*-NaOH, and then heated at 100°C for 3.5 h. The reaction mixture was adjusted to pH 2 with 5*M*-HCl and applied to a column (500 mm × 18 mm) of Zeo-Karb 225 (SRC 12) resin that had been equilibrated with sodium acetate-formate buffer (0.2*M* with respect to sodium acetate), pH 2.80. A

portion of the effluent was monitored continuously by using the Jaffé reaction. The appropriate fractions were pooled and applied to a column (70 mm × 30 mm) of Zeo-Karb 225 (SRC 10; H⁺ form). The column was washed with water and then rapidly eluted with an ice-cold solution of freshly distilled pyridine, 0.5*M* in water. The effluent was rapidly evaporated to dryness at a temperature below 35°C and the resultant solid was washed with water on a filter and dried at room temperature under vacuum. The product (113 mg) was a white solid, which darkened at 265°C and melted with decomposition at 295°C (Found: C, 47.1; H, 5.1; N, 16.2; C₁₀H₁₃N₃O₅ requires C, 47.1; H, 5.1; N, 16.5%).

Separation of diastereoisomers of 8-carboxy-2-carboxymethyl-3-oxo-2,3,5,6,7,8-hexahydro-1*H*-imidazo[1,2-*a*][1,3]-diazepine

The mixture of diastereoisomers (200 mg) was suspended in water (5 ml) and dissolved by dropwise addition of 2*M*-HCl. The solution was applied to a column (500 mm × 18 mm) of Zeo-Karb 225 (SRC 12) resin that had been equilibrated with 0.5*M*-HCl. The column was eluted with 0.5*M*-HCl and a portion of the effluent was monitored continuously by the Jaffé reaction. The fractions containing the diastereoisomers were pooled separately and the compounds were recovered by evaporation under vacuum at temperatures below 35°C.

Each compound was redissolved in 5*M*-HCl and divided into three portions. Two of these were used for optical-rotation measurements and the third was neutralized and desalted on a Zeo-Karb 225 (SRC 10) column by elution with pyridine. The weight of compound recovered was used for calculating the specific rotations.

Isolation and assay of hexahydroimidazodiazepines from patient's urine

A sample (50 ml, equivalent to 62 min of excretion) from a 24 h collection was applied to a column of Zeo-Karb 225 (SRC 10; H⁺ form) resin. The column was washed with water and the hexahydroimidazodiazepines were eluted with 0.5*M*-pyridine. The pyridine solution was evaporated to dryness and the residue chromatographed on a column of Zeo-Karb 225 (SRC 12) in pH 2.8 buffer as described above. A portion of this effluent was monitored continuously by the Jaffé reaction. The sensitivity of the monitoring system was determined at intervals during the run by disconnecting the column temporarily and running standard solutions of the hexahydroimidazodiazepine through the system. The total hexahydroimidazodiazepine content of the urine was calculated from the integrated areas under the curves by using the sensitivity results and the effluent splitting ratio.

The other portion of the effluent was collected in a fraction collector and the appropriate fractions were desalted and examined by i.r. spectrometry.

A pure sample of anhydride (I) was also subjected to the preliminary desalting step (on the SRC 10 column). Paper electrophoresis showed that no hexahydroimidazodiazepines were formed during this procedure. Neither pure argininosuccinic acid nor pure anhydride (I) gave any hexahydroimidazodiazepine peak on chromatography on the SRC 12 column at pH 2.8.

Results and Discussion

The compounds under investigation do not give colours with ninhydrin under the usual conditions. Ratner & Kunkemueller (1966) used ^{14}C -labelled precursors and detected the ninhydrin-negative compounds by their radioactivity. Westall (1960) initially noted the presence of a ninhydrin-negative substance (substance Y) in the urine extracts by the displacement of an adjacent ninhydrin-positive spot on paper chromatographs. He later found that this compound reacted with the nitroprusside–ferricyanide reagent to give a red colour. A more general and convenient detection method is to apply the conventional ninhydrin–collidine–acetic acid reagent and then to heat the paper to 140°C for a few minutes. The compounds under investigation give a well-defined yellow colour. This is also given by many of the model compounds, including the five- and six-membered lactams of guanidinosuccinic acid. When the patient's urine was examined, the position of the yellow spots coincided with the nitroprusside–ferricyanide-positive material, so that there is little doubt that this method was detecting the compound Y mentioned by Westall (1960).

Urine from the patient was subjected to high-voltage paper electrophoresis at pH 5.3 and 2.0. At pH 5.3 three spots were seen, one moving towards the anode with mobility 1.3 times that of argininosuccinic acid, and a poorly separated pair with mobility 2.2 times that of argininosuccinic acid. If the urine was left at pH 2.0 for a few hours before electrophoresis at pH 5.3, only the slower-moving spot was present, and if the urine was left at pH 10 for a few hours before electrophoresis, the faster-moving pair of spots predominated. The two sets of components separable at pH 5.3 are thus mutually interconvertible, and they were termed the acid-stable and alkali-stable components respectively. This ready interconversion caused several difficulties in attempting to isolate these compounds by column chromatography. At pH 2.0, one major yellow spot was seen, with a mobility 0.62 times that of argininosuccinic acid itself, together with traces of a substance of lower mobility streaking towards the major spot. This pattern was unaffected by leaving the urine to

stand at pH 2 or 10 before electrophoresis, so that the minor spot seen on electrophoresis at pH 2 is not residual alkali-stable component.

Chemical formation from argininosuccinic acid and its anhydrides

Studies by Westall (1960) and by Ratner & Kunkemueller (1966) had indicated that although anhydride (II) was relatively stable, argininosuccinic acid, and particularly anhydride (I), showed considerable loss of ninhydrin-positive material on heating at neutral pH values. The products of these reactions have now been examined by high-voltage electrophoresis. After 10 min at 100°C and pH 7.0, anhydride (I) gave high yields of yellow spots having the same electrophoretic mobility at pH 2.0 and 5.3 as those found in the patient's urine and showing the same pH-dependent interconversions. Argininosuccinic acid reacted more slowly, giving maximum yields of yellow spots after 4 h. These results agree with Westall's (1960) semi-quantitative studies but differ from those of Ratner & Kunkemueller (1966), who found that 40% of the argininosuccinic acid was converted into a ninhydrin-negative material after heating for 10 min at 100°C at pH 7.0.

Attempts were made to separate the mixture of yellow spots obtained from anhydride (I) on a quaternary ammonium resin (De-Acidite FF) in the acetate form by a method similar to that described by Ratner & Kunkemueller (1966). The argininosuccinic acid and anhydrides were eluted as expected, but the yellow spots did not form sharp peaks. Much of the material was eluted by 1M-acetic acid as a broad tailing peak, and complete removal from the column required 3M-acetic acid or 0.1M-HCl. This behaviour would be expected from the properties of the compounds in the mixture. Thus the alkali-stable component is the more strongly acidic (shown by its behaviour on electrophoresis) and would be strongly bound to the column. It would be progressively converted into the less tightly bound acid-stable spots as the pH was lowered, resulting in the observed tailing.

Chromatography of the yellow-spots mixture on a strongly acidic ion-exchange resin (Zeo-Karb 225) at pH 2.8 gave well-defined peaks corresponding to the acid-stable derivatives, but revealed a rather complex situation (Fig. 1). The two major components (peaks B and C) were slowly interconverted on the column, as indicated by the high base-line between them. Rechromatography of isolated peaks B and C confirmed this, though the two major components had identical electrophoretic mobilities at pH 2.0 (Fig. 2). A minor pair of interconverting substances was also present, one component eluting in peak A and the other overlapping with one of the major components in peak B. Thus peak B¹ showed a

Fig. 1. Separation of yellow-spots mixture on strongly acidic ion-exchange resin

A column (1.0cm $\times$ 50cm) of Zeo-Karb 225 was used with pH2.80 buffer. Other experimental details were as for the preparative runs. Samples applied to the column were: (a) reaction mixture from heating anhydride (I) at 100°C, pH 7.0, for 10 min; (b) peak B¹, partially desalted by acidifying it with conc. HCl and evaporating it to small volume to precipitate NaCl, filtering, then evaporating to dryness and dissolving in water; (c) peak C¹, treated similarly to peak B¹.

second yellow-spot component on electrophoresis at pH2.0, and this was still present in B² obtained by rechromatography of B¹. Rechromatography of C¹ gave B³, which contained only the major component. Peak A¹, from the chromatography of the original reaction mixture, contained also a ninhydrin-positive derivative that contributed much of the colour in the Jaffé reaction. Peak A², from the rechromatography of peak B¹, contained only yellow-spot substances (Fig. 2). The minor pair of components separated on electrophoresis at pH2 and the streaking between them indicated interconversion on the paper. The properties of the various compounds are summarized in Table 1.

Isolation of major products

Because of their ready interconversion, the major products were at first isolated as a mixture by desalting the appropriate fractions from the Zeo-Karb

Fig. 2. Electrophoresis of peaks obtained from the ion-exchange column (Fig. 1)

Electrophoresis was at pH2.0 and the spots were detected by heating with ninhydrin–collidine–acetic acid. Hatched spots gave blue or blue–green colours at 100°C, open spots gave yellow colours at 140°C. (a) Peak A¹; (b) peak A²; (c) peak B¹ or B²; (d) peak B³; (e) peaks C¹, C² or C³; (f) marker of aspartic acid.

225 column. The resulting solid had an elemental analysis corresponding to C₁₀H₁₃N₃O₅, i.e. an argininosuccinic anhydride that has lost the elements of ammonia with the formation of a double bond or an additional ring. Hydrolysis with 3M-Ba(OH)₂ at 100°C for 24 h followed by electrophoresis showed ornithine and aspartic acid as the only ninhydrin-positive components. Similar behaviour was found by Westall (1960) for his compound Y. The original compounds are ninhydrin-negative under normal conditions, indicating that the ornithine α -amino group has been masked, but not lost, in the ring closure. These properties are met by four different compounds (ignoring for the time being the possibility of diastereoisomerism) (IV to VII) corresponding to the known anhydrides (I) and (II), and the two six-membered anhydrides that have not been isolated. Only compound (IV), which is 8-carboxy-2-carboxymethyl-3-oxo-2,3,5,6,7,8-hexahydro-1*H*-imidazo[1,2-*a*][1,3]diazepine, can be formed directly from anhydride (I) without opening the guanidino lactam ring. For simplicity the major yellow-spot product will be discussed in terms of this structure and the assumption will be justified later by reference to the properties of some of the minor components.

Table 1. *Chromatographic and electrophoretic properties of yellow-spot compounds*

For further details see the text.

	Column chromatography at pH2.8	Paper electrophoresis
Major acid-stable compounds (primary)	Resolved into two peaks (B and C) with interconversion	Single spot at pH2.0 Single spot at pH5.3
Minor acid-stable	Resolved into two peaks (A and B)	Gave two spots at pH2.0 with streaking; the faster spot overlaps the major acid-stable spot; not resolved from each other or the major acid-stable spot at pH5.3
Alkali-stable compounds	Not seen; probably very rapidly converted into acid-stable compounds	Not seen at pH2.0; gave two overlapping spots at pH5.3

N.m.r. spectra

The spectra of the hexahydroimidazodiazepine mixture, and of a number of other compounds, are summarized in Tables 2 and 3. The resonances for C-3, C-4 and C-5 are broader and more complex than in the anhydrides, suggesting restricted rotation due to ring formation in compound (IV). The C-2 resonance was shifted downfield by comparison with the anhydrides and overlapped the C-8 resonance. The *N*-proton resonances were also consistent with the proposed structure. The triplet due to the α -proton seen in ornithine is missing and instead there are two peaks due to a proton each on N-c and N-d.

Potentiometric titration

The titration curve of the solid hexahydroimidazodiazepine mixture indicated three dissociations, with pK values 2.31, 3.60 and 8.73. The values of pK_1 and pK_2 corresponded to the original ornithine carboxyl

group and the β -carboxyl group of the aspartate residue respectively. The value of 8.73 for pK_3 might be expected for an $N^2,3$ -disubstituted glycohydrazide (Matsumoto & Rapoport, 1968; Lee & Pollitt, 1970).

After the end-point had been reached, a fourth equivalent of KOH was added and the solution was left at 25°C under N_2 for 3 h. This treatment converted an analogous solution largely into the alkali-stable (more acidic) component. The mixture was then rapidly back-titrated with HCl. There was only a slight inflexion near the original pK_3 value, but the compound now showed three acidic groups in the pK range 2–4, indicating ring opening of the guanidino lactam ring to form the compound (VIII).

The compound (IV) mixture had an u.v.-absorption maximum at pH 12 of 235 nm (ϵ 2700). The change in spectrum on ring opening gave a half-life for compound (V) at pH 12 of approx. 60 min, which agrees with the acylimino type of structure suggested (Lee & Pollitt, 1970).

Table 2. *N.m.r. spectra of argininosuccinic acid and derivatives in trifluoroacetic acid solution: C-bonded protons*

The τ values are in parts per million from tetramethylsilane. The figures in parentheses are the number of protons observed. Abbreviations: m, multiplet; t, triplet; d, doublet; s, singlet; b, broad.

Compound	Proton ...	τ value				
		C-2	C-3, C-4	C-5	C-8	C-9
Argininosuccinic acid*		5.54 (m, 1)	7.77 (m, 4)	6.48 (m, 2)	5.11 (m, 1)	6.64 (t, 2)
Anhydride (I)*		5.51 (m, 1)	7.72 (m, 4)	5.97 (m, 2)	5.20 (m, 1)	6.62 (m, 2)
Anhydride (II)*		5.53 (t, 1)	7.76 (m, 4)	6.33 (t, 2)	5.15 (t, 1)	6.67 (d, 2)
Major acid-stable compounds (IV)		5.16 (t, 1)†	7.45–7.74 (m, 4)	5.3–6.6 (m, 2)	5.16 (t, 1)†	6.68 (d, 2) $J = 4\text{ Hz}$
Minor acid-stable compounds (V)		4.59 (m, 1)	7.16–7.83 (m, 4)	6.23 (b, 2)	5.16 (t, 1) $J = 4.5\text{ Hz}$	6.67 (t, 2) $J = 4.5\text{ Hz}$
Alkali-stable compounds (VIII)		5.49 (b, 1)	7.77–8.04 (b, 4)	6.55 (b, 2)	5.18 (b, 1)	6.70 (b, 2)
Compound (IX)		5.54 (t, 1) $J = 5\text{ Hz}$	7.82 (m, 4)	6.16 (m, 2)	5.40 (t, 1) $J = 4\text{ Hz}$	6.75 (d, 2) $J = 4\text{ Hz}$
<i>N</i> ² ,3-Dimethylglycocycamidine		6.70 (d, 3) $J = 5\text{ Hz}^\ddagger$		6.65 (s, 3)	5.45 (s, 1)	

* From Kowalsky & Ratner (1969).

† These peaks are overlapping triplets. The spectrum is simplified by deuterium exchange (see the text).

‡ Probably spin-spin splitting with proton on N-c.

Semi-quantitative electrophoresis experiments indicated that the rate of formation of the alkali-stable compounds from the hexahydroimidazodiazepine (IV) increased with increasing pH, but that the reverse reaction (cyclization) is fastest between pH2 and pH3.

A rather poor n.m.r. spectrum was obtained from a barium salt of compound (VIII) dissolved in trifluoroacetic acid (Tables 2 and 3). An additional proton was now present on N-b and the *N*-proton resonances were shifted upfield from those of compound (IV) so that they resembled more closely those of the free guanidino group of argininosuccinic acid. The positions of the C-8 and C-9 resonances differed little from those of the compound (IV); analogously there was little change in these resonances on the formation of the anhydrides from argininosuccinic acid. In this series of compounds, both C-2 and C-5 resonances moved upfield on ring opening, as opposed to only C-5 resonance in the argininosuccinic acid anhydrides, as in compound (IV) both C-2 and C-5 are adjacent to the guanidino function.

Deuterium exchange and separation of diastereoisomers

Solutions of the compound (IV) mixture in 2M-²HCl-²H₂O showed no change in n.m.r. spectrum after 48 h at room temperature. In ²H₂O at pD5, however, the hydrogen atom on C-8 was rapidly replaced by deuterium; the doublet due to the C-9

protons was replaced by a singlet and the C-8 peak disappeared. The rest of the spectrum was unaltered. Similar exchange is shown by anhydride (I) and by 3,5-dimethylglycocycamidine.

This ready exchange, which is presumably accompanied by inversion of configuration at C-8, suggested that the two slowly interconverting components of the compound (IV) mixture were diastereoisomers. Because deuterium exchange appeared to be slow in strongly acidic solutions, the separation on a sulphonic acid column in the H⁺ form was attempted. Elution with 0.5M-HCl gave two peaks that were almost completely resolved. The two separated peaks had different optical rotations and both showed slow mutarotation in acetate buffer at pH5 to give a final rotation close to that of the original mixture of the two components (Table 4). The n.m.r. spectra of the two separated components (taken in 2M-²HCl-²H₂O, because the hydrochlorides are insoluble in trifluoroacetic acid) were indistinguishable from each other and from that of the original mixture.

Minor acid-stable compounds

The reaction mixture from heating anhydride (I) at pH7 contained a pair of interconvertible minor spots in addition to the major pair just discussed. In the crude preparation mixture, one of this minor pair is well resolved from the major components, but emerges with a further breakdown product (compound IX; see below) (Fig. 1a). Mixtures free of

Table 3. *N.m.r. spectra of argininosuccinic acid and derivatives in trifluoroacetic acid solution: N-bonded protons*

The τ values are in parts per million from tetramethylsilane. The figures in parentheses are the number of protons observed. Abbreviations: d, doublet; s, singlet; b, broad.

Compound	Proton ...	τ value			
		N-a	N-b	N-c	N-d
Argininosuccinic acid*		2.35 (s, 3)	3.37 (s, 2.3)		3.16 (d, 1.3)
Anhydride (I)*		2.42 (s, 3)		1.84 (s, 2)	1.17 (s, 1)
Anhydride (II)*		2.37 (s, 3.4)	1.63 (s, 1.2)		1.40 (b, 0.8)
Major acid-stable compounds (IV)				1.76 (s, 1)	1.50 (b, 1)
Minor acid-stable compounds (V)			1.80 (b, 1)		1.40 (b, 1)
Alkali-stable compounds (VIII)				2.76 (b, 3)	
Compound (IX)		2.56†			2.56†
<i>N</i> ² ,3-Dimethylglycocyamidine				2.20 (b, 1)	1.90 (b, 1)

* From Kowalsky & Ratner (1969).

† Total of 4 protons.

compound (IX) were obtained by rearrangement of the pure hexahydroimidazodiazepine mixture carefully via the alkali-stable compound (VIII). A solution of the mixed diastereoisomers of compound (IV) was adjusted to pH 13–14 by the addition of 0.5 M-NaOH and left at 20°C for 3 h. The solution was then adjusted to pH 2 by the addition of HCl, left for a few minutes to allow ring closure to complete and then chromatographed on a preparative column of Zeo-Karb 225 (SRC 12) resin exactly as described for the isolation of the hexahydroimidazodiazepine (IV). The ratio of major to minor compounds formed was about 10:1. The minor-compound mixture obtained in this way could not be crystallized, so that rigorous identification by elemental analysis was not possible. On treatment with 0.1 M-NaOH for 10 min at 100°C, followed by brief heating at pH 2, the compounds were largely converted back into the major compounds, indicating that no degradation had occurred, but only rearrangement.

The n.m.r. spectrum of a mixture of the minor compounds in trifluoroacetic acid showed the expected total number of C- and N-bonded protons (Tables 1 and 2). The C-8 and C-9 peaks were at chemical shifts identical with those of the primary compounds (IV), though the fine structures were a little dis-

torted. The C-3, C-4 and C-5 proton resonances were broad and complex, the C-5 resonance in particular overlapping those of both the C-9 and C-3, C-4 protons. Compared with the primary compounds, the C-5 resonance is shifted upfield and the C-2 is shifted downfield. At first sight, the shift of C-5 resonance appears analogous to that of C-5 in changing from argininosuccinic anhydride (I) to anhydride (II), and the C-2 is showing the reverse change. However, this is not a strict analogy, as anhydride (II) is *O*-protonated (Kowalsky & Ratner, 1969) whereas anhydride (I) and the hexahydroimidazodiazepines are *N*-protonated in acidic solution. It would be predicted that the C-5 protons in compound (IV) would be more shielded (lower τ values) than in compound (V) because of their closer proximity to the carbonyl group; a reverse situation would apply to the C-2 proton. A similar argument has been used by Hart *et al.* (1969) in assigning the structures of the alkaloids alchornine and isoalchornine. The only simple model available is *N*²,3-dimethylglycocyamidine. In this compound the difference in τ value between the two sets of methyl protons is very small, probably because the 3-methyl protons (adjacent to the carbonyl function) are free to rotate and the observed τ value is an average for positions near to the carbonyl function and positions near to the imino function. In the hexahydroimidazodiazepines the appropriate protons are constrained in a position close to the carbonyl function. Hence these results indicate that the assumption that the major components were the primary ones was justified and that the minor components are secondary and correspond to formula (V).

Table 4. Optical rotations of derivatives of argininosuccinic acid

The pH 5.0 buffer was 1 M-sodium acetate-acetic acid. For other details see the Experimental section.

Conditions ...	$[M]_D^{25}$			
	pH 5 (initial)	pH 5 (final)	5M-HCl	0.1M-NaOH
Compound IV mixture	83°	83°	69°	-12° (extrapolated)*
Separated diastereoisomers of compound IV {	263° -92°	112° 82°	237° -81°	— —
Compound IX	—	—	105°	1.4°

* Ring opens to give alkali-stable compounds, $[M]_D^{25}$ 288°, half-life 32min.

The u.v. spectrum of the minor compounds in 0.01 M-NaOH showed a shoulder at 235 nm. The half-life of the compound was about 50 min.

Although the minor spots are formally derivatives of argininosuccinic acid anhydride (II), they have not been obtained directly from it. Anhydride (II) appears stable except in strongly acidic or alkaline solution. It seems reasonable to assume that the presence of two secondary compounds reflects the presence of diastereoisomers, as with the major compounds. In addition to the two major secondary spots, at least two other more minor components are present in the rearrangement mixture. These are less mobile on electrophoresis at pH 2 than the other components and probably have six-membered structures, (VI) or (VII). Experiments with radioactively labelled derivatives indicated that they are present in about 1 part per 500 of the major product.

Partial degradation products

Westall (1960) isolated a crystalline substance Z after hydrolysing yellow-spot mixtures with 0.5M-NaOH under reflux for 4 h. However, the degradation of the hexahydroimidazodiazepines by alkali is very complex, as all the C-N bonds of glycohydrazines are labile and electrophoresis of the desalted reaction mixture showed that several other products were present also. We therefore turned our attention to another partial degradation product that is formed from the primary yellow spot by heating in solution at any pH below 8. It is also present in the reaction mixtures from the preparation of the yellow spot

from argininosuccinic acid anhydride (I) (in peak A¹, Fig. 1a) and traces are present in the alkaline hydrolysis mixture also. A pure sample of this compound was obtained by refluxing anhydride (I) with 2M-HCl for 4 days. It had an analysis corresponding to C₁₀H₁₅N₃O₆, i.e. it was isomeric with Westall's (1960) compound Z. It was, however, clearly distinguishable on electrophoresis at pH 2 and pH 5.3. It gave a normal blue colour with ninhydrin, indicating that it contained a free amino group. The n.m.r. spectrum (Tables 2 and 3) was very similar to that of anhydride (I), except that the position and number of N-proton resonances had changed. The pK values (2.13, 3.79 and 9.26) indicated an α-amino acid grouping and ruled out structures containing ω-amino groups. These results suggested that the product had the structure (IX) and was formed directly by partial hydrolysis. A product with identical chromatographic and electrophoretic properties and i.r. spectrum was prepared from the primary yellow spots by heating at pH 7.

Stereochemistry

The primary hexahydroimidazodiazepines (IV) are a pair of diastereoisomers and the secondary compounds and alkali-stable forms probably show the same phenomenon. The two chemically prepared diastereoisomeric forms show nearly equal and opposite mutarotations and the final product still has a definite rotation. This indicates that the configuration at C-2 does not affect the equilibrium of the two forms at C-8, and also C-2 is not racemized. We have not proved conclusively the absolute configuration at C-2 or C-8 in these diastereoisomers. Chemical considerations suggest that C-2 probably retains its original configuration in the direct formation of the imidazodiazepines from anhydride (I), the ammonia being derived from the guanidino group by nucleophilic displacement on the guanidino carbon atom. The biological formation of the compound is not necessarily by the same route, however.

The ring opening of the imidazodiazepines to give compound (IX) probably also occurs with retention of configuration. Compound (IX), derived from the chemically prepared diazepine mixture (of more or less equal amounts of diastereoisomers), showed a strong positive rotation in 5M-HCl, changing to an almost zero rotation in 0.1M-NaOH (Table 4). This is similar to the behaviour of L- α -amino carboxylic acids and supports the idea of retention of configuration at C-2.

Occurrence in urine

The hexahydroimidazodiazepine derivatives formed *in vitro* from argininosuccinic acid and anhydride (I) were similar in chromatographic and electrophoretic properties to those found in the patient's urine. For direct comparison, some of the yellow-spot material was isolated directly from the urine by column chromatography. The two major peaks corresponded to an excretion of 0.57g of total (acid- and alkali-stable) diazepine derivatives/24h. This estimate neglects the secondary derivatives that will have been formed by cyclization of the alkali-stable compounds, and probably contribute another 5–10%. The argininosuccinic acid excretion of the patient over the same period was 6.8g/24h. Thus the hexahydroimidazodiazepines amount to about 9% of the argininosuccinic acid production. Anhydride (I) is present in fresh urine from argininosuccinicaciduria patients at about 7% of the concentration of argininosuccinic acid (Ratner & Kunkemueller, 1966). The rate of formation of diazepine derivatives

from argininosuccinic acid *in vitro* is too slow to account for this excretion; either a very large, slowly moving, pool of argininosuccinic acid or anhydride (I) in some parts of the body, or some catalytic mechanism, must be postulated.

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