Separation and Properties of Argininosuccinate and Its Two Anhydrides and Their Detection in Biological Materials*

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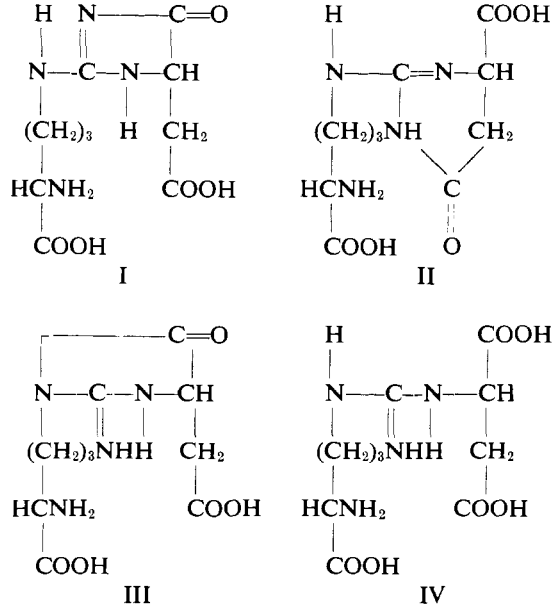
ABSTRACT: A chromatographic procedure is described for the estimation of argininosuccinate and its two related anhydrides involving fractional elution from Dowex-1-acetate followed by Amberlite 1R-120 for the further characterization of anhydride I. The compounds do not undergo change during column exposure. The methods have been used for preparative purposes and for the analysis of biological materials. The procedure has been specifically applied to the analysis of argininosuccinic acid and its anhydrides in the urine of a child afflicted with argininosuccinic aciduria. Interconversion among the three compounds has been studied as a function of pH and temperature. Anhydride II is formed in small amounts (probably from argininosuccinate) under a variety of conditions and, once formed, cannot be converted back to either of the related compounds except in trace amounts. Argininosuccinate and anhydride I readily undergo interconversion at 25°. In general, argininosuccinate predominates at pH 7 and above, whereas anhydride I predominates at acid pH. At 100°, extensive side reactions, associated primarily with anhydride I, prevented an evaluation of the interconversion, except at acid pH.

Anhydride I also undergoes oxidative degradation under mild conditions so as to form arginine, pyruvate, and CO_2, and the mechanism of this oxidation has been established. A revision of the structure originally proposed for anhydride I has been suggested, based on these properties. Anhydride II, originally described by Westall, has also been studied. This compound contrasts with anhydride I with respect to heat lability, ease of oxidation, acid dissociation, and absorption in the ultraviolet region.

Earlier work (Ratner *et al.*, 1953b) has shown that argininosuccinate readily undergoes anhydride formation in acid solution to form a compound (anhydride I) for which a structure having a five-membered ring was provisionally assigned as the more likely of several possible ring structures. It was shown that cleavage of this anhydride to argininosuccinate is favored at alkaline pH and it was suggested, as the result of a preliminary survey using enzymatic analysis, that ring formation and cleavage varied with pH and temperature as in the interconversion of creatine and creatinine. Reversible anhydride formation was later reexamined and confirmed by Westall (1960) with the aid of paper electrophoresis. He also found and examined a second anhydride which is referred to here as anhydride II.

Further experiments with these compounds revealed that still other changes take place at neutral and alkaline pH, particularly at elevated temperatures. It was essential to the further study of compounds showing such great lability that the fractionation conditions used should not contribute to any of these changes during the analytical procedures. The method described here, which makes use of fractionation on Dowex-1-acetate followed by Amberlite 1R-120, satisfies these requirements. It results in a separation of argininosuccinate, anhydride I, and anhydride II. ^{14}C-Labeled compounds have been prepared to facilitate the detection of new products and to gain a more complete picture than ninhydrin estimation alone could provide. The fractionation procedures are applicable to materials from biological sources and have been used, by way of illustration, for the analysis of the urine of a child afflicted with argininosuccinic aciduria.



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The structures originally proposed for anhydrides I and II (formulas I and II, respectively) as being the most likely of several theoretically possible structures (Ratner *et al.*, 1953b; Westall, 1960) was based on information available at the time. A detailed study of the extent of interconversion of these compounds with changes in pH and temperature has now been carried out with the aid of the new procedures. The results are in general agreement with the original conclusions for interconversion at 25° but they also show that anhydride I is much less stable and anhydride II much more stable than was to be expected from the structures favored previously. To gain further insight into their behavior, a detailed comparison of the physical and chemical properties of anhydrides I and II under a variety of conditions has been carried out. The results have led to the formulation of a revised structure for anhydride I.

Experimental Section

Materials. Dowex-1-acetate, purified grade, was purchased from Bio-Rad and Amberlite 1R-120-Na^+ was purchased from Mallinkrodt as Amberlite CG-120, analytical grade. Ninhydrin was purchased from Pierce Chemical Co. and lactic dehydrogenase from Boehringer Mannheim Corp.

Uniformly labeled L-[^{14}C]arginine, L-[^{14}C]glutamate, L-[^{14}C]aspartate, and L-[4-^{14}C]aspartate were purchased from New England Nuclear Corp. and Na^{14}CN was purchased from Tracer Laboratory. L-Leucine, L-arginine·HCl, and L-ornithine·HCl were purchased from Calbiochem or Mann Research Laboratories.

Analytical Methods. Ninhydrin color was estimated, usually in 0.1-ml aliquots of each 5-ml fraction, by the methods of Moore and Stein (1948, 1954) using the citrate-buffered reagent for the Dowex-1-acetate peaks and the more heavily acetate-buffered reagent for the Amberlite fractions. Arginine·HCl was used as standard. It was found to give the same color value as leucine with both buffers. Under these conditions, the ninhydrin color value was 1.17 for argininosuccinic acid, 1.12 for anhydride I, 1.00 for anhydride II, 1.05 for glutamic acid, and 0.88 for aspartic acid. Specific radioactivity was based on the color values given above.

Arginine was estimated by the Sakaguchi method as modified by Van Pilsum *et al.* (1956) and anhydride I by a modification (Ratner *et al.*, 1960) of the creatinine method of Van Pilsum *et al.* (1956). NH_3 was estimated by the Conway procedure.

Radioactivity was estimated in a Nuclear-Chicago gas flow counter having an efficiency of *ca.* 15%. Self-absorption corrections were necessary only when samples containing citrate buffer were plated. All ^{14}C values are given without correcting for the counting efficiency. The total recovery of ninhydrin color and of radioactivity was always within at least 5% of the amount placed on the column.

Preparation of Ion-Exchange Columns. Dowex-1-acetate, 8% cross-linked, 200–400 mesh, was treated with 3 M sodium acetate as described (Hirs *et al.*, 1954) and stored under 0.5 N acetic acid. Before use, the columns, 25 × 1 cm in size, were treated with 75 ml of 1.0 N acetic acid followed by 150 ml of 0.05 N acetic acid. The columns were reused after regeneration with 30 ml each of 3 M sodium acetate and 1.0 N acetic acid, and equilibration with 0.05 N acetic acid as before. To prevent high blank readings with ninhydrin, the acetic acid solutions were protected at all times from NH_3 contamination by H_2SO_4 traps.

Amberlite 1R-120-Na^+, 200–400 mesh, was freed of fines by decantation, sized, washed with acid and alkali as described by Moore *et al.* (1958), and stored under water. Prior to use the columns, which were 15 × 1 cm in size, were treated with 10 ml of 0.35 N NaOH, rinsed with water, and then equilibrated with 80 ml of the first eluent, either 0.1 N or 0.2 N citrate buffer, pH 4.25, as indicated in the appropriate figure. Equilibration and fractional elution of both columns were carried out at room temperature.

Conditions for Analytical Chromatography. The compounds investigated were kept stored at 0° under desiccation. Most solutions were made up just prior to use, or were kept at −20° for no longer than 1 week. Solutions of argininosuccinate were always stored at pH 8 or 9. All solutions were neutralized to pH 7 or 8 just prior to application to the Dowex-1 column. The urine samples to be analyzed were taken from a pooled 24-hr collection kept frozen during storage and transportation. Samples were neutralized and analyzed the day collection was completed wih the exception noted. All samples (usually brought to 5 or 6 ml) were allowed to run through the column by gravity. After rinsing in the sample with three 1-ml portions of water, elution was carried out with 75 ml of 0.05 N followed by 50 ml each of 0.10 and 0.5 N acetic acid at a flow rate of 0.5 ml/min. The effluent was collected in 5-ml fractions. Any variations in the eluting conditions are indicated in the appropriate figures. The anhydride I fraction (6–32 ml), which has a pH of *ca.* 5, was passed through the Amberlite column by gravity. This column was eluted with 100 ml of 0.1 N sodium citrate buffer, pH 4.25.

Preparation of Argininosuccinic Acid and Anhydrides I and II. The barium salt of L-argininosuccinic acid was prepared enzymatically from arginine and fumaric acid as previously described and assayed enzymatically (Ratner *et al.*, 1953a,b; Havir *et al.*, 1965). Anhydride I was prepared from this barium salt as before (Ratner *et al.*, 1953b) with the modification that contaminating fumaric acid was removed by passage through Dowex-1-acetate. For this purpose 3 g of the salt dissolved in 5 ml of water was treated with a slight excess of 1 N H_2SO_4. After centrifugation, the supernatant solution was held at room temperature for 72 hr, diluted to 25 ml, passed through a column of Dowex-1-acetate, and eluted with 0.05 N acetic acid as described above. In this case the anhydride emerged in the 6–55-ml fraction. After concentration *in vacuo* at 35°, the residue was taken to dryness over KOH *in vacuo*, dissolved in 20 ml of water, and filtered. After the slow addition of alcohol as described (Ratner *et al.*, 1953a), the anhy-

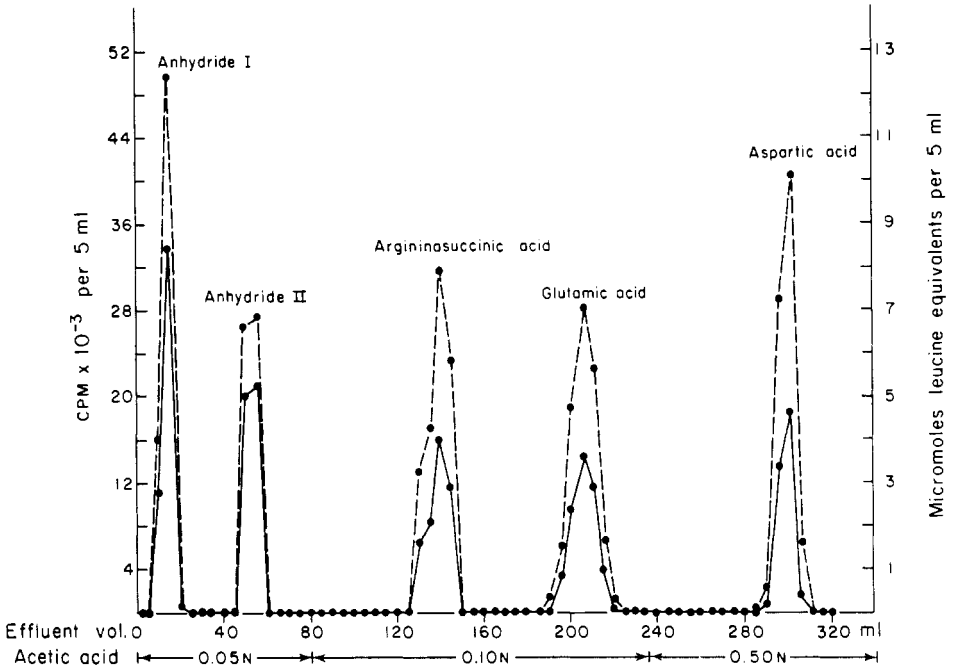


FIGURE 1: Chromatographic analysis of an amino acid mixture containing argininosuccinate and its anhydrides by fractional elution from a 25 × 1 cm column of Dowex-1-acetate. The starting mixture contained 10 μmoles of each compound. The labeling and specific radioactivity were as follows: [amidine-^{14}C]anhydride I, 6300; [aspartate-4-^{14}C]-anhydride II, 5500; [arginine-U-^{14}C]argininosuccinate, 4500; [U-^{14}C]glutamate, 8400; [4-^{14}C]aspartate, 7500. The fractional elution and analyical procedures were carried out as described under Experimental Section. Radioactivity (●- - -●); ninhydrin (●—●).

dride crystallized out in 88% yield (1.34 g). The preparations were analyzed by ninhydrin estimations and also by a specific colorimetric method (Ratner *et al.*, 1960).

To prepare anhydride II, a solution of the barium salt of argininosuccinic acid was brought to pH 6.0 with sulfuric acid and centrifuged, and the concentration was adjusted to 0.01 M with CO_2-free water. The solution was then heated in 10-ml portions in glass-stoppered tubes for 1 hr at 100°; the pH was then 8.3. Each 10-ml portion (the maximum load per column for this purpose) was passed through a column of Dowex-1-acetate and eluted with 0.05 N acetic acid as described above. Anhydride I and some breakdown products emerged in the throughput without retardation, and anhydride II emerged between 45 and 57 ml. The anhydride II fractions from seven to eight such columns were combined, concentrated to a small volume *in vacuo* at 30°, and taken to dryness over KOH *in vacuo*. After accumulating several millimoles, the crude compound was dissolved in 5 ml of water and the solution was centrifuged and adjusted to pH 7.0. This material was then passed through Dowex-1-acetate in the same manner as before at a maximum load of 100 μmoles/column. Acetic acid was removed as before; on dissolving the residue in 5 ml of water, alcohol was slowly added. The anhydride crystallized out in 50% aqueous alcohol in thick prisms and was recrystallized in the same way. The elemental analysis indicated that it contained 1 mole of water of crystallization.

Preparation of Isotopic Compounds. Na^{14}CN was converted to ^{14}CNBr and then to $NH_2{}^{14}CN$, and this was converted to [carbamyl-^{14}C]-*O*-methylisourea. L-[Amidine-^{14}C]arginine [prepared from L-ornithine and isotopic *O*-methylisourea (Kurtz, 1949)] was used for the preparation of L-[amidine-^{14}C]argininosuccinate and anhydride I as described above. Both compounds had a specific radioactivity of 6300 cpm/μmole. Both compounds were also prepared from uniformly labeled L-[^{14}C]arginine and had a specific radioactivity of 4400 cpm/μmole.

Two preparations of anhydride I were made from argininosuccinate containing ^{14}C in the aspartate moiety, the latter having been prepared enzymatically with argininosuccinate synthetase (Ratner *et al.*, 1953b). L-[4-^{14}C]Aspartate and uniformly labeled L-[^{14}C]-aspartate were used as starting materials. The specific radioactivity of the anhydrides was found to be 7100 and 8400 cpm/μmole, respectively. Anhydride II was isolated as a side product from column fractions and, after dilution, the specific activity of the preparation with ^{14}C in the C-4 position of the aspartate chain was 5500 cpm/μmole.

Conditions for pH and Temperature Effects. For these experiments, 1 ml of a solution containing 25 μmoles of the barium salt of [^{14}C]argininosuccinate was brought to the desired pH with sulfuric acid, 100 μmoles of potassium phosphate buffer of the indicated pH was added, and the mixture was clarified by centrifugation.

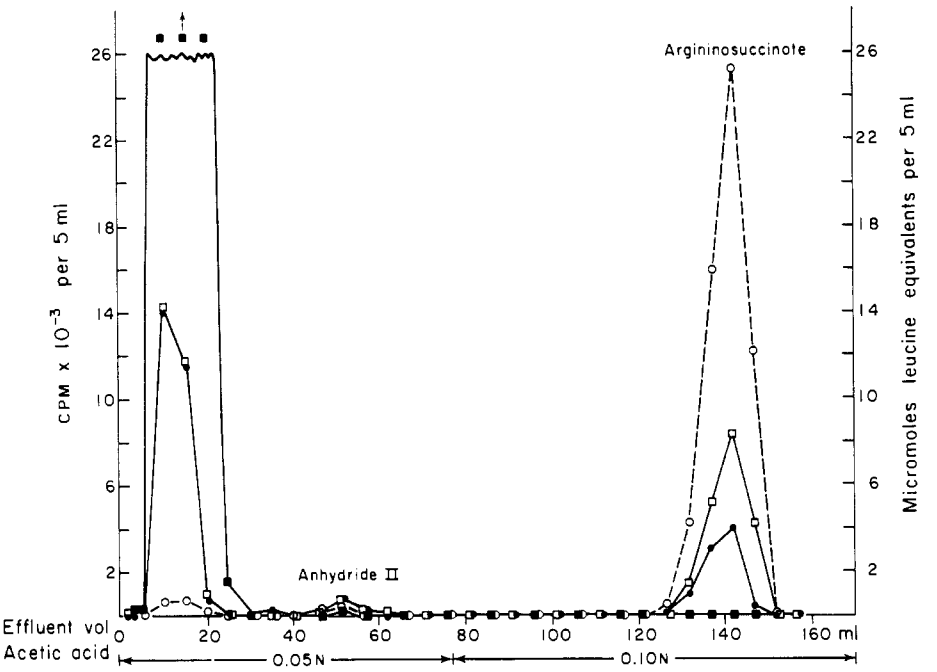


FIGURE 2: Analysis of argininosuccinate in urine of individual with argininosuccinic aciduria by fractional elution from a 25 × 1 cm column of Dowex-1-acetate. Normal urine, 5 ml, ninhydrin (■—■). Patient's urine, 0.2 ml, no additions, ninhydrin (●—●). Patient's urine, 0.2 ml to which 9.25 μmoles of [amidine-^{14}C]argininosuccinate, containing 6300 cpm/μmole, was added; ninhydrin (□—□); radioactivity (O- - -O).

A 2.0-ml sample, containing 20 μmoles of the amino acid, was then incubated in a glass-stoppered tube for the time and temperature indicated. The pH was taken again, at the end, in a companion tube. For the incubations at acid pH, no buffer was added after adjustment to the desired pH. After the experimental period, the pH was brought to 7 or 8 with NaOH solution and the mixture was analyzed by column chromatography as described above with the modification that, in the elution, 0.05 N acetic acid was followed by 50 ml of 1.0 N and the Amberlite column was eluted with 0.2 N sodium citrate buffer, pH 4.25. The amount of each amino acid was calculated from the total radioactivity of the peak and the specific radioactivity of the starting compound.

Conditions for Oxidation of Anhydride I. To 24 μmoles of anhydride I in 5.2 ml were added 0.24 ml of a 5% solution of *o*-nitrobenzaldehyde in 95% ethyl alcohol and 0.4 ml of 0.64 N NaOH solution. After standing at room temperature for 15 min, 1.0 ml of a mixture of sulfuric acid and phosphate buffer (0.5 M with respect to pH 7.0 potassium phosphate buffer and 0.25 N with respect to sulfuric acid) was added to bring the pH to 7.0. The tube was stoppered, heated for 10 min in a boiling water bath, and quickly brought to room temperature. A 6-ml aliquot (equivalent to 21 μmoles) was transferred quantitatively to the first column with three rinsings and fractional elution was carried out as described under "analytical chromatography."

Isolation of Pyruvate and Arginine after Oxidation of Anhydride I. Oxidation of anhydride I was carried out as described above, except that it was scaled up fivefold, the phosphate buffer was omitted in the neutralization, and the mixture was diluted to 100 ml before it was passed through a column of Dowex-1-Cl, 200–400 mesh, 8 × 1 cm in size, and rinsed in with 20 ml of water. On elution with 0.1 N H_2SO_4, pyruvic acid appeared in the first 20 ml in 38% yield and was estimated enzymatically with lactic dehydrogenase (Kornberg and Pricer, 1951). Eight such batches were combined and rechromatographed to remove a yellow impurity. The pyruvate fraction was neutralized with KOH solution, treated with 50 mg of semicarbazide·HCl, and concentrated to 1 ml. The crystalline semicarbazone amounted to 80% of the pyruvate found. After recrystallization, the melting point was 207–208.

Anal. Calcd for $C_4H_7N_3O_3$: C, 33.10; H, 4.86; N, 28.9. Found: C, 32.82; H, 4.92, N, 28.68.

The throughput from these columns was used for the isolation of arginine. Each 100 ml was passed through a column of Amberlite 1R-120, 15 × 1 cm in size, previously equilibrated with 0.1 N pyridine acetate, pH 3.28 (Canfield, 1963), instead of citrate. The column was eluted with 80–90 ml of 0.3 N pyridine acetate buffer, pH 4.25, followed by 1 N buffer, pH 4.98. Arginine emerged between 60 and 90 ml of the latter. After removing the pyridine acetate *in vacuo*, crystalline arginine·HCl in 30% yield was obtained in the usual manner and after one recrystallization gave ninhydrin and Sakaguchi values within 2% of theory.

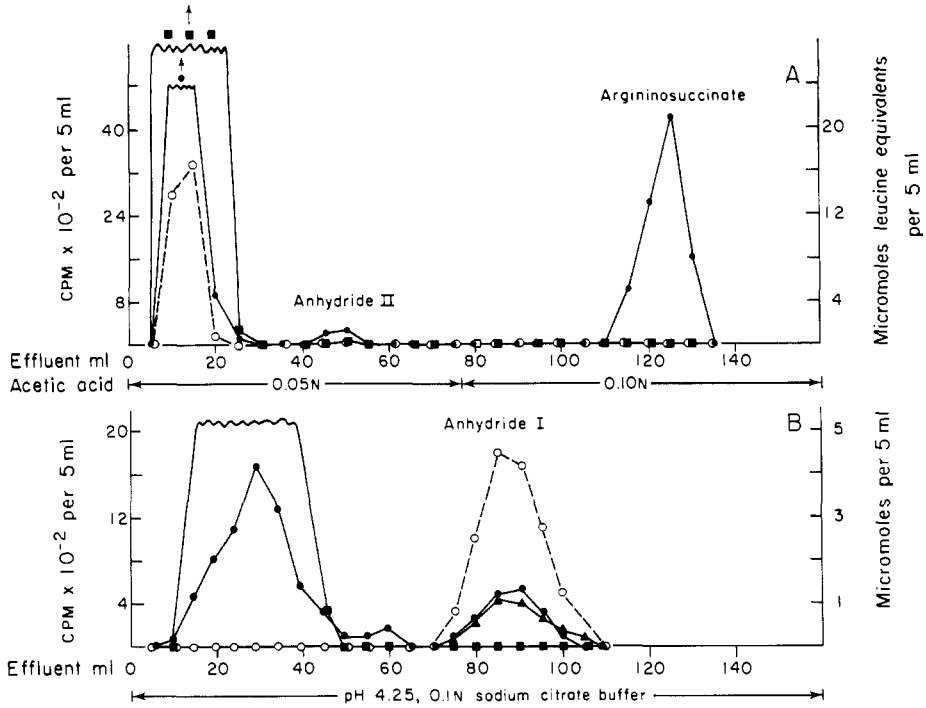


FIGURE 3: Analysis of anhydride I of argininosuccinate in urine of afflicted individual by fractional elution from a 25 × 1 cm column of Dowex-1-acetate (A) followed by a 15 × 1 cm column of Amberlite-1R-120 (B). Normal urine, 5 ml, ninhydrin (■—■). Patient's urine, 1.0 ml, to which 1.0 μmole of [amidine-^{14}C]anhydride I had been added containing 6300 cpm/μmole, radioactivity (O- - -O); anhydride I (▲—▲); ninhydrin (●—●). The last two estimations are expressed as anhydride I equivalents and leucine equivalents, respectively.

Results

Detection and Estimation of Argininosuccinate and Its Anhydrides in Biological Materials

Stability during Chromatography. The separation of argininosuccinate from anhydrides I and II as well as from glutamic and aspartic acids by fractional elution from Dowex-1-acetate, suggested by the paper of Hirs *et al.* (1954), is shown in Figure 1. The latter two amino acids were included in the mixture since they are frequently encountered in biological materials. Anhydride I, the least acidic of the five, emerged with very little retardation, and when further characterization was desired, this fraction was passed through a column of Amberlite 1R-120 (Moore *et al.*, 1958) and was subsequently eluted with citrate buffer (not shown in the figure). The remaining four compounds emerged well separated on successive elution with 0.05, 0.10, and 0.50 N acetic acid.

The homogeneity of the peaks was thoroughly examined so that all possibility of interconversion or losses due to other changes during passage down the column could be rigorously excluded. Amino acids labeled with ^{14}C were used to increase the sensitivity and to provide criteria independent of ninhydrin. It was established that when argininosuccinate or one of its anhydrides alone or in mixtures was adsorbed and eluted, each compound emerged with the same specific radioactivity as that of the starting material (Figure 1). Furthermore, the elution peak was in each case found to be homogeneous as indicated by the fact that the values found for the specific radioactivity of the fractions within each peak were in good agreement. Such results show that exposure to the column did not produce any changes. It was found, however, that each compound contained 1 or 2%, or less, of the other two as impurities (see below). These were formed during preparation of the compounds, not during the analytical chromatography.

The column conditions proved convenient for preparative or isolation purposes since the eluting medium could be easily removed. The methods were particularly valuable when small amounts of the ^{14}C-labeled compounds were being prepared, since it was necessary to have preparations of the anhydrides that were essentially free of mutual contamination. Even in the case of argininosuccinate, good recovery could be obtained if the acetic acid was quickly removed from the effluent fractions.

Estimation of Argininosuccinic Acid and Related Anhydrides in Urine. The conditions given in Figure 1 proved applicable to the analysis of argininosuccinate in urine. When 5.0 ml of normal urine was applied to the column, no ninhydrin-positive material could be found in the argininosuccinate region (Figure 2). The elution pattern for the 0.2 ml of urine obtained from a male patient, 15 years of age, suffering from arginino-

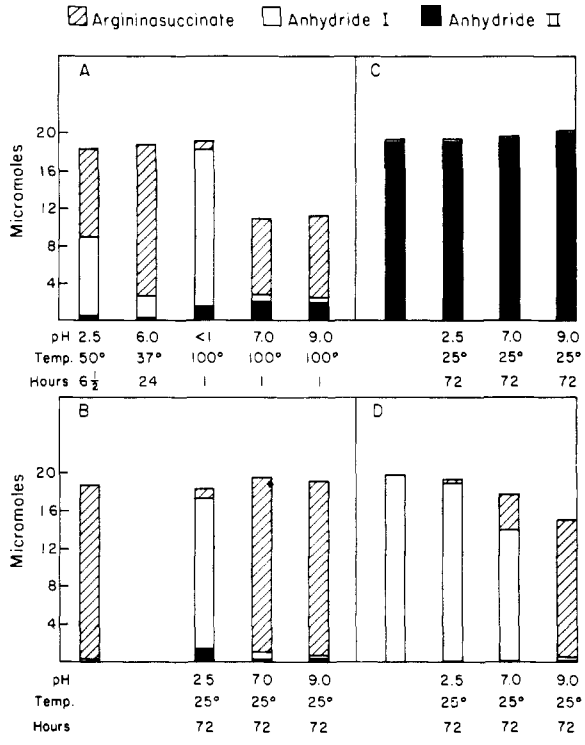


FIGURE 4: Composition of mixtures formed by exposing argininosuccinate or its anhydrides to various changes of pH and temperature. The incubations and analyses were carried out as described under Experimental Section. Values are expressed in micromoles, calculated from the radioactivity of the fraction and the specific radioactivity of the compounds at the start. The labeling and radioactivity for the first two compounds are the same as were used for Figure 1. [Arginine-U-^{14}C]anhydride I had 4400 cpm/μmole.

succinic aciduria[1] is shown in the same figure and also the results of a parallel run, carried out after adding 9.25 μmoles of [^{14}C]argininosuccinate to a second 0.2-ml sample. It can be seen that the excreted and added argininosuccinate emerged together in a homogeneous peak. The specific radioactivity found was 3460 cpm/μmole as compared to 6300 for the compound added. Calculating from the isotope dilution, this corresponds to a value of 7.59 μmoles of preexisting argininosuccinic acid in the 0.2 ml-sample. This value agrees well with the value of 7.40 μmoles obtained from the ninhydrin value for this peak when no addition was made. The value obtained by direct enzymatic assay was 7.47 μmoles. When corrected for the small volume change due to neutralization, this corresponds to a 24-hr excretion of 8.8 g of argininosuccinic acid.[2] The disease associated with the excretion of this amino acid (and with mental deficiency) was first described by Allan *et al.* (1958). Westall (1960) established the identity of the amino acid and reported an average excretion of about 3 g daily for a male child, 8 years of age, one of the two cases described by Allan *et al.* (1958).

Excretion of Anhydrides I and II. The first 0.2-ml sample of urine taken for analysis was further examined for the presence of anhydride I with the aid of the Amberlite column. It was found in the effluent from this column to the extent of *ca.* 10% of the argininosuccinate excretion. It was of interest to determine whether the anhydride was excreted as such or was formed during storage of the urine. A single, freshly excreted specimen was, therefore, obtained, frozen immediately after collection, and analyzed within a few hours. A 1.0-ml sample was taken for analysis after the addition of 1.0 μmole of [^{14}C]amidine-labeled anhydride I. As shown in Figure 3, the preexisting and added [^{14}C]anhydride I emerged together from the Dowex-1 and Amberlite columns.

The amount of preexisting anhydride I calculated from the change in specific radioactivity (6300 before and 1660 after elution) corresponded to 2.80 μmoles.[3] The specific radioactivity was based on the specific colorimetric method (Ratner *et al.*, 1960) for estimating the anhydride. The separation of anhydride I from other amino acids was effective since estimation by ninhydrin gave values for anhydride I that were only 2 or 3% higher.[4] The argininosuccinate content of the same 1.0-ml sample of urine (Figure 3) amounted to 43.5 μmoles. The amount of anhydride I excreted was, therefore, 6.8% of the free acid. This was appreciably more than could have formed as a result of the brief manipulations at room temperature before application to the column. Possibly the anhydride was formed at the slightly acid pH of the bladder (see section below on effect of pH).

The presence of a small peak in the anhydride II region (Figure 3) suggests that this anhydride might also have been excreted preformed. No attempt was made, however, to establish this conclusively.

Comparison of Chemical and Physical Properties of Anhydrides I and II

As will be shown in a later section, anhydride I is much more susceptible to hydrolytic cleavage and to changes caused by heating than is anhydride II. Because the great difference in behavior is difficult to understand on the basis of the structures provisionally assigned to these anhydrides (Ratner *et al.*, 1953b; Westall, 1960), comparison of a number of chemical

[1] We are greatly indebted to Dr. Mary Efron for providing these specimens.

[2] This case, P. A., has been intensively studied by H. W. Moser, M. L. Efron, H. Brown, R. Diamond, and C. G. Neumann (1966, to be published). These authors found that the daily excretion ranged from *ca.* 4 g on a low protein diet to *ca.* 9 g on a high protein diet.

[3] The amount of preexisting anhydride I, estimated colorimetrically, was the same (2.75 μmoles) before and after the addition of the 1.0 μmole of isotopic anhydride I.

[4] This discrepancy was a few per cent greater after storage of the urine.

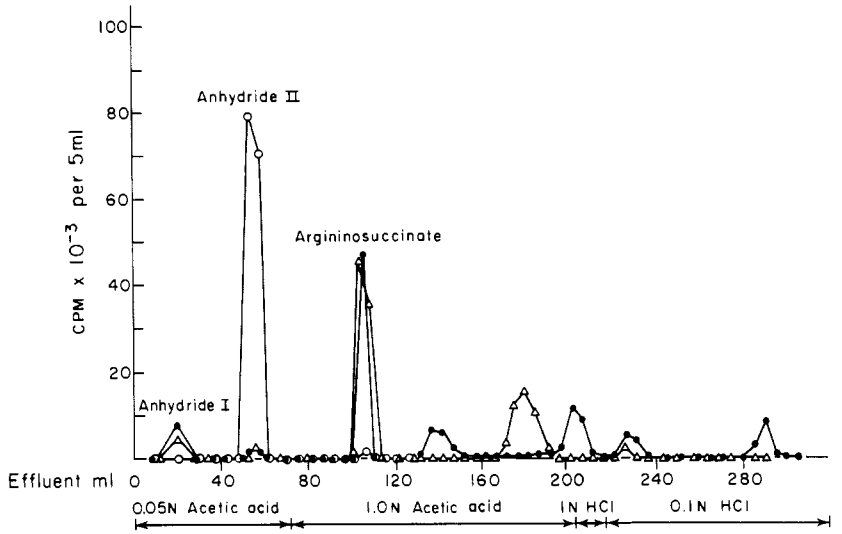


FIGURE 5: Products formed by heating argininosuccinate and its two anhydrides at pH 7 for 10 min at 100° and analyzed by fractional elution from a 25 × 1 cm column of Dowex-1-acetate. The eluate from the Amberlite column contained ^{14}C amounting to <1 μmole in each case. The NH_3 amounted to 2.70, 12.6, and 0.0 μmoles, respectively, for argininosuccinate, anhydride I, and anhydride II. The compounds all contained ^{14}C in the C-4 position of the aspartate moiety. Since they differed in specific radioactivity the data have been recalculated, for comparison, for a uniform value of 7100 cpm/μmole. Argininosuccinate (Δ—Δ); anhydride I (●—●); anhydride II (○—○).

and physical properties was made to provide further insight into this problem.

Preparation of Anhydride II. Anhydride II was first detected by Westall (1960) on two-dimensional paper chromatograms for which he used a solution of argininosuccinate left standing at room temperature for several days. He also isolated the compound in low yield from the products of alkaline treatment of argininosuccinate by fractionation on Dowex-2, and found that the isoelectric point was more acidic than that of anhydride I (4.2 as compared to 5.7).

For the present study, this anhydride was prepared as described in the Experimental Section by heating argininosuccinic acid (pH 6–8) at 100° for 1 hr. The yield was *ca.* 10%, and this could not be increased, although many variations in pH and temperature of heating were examined. These could be carried out on a small scale with [^{14}C]argininosuccinate or [^{14}C]anhydride I. The amount formed was appreciably lower than was found by Westall (1960) after heating argininosuccinate at 100° for 1 hr. Anhydride II prepared as described above crystallized out in hexagonal prisms. The preparation resembled the one prepared by Westall[5] in all respects except that analysis showed that our compound contained 1 mole of water of crystallization.

Effects of pH and Temperature on Argininosuccinate and Anhydrides I and II. In the present study the three compounds, argininosuccinate and anhydrides I and II, were estimated after exposing 20 μmoles of each of these compounds to varying conditions of pH and temperature. The results are shown in Figure 4. The bars on the extreme left give the composition of a freshly prepared solution of the starting compound. They show that each contained very small amounts of the other two as contaminants. The values given for the incubated samples have not been corrected for these impurities.

Argininosuccinate kept at pH 9.0 or 7.0 for 72 hr at 25° (Figure 4B) showed very little tendency toward anhydride formation. However, exposure to pH 2.5 led to the formation of anhydride I to the extent of 80% and of anhydride II to 7.5%. Ring closure was slow, for only 55% of anhydride I was formed after 24 hr.

The results shown for pH 6.0 represent the composition after a 24-hr incubation at 37°. This was carried out to simulate physiological conditions in the bladder. Conversion to anhydride I took place to the extent of 12.7% in 24 hr.

When anhydride I was taken as the starting compound (Figure 4D) *ca.* 75% was converted to argininosuccinate at 25° and pH 9.0, and only traces of anhydride I remained. The loss was due to the formation of other compounds by side reactions. Anhydride I was somewhat more stable at pH 7.0, and still more so at pH 2.5. As may be seen, ring cleavage diminished as the pH was lowered.

Anhydride II did not undergo conversion to the other compounds when exposed at 25° to pH 9.0, 7.0, or 2.5 (Figure 4C). The recovery amounted to at least 95% in all three cases, thus indicating that this anhydride, in contrast to anhydride I, resists ring cleavage at pH

[5] We are greatly indebted to Dr. R. G. Westall for sending us a generous amount of his preparation for comparison purposes.

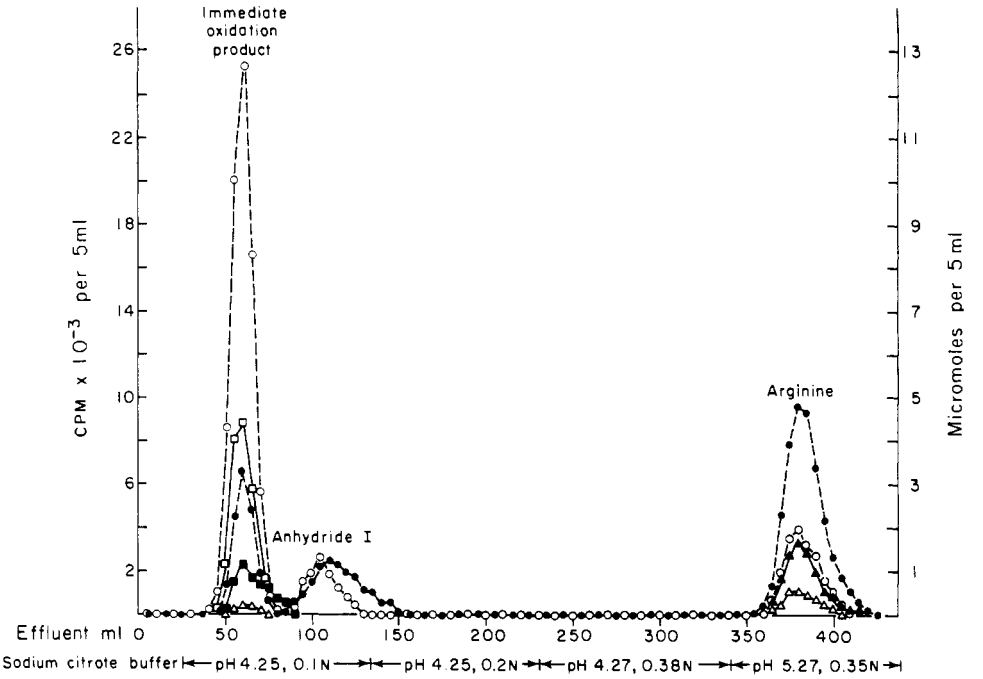


FIGURE 6: Oxidation products formed from anhydride I and analyzed by fractional elution from a 25 × 1 cm column of Dowex-1-acetate followed by a 15 × 1 cm column of Amberlite 1R-120. The elution pattern from the Amberlite column only is shown. Oxidation and analysis were carried out as described in Experimental Section with 21 μmoles of [amidine-^{14}C]anhydride I containing 6300 cpm/μmole. Oxidation without hydrolysis, ninhydrin (□—□); Sakaguchi (△—△); radioactivity (○- - -○). Oxidation followed by hydrolysis at 25° for 17 hr, ninhydrin (■—■); Sakaguchi (▲—▲); radioactivity (●- - -●). The colorimetric values are expressed as leucine or arginine equivalents, respectively.

9.0 and does not undergo the secondary changes mentioned above. As shown in B and D, the formation of anhydride II from argininosuccinate or from anhydride I at 25° was in most instances scarcely discernible.

On heating argininosuccinate at 100° in 0.25 N sulfuric acid, conversion to anhydride I occurred very rapidly, amounting to 90% in 1 hr (Figure 4A). This probably represents the limit of conversion since most of the remainder was anhydride II. The good recovery indicates that the compound was stable in acid solution. However, the changes which took place on heating at neutral pH for 1 hr were much more complex, as indicated by the low recovery (Figure 4A). The losses were due to transformation to new compounds and such changes were appreciable even after a few minutes. The elution pattern obtained after heating argininosuccinate for 10 min at 100° at pH 7.0 is given in Figure 5. As much as 40% of the argininosuccinate had disappeared. Although the loss might have been due to conversion to anhydride I, only traces of anhydride I could be found and a new compound was formed which was eluted after argininosuccinate.

Exposure of anhydride I to the same conditions led to practically complete disappearance of this compound (Figure 5). Only *ca.* 30% could be accounted for by conversion to argininosuccinate. The rest was located among the more acidic compounds which emerged on further elution of the column with 1.0 N acetic acid and 0.1 N HCl. Four new radioactive peaks were found representing compounds which were all ninhydrin negative. Some deamination must have occurred during the heating, for an appreciable amount of NH_3 was eluted from the Amberlite column. The great instability of anhydride I may account for its absence from among the products encountered on heating argininosuccinate at pH 7.0. The elution pattern was the same whether the anhydride was labeled in the ornithine or in the aspartic portion of the molecule, indicating that the carbon skeleton remained intact. By contrast, exposure of anhydride II to the same 10 min of heating at pH 7.0 led to complete recovery (Figure 5). The results again demonstrate the extreme instability of anhydride I as compared to anhydride II.

Oxidation of Anhydrides I and II. It has been previously shown that anhydride I is susceptible to oxidative cleavage by *o*-nitrobenzaldehyde in alkaline solution at 25°. A Sakaguchi-positive compound was found which proved to be arginine and this has been made use of for the quantitative estimation of this anhydride (Ratner *et al.*, 1960). Preliminary exploration of the reaction products indicated that pyruvic acid and CO_2 were among the other products of oxidation.

The mechanism of the oxidation was investigated with ^{14}C-labeled preparations. Chromatographic fractionation of the mixture obtained after oxidation followed by the usual 10-min hydrolysis at 100° showed

TABLE I: ^{14}C-Labeled Oxidation Products of Anhydride I without and with Hydrolysis.[a]

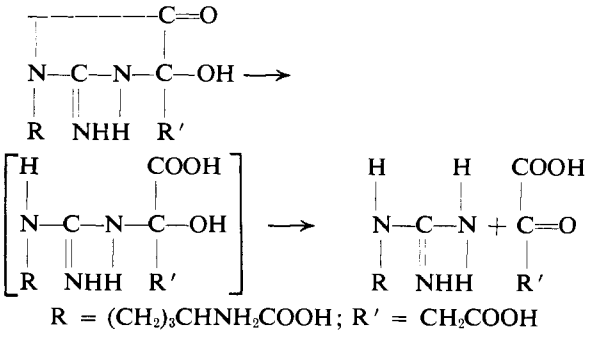
Products Found	[Amidine-^{14}C]Anhydride I			[Aspartate-4-^{14}C]Anhydride I	
	Hydrolytic Treatment			Hydrolytic Treatment	
	None (μmole)	25°, 17 hr (μmole)	100°, 10 min (μmole)	None (μmole)	100°, 10 min (μmole)
Dowex-1-acetate					
Anhydride II	1.56	0.76	0.27	1.22	0.25
Argininosuccinate	1.68	2.26	2.13	1.02	1.10
Unidentified	0.80	1.84	4.25	0.53	1.20
Amberlite 1R-120					
Primary product	12.40	3.27		11.33	
Anhydride I	1.71			3.25	
Unidentified		3.14	5.72[b]		0.20
Arginine	3.17	9.00	8.52		
Recovery of ^{14}C	21.32	20.27	20.89	17.35	2.75
NH_3	0.27	3.36	8.45	0.22	6.51

[a] [Amidine-^{14}C]anhydride I (6300 cpm/μmole) and [aspartate-4-^{14}C]anhydride I (7100 cpm/μmole) were prepared, oxidized, and hydrolyzed as described under Experimental Section, except when hydrolysis was varied as indicated. An aliquot corresponding to 21 μmoles was applied to the columns. The eluting schedule and other conditions were the same as for Figure 6. Estimation of radioactivity and ninhydrin was carried out on each tube as described above. In addition, Sakaguchi and anhydride estimations were carried out on the fractions from the Amberlite columns. The values estimated colorimetrically are not included. [b] This value includes all of the radioactivity in the first 120 ml of effluent; none of the primary oxidation product remains.

peaks corresponding to six or more compounds. *Ca.* 50% of these products were represented by unidentified peaks presumably unrelated to the oxidation mechanism. In order to eliminate these side reactions, column chromatography was carried out immediately after oxidation, and before hydrolysis. The elution pattern (Figure 6) showed the presence of a new compound which was not retained on Dowex-1. It emerged from the Amberlite column between 50 and 75 ml, immediately in advance of anhydride I. The behavior of this material indicated that it was the immediate product of the oxidation. It was formed in *ca.* 75% yield (correcting for some hydrolysis during manipulation). When, in a parallel experiment, the oxidation mixture was allowed to stand at 25° for 17 hr before chromatography (Figure 6), most of the immediate oxidation product had disappeared and the arginine peak was appreciably increased. The preparation of anhydride I used for these experiments contained ^{14}C located in the amidine carbon of the arginine moiety. Other experiments, described below, showed that pyruvate and CO_2 were liberated at the same time.

The immediate oxidation product is thus seen to be an unstable compound which slowly undergoes hydrolysis at room temperature to arginine, pyruvate, and CO_2. The quantitative data, summarizing these results, are given in Table I. The data in the third column show that the unidentified compounds found after "hydrolysis" at 100° were not formed at 25°, thus indicating that their presence was indeed due to side reactions. The immediate oxidation product does not give color with the Sakaguchi method before hydrolysis and therefore must still retain the same amidine substitution as anhydride I. If the anhydride grouping had been hydrolyzed during oxidation, a more acidic compound would have been formed which would have been retained on Dowex-1. All of these observations suggest that oxidation takes place at the α carbon of the aspartate moiety to form a "pseudo-base," as shown in Scheme I, which then hydrolyzes at the C-1 carboxyl

SCHEME I



R = $(CH_2)_3CHNH_2COOH$; R′ = CH_2COOH

and rearranges to arginine and oxaloacetic acid. The oxaloacetate then undergoes a β decarboxylation with the loss of the C-4 carbon of aspartate so as to form pyruvic acid and CO_2.

The mechanism of the oxidation is further supported by experiments with anhydride I labeled in the as-

TABLE II: Behavior of Anhydride I and Various Other Compounds toward Conditions of Oxidation and Hydrolysis.[a]

Products Found	Compounds Subjected to Oxidation				
	[Arginine-U-^{14}C]-Anhydride I (μmoles)	[Aspartate-U-^{14}C]-Anhydride I (μmole)	[Amidine-^{14}C]-Argininosuccinate (μmole)	[U-^{14}C]-Arginine (μmole)	[Aspartate-4-^{14}C]-Anhydride II (μmoles)
Dowex-1-acetate					
Anhydride II	0.32	0.25	0.64		18.16
Argininosuccinate	2.64	2.40	15.64		0.54
Unidentified	3.57	2.20	3.31		
Pyruvate		6.89			
Amberlite 1R-120					
Primary product					
Anhydride I			0.57		
Unidentified	5.50[b]	2.75[b]	0.70		2.00[b]
Arginine	8.38		0.00	20.50	
Recovery of ^{14}C	20.41	14.49	20.86		20.70
NH_3	7.78	9.3	3.40	0.37	0.00

[a] The preparations of anhydride I had 4400 and 8400 cpm/μmole, respectively. Argininosuccinate, arginine, and anhydride II had 6300, 4400, and 7100 cpm/μmole, respectively. All compounds were subjected to oxidation and 10-min hydrolysis at 100° as described under Experimental Section. The amounts, columns, eluting conditions, and various analytical procedures were the same as for Table I and Figure 6. The values given in the table represent ^{14}C-containing products only. The values estimated colorimetrically are not included. [b] These values include all of the radioactivity in the first 120 ml of effluent; none of the primary oxidation product remains.

partate moiety, either in the C-4 position or uniformly along the chain. The data in the second half of Table I give the results obtained with the first of these two preparations. The amount of the immediate oxidation product formed was about the same as before and much of the radioactivity was located and recovered. However, after the 10-min heating period *ca.* 87% of the original counts was lost. The amount of arginine formed after the heating was about the same in both experiments. Since the ^{14}C was located exclusively in the position where decarboxylation was expected, the loss of ^{14}C during the heating period is entirely consistent with the mechanism proposed.

A similar experiment was carried out with a preparation of anhydride I labeled uniformly in the aspartate moiety. The results found after oxidation and hydrolysis (Table II) show that in addition to side products, the main oxidation product that contained ^{14}C was pyruvate.[6] This fraction was converted to the 2,4-dinitrophenylhydrazone. The specific radioactivity found was 6200 cpm/μmole in agreement with theory for three of the four original carbons. Loss of radioactivity due to decarboxylation at the C-4 position of the aspartate moiety is reflected in a recovery of ^{14}C amounting to *ca.* 70%, as compared to 95% when the ^{14}C was located in the arginine moiety (Table II). Another experiment carried out with anhydride I labeled uniformly in the arginine moiety is also summarized in Table II. The distribution of radioactivity among the various products is seen to be about the same as was obtained when only the amidine carbon was labeled (Table I). Final identification of the main products of oxidation, arginine and pyruvic acid, was obtained by isolation from large scale runs as described in the experimental section.

The susceptibility to mild oxidation observed with anhydride I was not observed with anhydride II, with argininosuccinate itself, or with arginine, as shown in Table II. The preparation of anhydride II used for this experiment contained ^{14}C in the C-4 position, which is the one expected to be most labile. The compounds were recovered largely unchanged and what changes were found may be attributed to the effects of the 10-min heating period at pH 7.

Dissociation Constants and Ultraviolet Absorption of Anhydride II. The titration curve for our preparation of anhydride II and for a preparation kindly supplied by Dr. Westall (1960) were identical, as shown in Figure 7. The data were obtained and plotted as previously described (Ratner *et al.*, 1953b). The values found for the dissociation constants were 2.00 for pK_1, the α carboxyl of the ornithine residue; 3.30 for pK_2, one of the carboxyls of the aspartic residue; 5.15 for pK_3, the guanidino anhydride group; and 9.50 for pK_4, the α-amino group of the ornithine residue. The values for three of

[6] Losses were encountered in the recovery of pyruvate due to side reactions during the heating period. Such side products, since they were radioactive, were partially recovered among the unidentified fractions.

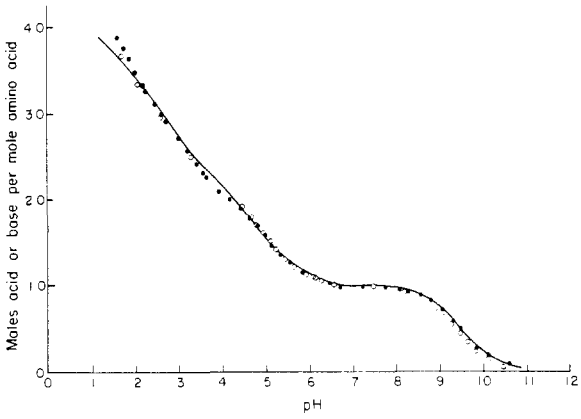


FIGURE 7: Constructed titration curve and observed acid and base binding of argininosuccinic acid anhydride II. The preparation made as described in the text (●—●); the preparation made by Westall (○—○).

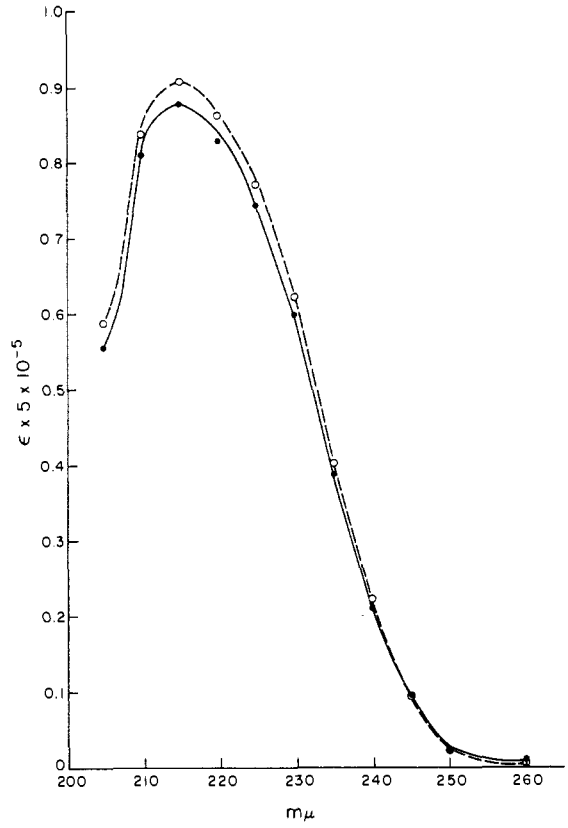


FIGURE 8: Absorption spectrum of argininosuccinic acid anhydride II. The preparation made as described in the text (●—●); the preparation made by Westall (○- - -○). The solutions were 0.05 mM with respect to the compound and 10 mM with respect to potassium phosphate buffer, pH 7.4.

these, pK_1, pK_2, and pK_4, were very close to those found for anhydride I (Ratner *et al.*, 1953b). However, pK_3, representing the dissociation of the guanidino anhydride group, had a value of 5.15, which is similar to that for creatinine or guanidinosuccinic anhydride but is appreciably lower than the corresponding value of 8.10 for anhydride I. The lower isoelectric point for anhydride II, 4.2 as compared to 5.7 for anhydride I, is therefore due to the low value for pK_3, and not to any difference in the dissociation constant for the aspartate carboxyl, pK_2.

The ultraviolet absorption curve for anhydride II is shown in Figure 8. The compound exhibits a maximum at 215 mμ, thus contrasting with anhydride I which showed only end absorption. Anhydride II resembles creatinine and guanidinosuccinic acid anhydride in that both of these compounds show absorption bands in the short ultraviolet region (Ratner *et al.*, 1953b). The preparation of anhydride II made by Westall (1960) exhibits an absorption spectrum that coincides with the preparation made here (Figure 8).

Discussion

Argininosuccinate and Its Anhydrides in Biological Material. The methods previously used for the estimation of argininosuccinate have suffered from various drawbacks. Estimation by enzymatic assay (Ratner *et al.*, 1953b) has been of limited usefulness in that only one of the three compounds can be estimated directly. The use of cationic exchangers, as Westall (1960) has shown, leads to extensive anhydride formation during the passage of argininosuccinate down the column. Armstrong *et al.* (1964) and Coryell *et al.* (1964) have attempted to circumvent this disadvantage by converting the argininosuccinate to anhydride I at acid pH before application to the column. However, as the present results show, the possibility of losses due to incomplete conversion, the instability of anhydride I at elevated temperatures, and the less favorable conditions for the separation from other neutral amino acids are difficulties which the present method avoids.[7]

The present results confirm Westall's original observation (1960) that normal individuals do not excrete argininosuccinate although it is a metabolic intermediate. It cannot be excluded completely, however, that a further increase in the sensitivity of the procedure, such as would be gained by automation of the column, might lead to the detection of trace amounts.

No interference was encountered in obtaining a quantitative recovery of argininosuccinate when added to a 5-ml sample of normal urine. The capacity of the column is probably sufficient to handle a sample size several times larger. The method is consequently also well suited to the detection of very small amounts of argininosuccinate in the urine of individuals suspected of being heterozygous for the genetic defect, such as were

[7] It is necessary, however, to take extreme precautions in guarding against even the slightest change during storage for the 24-hr collection period and to avoid delay in the analysis.

found by Coryell *et al.* (1964) among relatives of an individual afflicted with argininosuccinic aciduria.

Interconversion of Argininosuccinate and Its Anhydrides. The pH dependence of the argininosuccinate, $\rightleftarrows$ anhydride I interconversion at 25° supports in general the conclusions of preliminary studies (Ratner *et al.*, 1953b; Westall, 1960). However, at 100°, interconversion could only be evaluated at acid pH. At higher pH values extensive side reactions and degradative changes associated primarily with anhydride I prevented an evaluation of the rates of interconversion or of the equilibrium position.

In spite of the stability of anhydride II, the conditions that influence the rate of formation are poorly understood. Our results for 100° differ from those of Westall (1960) who made use of paper electrophoresis to follow the changes. The results obtained thus far suggest that this anhydride is formed directly from argininosuccinate rather than from anhydride I. The rate of formation probably increases with temperature and with a lowering of the pH, but the rapid changes which these two compounds undergo as a result of simultaneous side reactions interfered with our evaluation of the factors that control the formation of anhydride II.

Structure of Anhydrides I and II. The configuration originally suggested for anhydride I (formula I) represented a choice of several theoretical possibilities and was based on the information then available (Ratner *et al.*, 1953b). The further knowledge of its behavior and the contrast with anhydride II in heat lability and susceptibility to oxidation described above are difficult to reconcile with formula I; these properties are more consistent with the behavior suggested by the configuration shown in formula III. The work of Zervas *et al.* (1957, 1961) on acyl derivatives of arginine has shown that the δ nitrogen atom is more reactive than was previously supposed. It can be acylated under relatively mild conditions and also can undergo internal acylation with the α carboxyl. Furthermore, the amidine group of the piperidone compound thus formed has become labilized to the extent that rupture of the bond between the δ N and the amidine C takes place very easily (Zervas *et al.*, 1961).

The fact that the pK of the guanidino anhydride group of anhydride I has a value that is higher than that found for anhydride II (8.10 as compared to 5.15) is also more consistent with structure III. Zervas *et al.* (1957) have pointed out that arginine derivatives containing one acyl substituent at the guanidino group still retain basicity in association with this grouping. The resemblance lies in the less restricted possibilities for resonance for structure III as compared to I and II.

If further evidence should continue to support the proposed formula III for anhydride I, then structure I, as well as structure II, should be given consideration for anhydride II. It is difficult to distinguish between them on the basis of information available at the present time. Westall (1960) originally suggested formula II for anhydride II on the supposition that the more acidic isoelectric point might be due to an anhydride linkage involving the C-4 carboxyl of the aspartate moiety rather than the C-1 carboxyl. Since the dissociation constant for the aspartate carboxyl has been found to be the same for both anhydrides, this reason for giving preference to II has been removed. Further studies on both anhydrides are in progress.

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