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Argininosuccinic Aciduria: Identification and Reactions of the Abnormal Metabolite in a Newly Described Form of Mental Disease, with Some Preliminary Metabolic Studies

By R. G. WESTALL

Medical Unit, University College Hospital Medical School, London, W.C. 1

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Allan, Cusworth, Dent & Wilson (1958) have reported a new form of severe mental disease which they believe to be due to an inborn error of metabolism. The authors described a family with four children in which two of the sibs were normal and the other two were seriously mentally retarded. Paper-chromatographic examination of the urine of the family showed that the two affected children were excreting large amounts of a ninhydrin-reacting substance which was absent in the urine of the rest of the family. Moreover, further tests on the affected children showed a relatively high concentration of the abnormal metabolite in the cerebrospinal fluid and a somewhat lower concentration in the plasma. The unknown substance could not be identified as any of the substances usually found in normal urine, nor with any of the compounds found only in trace amounts (Westall, 1955). Hence at the time when the full report of the case was published the particular metabolite was unidentified but the authors were of the opinion that it was probably a peptide of a type resistant to acid hydrolysis.

Further work on this substance (reported briefly by Westall, 1958) has now established that it is argininosuccinic acid. This acid was first postulated on theoretical grounds to be an intermediate in the pathway of synthesis of arginine from citrulline and aspartic acid in the presence of liver enzymes (Ratner & Pappas, 1949). It was later synthesized, isolated and characterized from a similar system (Ratner, Petrack & Rochovansky, 1953). These authors demonstrated that the reaction takes place in two stages. In the first, argininosuccinic acid is formed from citrulline and aspartic acid under the action of a 'condensing enzyme' present in mammalian liver and kidney; this requires adenosine triphosphate. The second stage requires the presence of a 'splitting enzyme', which catalyses the breakdown of argininosuccinic acid to form arginine and fumaric acid. They were able to prepare argininosuccinic acid from both of these systems since the splitting-enzyme system is reversible. The natural accumulation of argininosuccinic acid in mammalian tissues or body fluids has not been demonstrated before and it may be

assumed that under normal conditions it occurs only transiently. However, it has been found in pea-meal extracts (Davison & Elliott, 1952) and a mutant strain of *Neurospora crassa* has been described which accumulates argininosuccinic acid in the mycelium (Fincham & Boylen, 1955).

In the present paper details are given of the formal identification of argininosuccinic acid obtained for the first time from human material.

RESULTS

Chemical studies

Isolation of argininosuccinic acid from the patient's urine. Several attempts were made to isolate the unknown substance by using a system of coupled ion-exchange-resin columns (Westall, 1952), but the results were extremely confusing and it became obvious that the substance being studied was being degraded during fractionation. However, it was possible to isolate a white crystalline substance which moved to a different position on paper chromatography from that taken up by the original unknown substance. This newly isolated substance was stable to acid hydrolysis but gave aspartic acid and ornithine after alkaline hydrolysis. This information provided the clue and led to the recognition that the original substance was probably argininosuccinic acid (ASA) and that the isolated crystalline substance was one of the cyclic forms described by Ratner *et al.* (1953).

As soon as it was supposed that the unknown substance was ASA a simple method for its isolation was immediately available. Ratner *et al.* (1953), in their studies on the biosynthesis of ASA, successfully isolated it from tissue homogenates as the barium salt, which is soluble in water but virtually insoluble in aqueous 75% ethanol. This method works equally well for isolation of ASA from urine, and 160 g. of the crude barium salt was obtained from 25 l. of urine from one of the affected sibs (K.R.). As Ratner *et al.* (1953) pointed out, ASA does not in practice take up the expected 3 equiv. of barium but has a variable content of between 2 and 2.5 equiv. of barium when precipitated in the presence of excess of $\text{Ba}(\text{OH})_2$ at about pH 10–11. ASA is conveniently stored as the barium salt, which seems to be stable if kept dry. The production of the free base presents some difficulty since it forms cyclic compounds spontaneously in aqueous solution. The anhydride formation is favoured by high temperatures and acidic conditions. Hence if an aqueous solution of ASA remains at room temperature for a short time considerable cyclization takes place, since the isoelectric point of the free ampholyte is about pH 3.4. The time required to remove barium quantitatively as BaSO_4 and to produce a

freeze-dried powder leads to the formation of about 10% of anhydride. However, removal of barium by exchange with NH_4^+ ion on a cation-exchange-resin column at 2° and freeze-drying of the effluent produced a much purer sample of ASA. This method of producing the free base is an apparent contradiction of the statement made earlier that ASA is unstable on ion-exchange resins. This can be explained by the fact that the conditions were carefully selected to avoid cyclization. The temperature was kept at 2°, the amount of resin used was only slightly more than that required to remove barium and exchange with NH_4^+ ion. Thus ASA did not exist as the free acid until NH_3 was removed by volatilization during the freeze-drying. Subsequent analysis gave no evidence for ammonium salt formation. The passage of the solution through the resin column was completed in 4–5 min. Unfortunately, in spite of the limited anhydride formation it has still not been possible to secure crystalline ASA. Therefore the example of Ratner *et al.* (1953) has been followed and the anhydride forms, which crystallize readily from aqueous ethanol, have been used for elementary analyses.

Behaviour of argininosuccinic acid on filter-paper chromatograms. Fig. 1 (a) represents the standard two-way paper chromatogram most commonly used for screening urines to detect amino acidurias of various types (Dent, 1951). Aspartic acid, glutamic acid and glycine were put in as markers since the lutidine–water solvent runs off the leading edge of the paper and absolute R_f values do not apply. When the purified barium salt of ASA, obtained by repeated precipitation of the crude material, was run in this system a single spot was obtained in the position indicated. A sample of Dr Ratner's barium salt of ASA moved to the same position. When, however, free ASA, regenerated from the barium salt, was dissolved in water and allowed to stand at room temperature before being examined by paper chromatography three well-defined spots were obtained. One corresponded with the original position of ASA and the others took up the positions marked as B and C. Ratner *et al.* (1953) were aware that ASA changed into a modified form, on standing in aqueous solution, which they postulated to be a cyclic anhydride. They isolated and analysed this substance. When a sample of this anhydride (this and the sample of barium argininosuccinate were kindly supplied by Dr J. B. Jepson from gift samples from Dr S. Ratner) was run in the same chromatographic system it moved to the position marked C. Thus it would appear that there are two modified, probably both cyclic, forms of ASA. It is understandable that Ratner *et al.* (1953) were not aware of this because they used one-way paper chromatograms

with phenol-NH₃ as the solvent and in this system the R_F values of ASA and the B substance differ only slightly.

Behaviour of argininosuccinic acid and its anhydrides on high-voltage paper electrophoresis diagrams. As has been shown above, ASA and its two 'cyclic' forms B and C can be separated on two-way paper chromatograms, but a quicker and more convincing separation can be achieved by high-voltage paper electrophoresis. The apparatus used was a slightly modified form of that described by Gross (1955). A Whatman 3MM paper strip 10 cm. wide and 52 cm. long was dipped in buffer and blotted with moderate pressure. The samples to

be analysed were spotted on the starting line, which was at various positions, according to the expected direction of migration of the substances. The paper strip was then placed between water-cooled metal plates 45 cm. long, but protected from contact with the plates by sheets of polythene 0.005 in. thick. An even pressure of 5 lb./in.² was then applied to the bottom plate by means of an inflated rubber bag. The upper plate, after assembly, was not free to move. The ends of the paper strip were then connected by means of wet filter-paper wicks to polythene buffer pots in which the electrodes from the power pack were immersed. For the separation of ASA and its anhydrides a pyridine-acetate buffer, pH 5.1, was used (pyridine, 25 ml.; acetic acid, 10 ml., diluted to 2.5 l.). Fig. 1 (b) shows a typical ninhydrin-developed strip illustrating the separation of ASA, B and C after running for 20 min. with a potential difference between the ends of the strip of 6 kv and passing a current measured at 60 ma. In this buffer system ASA (isoelectric point 3.4) has an appreciable net negative charge and moves towards the anode; B (isoelectric point 4.2) also has a slight net negative charge and moves less rapidly to the anode; C (isoelectric point 5.5) moves slightly towards the cathode.

Stability of argininosuccinic acid and its two cyclic forms. In order to study the behaviour of ASA in water more closely, a series of experiments were devised to study the effect of pH and temperature on solutions of ASA, B and C. A series of buffer solutions of pH 2-11 (Theorell & Stenhagen, 1939) were made up (0.1M) and these were mixed with equal volumes of 0.02M-solutions of the substances being investigated. One series was allowed to stand at room temperature (22°) for 48 hr. and a second series was heated at 100° for 1 hr. The percentage composition of the equilibrium mixtures was estimated by visual comparison of the spots obtained after electrophoresis of the samples with spots obtained from standard amounts of the substances run under the same conditions and the results, which are not claimed to have an accuracy greater than $\pm 10\%$, are presented graphically in Fig. 2. When ASA, in solution, is allowed to stand at 22° [Fig. 2 (a)], it is relatively stable, provided that the pH is adjusted to be more alkaline than 6, but under more acidic conditions it is converted into the B and C forms, predominantly the latter. After 1 hr. at 100° [Fig. 2 (c)], ASA is completely transformed to about equal amounts of B and C at acid pH; at pH 5-6 the equilibrium favours the B form and in the more alkaline range some ASA survives and the cyclization is only to the B form. When B is similarly treated it is unaffected throughout the pH range after 24 hr. at 22° [Fig. 2 (c)], but it is slightly changed after 1 hr. at

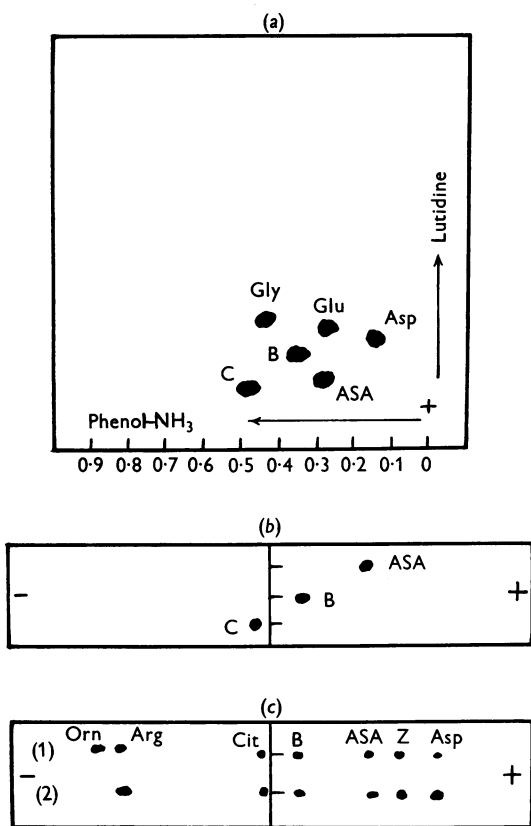


Fig. 1. (a) A two-way paper chromatogram with added marker substances showing the positions taken up by ASA and the two anhydrides B and C. (b) A paper-electrophoresis diagram showing the relative positions of ASA, B and C after running for 20 min. in pyridine-acetic acid buffer, pH 5.1, at 130 v/cm., 60 ma. (c) A paper electrophoresis diagram run as in (b) and showing the products obtained after hydrolysis of ASA with alkali: (1), 0.2N-Ba(OH)₂ for 16 hr. at 105°; (2), 0.5N-Ba(OH)₂ for 16 hr. at 105°. The samples were applied to the paper at the centre line. The positions of the substances were shown up by spraying with 0.1% ninhydrin in butanol.

100° [Fig. 2 (d)]; about 25% is changed to the C form at pH 2 and about 10% of ASA is regenerated at pH 11. The C substance is stable at pH 2–5 at 22° but is more readily converted back into ASA than B when the conditions become more alkaline [Fig. 2 (e)]. At 100°, C is still stable at pH 2–3 but at pH 5 the ring is opened rapidly with the formation of ASA, which presumably partly recyclizes to form B. It is curious that when C is heated at 100° at pH 5–7 there is a loss (30–40%) of total ninhydrin colour. Since the ninhydrin reaction is due to the amino group of the ornithine moiety it would seem that there may be some formation of intermediary substances involving some modification of the amino group. To summarize, ASA is more stable at alkaline pH provided that the temperature is not raised. The optimum conditions for forming the B anhydride is to heat ASA at pH 6; C, on the other hand, is best prepared by acidifying ASA and standing at room temperature.

Treatment of argininosuccinic acid and its anhydrides with strong acid. When ASA is refluxed for 24 hr. with 5.5N-HCl it is rapidly converted into the B and C forms and there is some further degradation (not exceeding 10%) leading to the formation of arginine, ornithine and aspartic acid. Similar experiments with B and C individually showed the same relative stability and the same products of degradation but there was a slight tendency for C to be more stable than B under these conditions.

Degradation studies on argininosuccinic acid with

alkali. As Ratner *et al.* (1953) have observed, ASA breaks down completely to ornithine and aspartic acid after refluxing with 3N-Ba(OH)₂ for 24 hr. They also stated that this was true also for their anhydride (C) although the liberation of NH₃ was slower and the final yield of aspartic acid was lower than for ASA. Repeat experiments under these conditions, where the products of hydrolysis have been examined by paper electrophoresis, have confirmed that ASA and C yield ornithine and aspartic acid only. Further, B also gave the same degradation products. Presumably B and C are first converted into the open ASA forms and further degradation proceeds from there. Reference to Fig. 2 (d) shows that B is more resistant to ring opening at alkaline pH than C [Fig. 2 (f)]. In discussing the route of degradation of ASA with alkali, Ratner *et al.* (1953) considered it might be by a simple multiple cleavage to give ornithine, aspartic acid, CO₂ and NH₃, but they stated that with milder conditions there might be some citrulline formed first which would later break down to yield ornithine. With alkali treatment of their anhydride (C) they had evidence for the formation of ureido acids and postulated breakdown by a route through ornithine and 5-(acetic acid) hydantoin, the latter being further degraded to ureidosuccinic acid and finally to aspartic acid, CO₂ and NH₃.

Degradative studies of ASA with varying strengths of alkali have shown that the series of

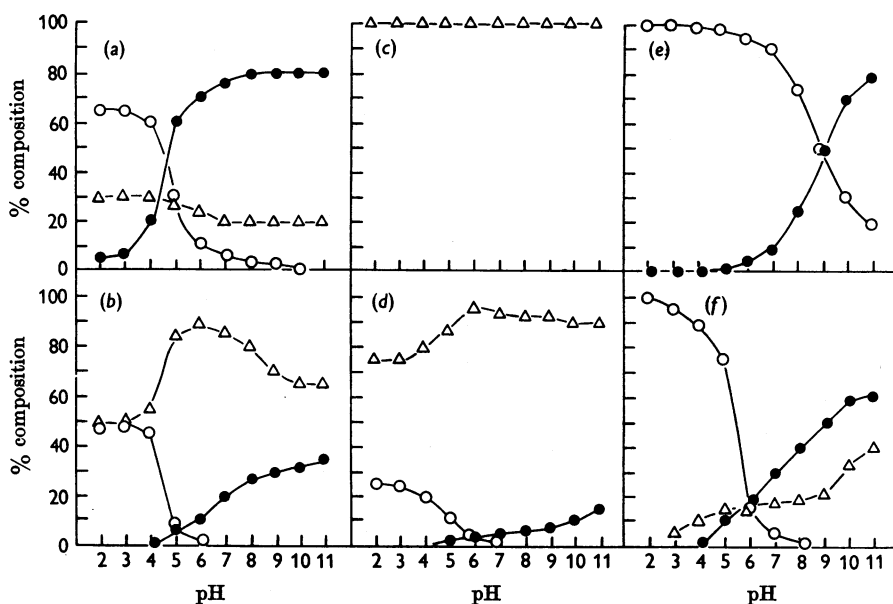


Fig. 2. Graphs showing the effect of temperature and pH on 0.01M-solutions of ASA (●), B (Δ) and C (○). (a) ASA stood at 22° for 48 hr. (b) ASA heated at 100° for 1 hr. (c) B stood at 22° for 48 hr. (d) B heated at 100° for 1 hr. (e) C stood at 22° for 48 hr. (f) C heated at 100° for 1 hr.

events that leads to the ultimate production of ornithine and aspartic acid is complex. Hydrolysis with 0.2N-Ba(OH)₂ for 16 hr. at 105° in a sealed tube gives a number of products: arginine, ornithine, citrulline, an unknown substance 'Z' and a trace of aspartic acid together with unchanged ASA and B. When 0.5N-Ba(OH)₂ was used for the same hydrolysis time no arginine was found and the amount of ornithine and aspartic acid was increased. Fig. 1 (c) illustrates the position taken up by these substances on the high-voltage electrophoresis diagram. The B and C forms, if treated similarly, give basically the same results but no trace of the Z substance was found. The occurrence of this unknown substance was difficult to explain until it was discovered that the sample of partially purified barium argininosuccinate used in the hydrolysis studies contained, as an impurity, another unknown substance 'Y'. Y behaves as an ampholyte, since it is retained on both anion- and cation-exchange resins and can be recovered without degrading. It does not give a colour with ninhydrin nor, in fact, with any of the routine spray reagents used in the chromatographic or electrophoretic studies. It was found by chance, since its presence on a particular electrophoresis diagram caused a distortion in the shape of the spot given by a known substance in a neighbouring position on the paper strip. It has now been found that its presence on paper electrophoresis diagrams can be shown by spraying with the alkaline nitroprusside-ferricyanide reagent (Kirby-Berry, Sutton, Cain & Berry, 1951). It gives a red spot which fades within 15 min. but it can still be seen some hours later since it gives a greenish fluorescence when viewed in u.v. light. Y has now been isolated but not fully characterized. However, it is converted into Z by mild alkaline hydrolysis, which explains the occurrence of Z in the hydrolysis studies; both Y and Z yield only ornithine and aspartic acid when

fully hydrolysed with 2N-NaOH for 24 hr. Hence it would seem that Y must have some close structural relationship with ASA.

Isolation of substances yielded by mild alkali treatment of argininosuccinic acid. In order to isolate some of the degradation products of ASA with alkali, and also the contaminating unknown substance Z, an hydrolysis of 10 g. of the crude barium salt was undertaken (as described in the Experimental section). The flow diagram of separation and the amino acid composition of the fractions is shown in Fig. 3. The early fractions contain ornithine only, followed by two fractions mixed with citrulline and then pure citrulline and so on. Z was easily isolated since it has a low solubility in water and, in fact, crystallized out as a fine white powder in the fraction tubes.

Quantitative determination of argininosuccinic acid. The method of Moore & Stein (1954) for the quantitative estimation of amino acids in body fluids with a column of the cation-exchange resin Dowex-50 provides a useful means of estimating ASA. It was used by Allan *et al.* (1958) for measuring the relative concentration of their unknown metabolite (now known to be ASA) in the blood plasma, cerebrospinal fluid and urine of their patients. Their more detailed analyses are now available (Cusworth & Dent, 1960). Further work on the chemistry of ASA has now led to a probable explanation of the behaviour of ASA on such a column. In the body fluids of these patients, within the range of physiological pH values, ASA exists predominantly in the open-chain form. However, in the early stages of the passage of the samples down the Moore & Stein column, the pH of the ambient solution is at 3.4 and is even more acid (2.0) than this when the sample is applied to the column. Under these conditions ASA forms an equilibrium mixture with its two anhydrides (called for convenience B and C) in the approximate ratio of 2:3:5. Since these three substances have different isoelectric points (ASA 3.4, B 4.2, C 5.5), ASA, being more acidic, moves ahead down the column and this leads to a new equilibrium with further production of more B and C. Hence the quantity of free ASA is soon decreased to minimal amounts because, under the conditions of the column procedure, there is no regeneration of ASA from the separated anhydrides. This all takes place quite rapidly since fairly sharp twin peaks are seen on the column flow diagram, which can be shown to correspond with the cyclic forms B and C. They occur near the position taken up by leucine and isoleucine and in the urine samples from the patient under study the B and C peaks are so large that the smaller leucine and isoleucine peaks were frequently overlapped. Paper-chromatographic analysis of the fractions from the column showed

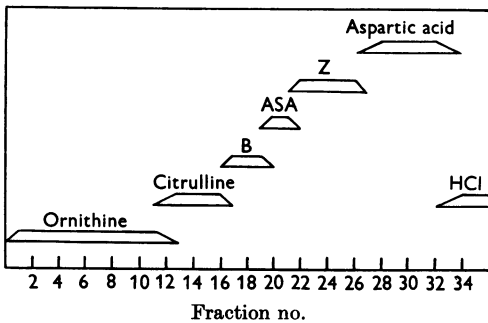


Fig. 3. Amino acid composition of the fractions obtained after displacement with 0.1N-HCl from a column of Dowex-2 ion-exchange resin. The mixture applied to the column was a partial hydrolysate of ASA with 0.5N-NaOH.

that the first of the two peaks was due to the B form and the second showed the C form with a small amount of free ASA. The occurrence of the small quantity of the open-chain form was probably an artifact and it was probably regenerated from C during the desalting of the fractions on small ion-exchange columns before the paper-chromatographic analysis. The explanation of the behaviour of ASA on the Moore & Stein column outlined above was further corroborated by carrying out test runs on the columns with isolated ASA (free base) and the B and C anhydrides. The ninhydrin colour values of B and C are 94 and 100% respectively of that given by leucine. A typical flow sheet of the Moore & Stein analysis of the urine of a child with argininosuccinic aciduria is shown in Fig. 4.

Clinical metabolic studies

Effects of dietary change on the excretion of argininosuccinic acid. Since ASA is an intermediate in the urea cycle it might be expected that variation in the quantities of the substances made available to the cycle could cause variation in the excretion of ASA.

The patient was an 8-year-old male child [previously described as case 2 of Allan *et al.* (1958)] admitted to the Metabolic Ward of University College Hospital, London, under the care of Professor C. E. Dent. The child was fed with a mixed constant diet of his own choice containing 30 g. of protein/day. The dietary supplements were fed in addition to the basic diet and were given in an orange-juice drink, one-quarter of which was consumed at each of four meals during the day. Urine samples (24 hr.) were collected throughout the study. L-Arginine was added at 2 g./day over a period of 3 days and the urine collected on the third day was selected for analysis. L-Aspartic acid, (5.0 g.) L-ornithine (4.0 g.) and L-citrulline (4.0 g.) were fed for one day only but as the last two substances are not found in the normal diet in appreciable quantities, a small test dose was given on the previous day in case of any ill-effects. The child was put back on the basic diet for several days between each experimental feed. As a final trial the protein level of the basic diet was dropped to 10 g./day over a period of 5 days, other dietary constituents being kept as constant as possible. The urine sample collected on the fifth day was selected for analysis.

The child remained physically well and active throughout the period of study and there appeared to be no appreciable change in his mental state. The results are shown in Table 1. ASA was determined

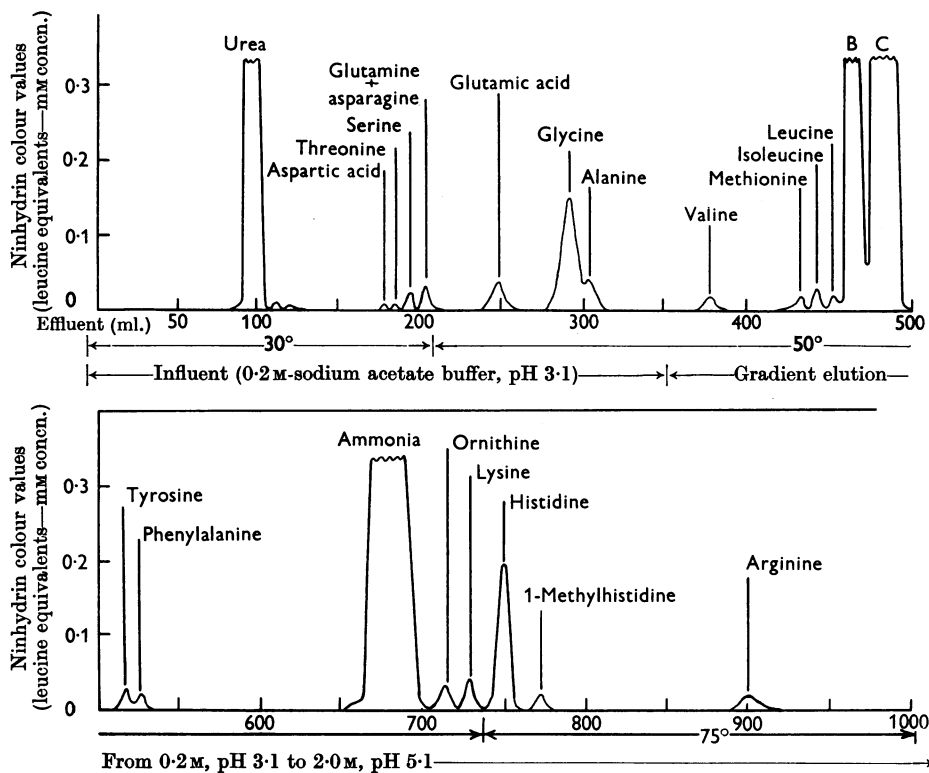


Fig. 4. A Moore & Stein column 2 flow diagram of the amino acid analysis of 2 ml. of urine (from total 24 hr. urine volume of 770 ml.) from patient K.R. Peaks B and C represent the anhydride derivatives of ASA.

Both cyclic forms, apart from some conversion of B into C, are almost completely stable to acid hydrolysis. On the other hand, in alkaline conditions the rings are opened, more slowly with B than with C, to regenerate ASA, which is then susceptible to further breakdown. The degradation is complex and presumably is related to the resonating effect of the double bond, which causes a variation in the site that is the weakest link in the chain. Arginine and citrulline have both been detected after mild alkaline hydrolyses but the formation of arginine by hydrolysis would also yield malic acid, which ultimately would affect the final yield of aspartic acid. Ratner *et al.* (1953), however, found that ASA, after strong alkaline hydrolysis, gave ornithine and aspartic acid in close to theoretical yield. To explain this it must either be postulated that the mechanism of breakdown is different under strong alkaline conditions or that the cleavage to form arginine is not hydrolytic and that direct cleavage forms fumaric acid, which later reacts with free NH_3 to form aspartic acid. The formation of aspartic acid from fumaric acid and ammonium carbonate heated in aqueous solution in a closed tube can readily be demonstrated.

With regard to the biological significance of these curious substances derived from ASA, it may well be that they are only chemical artifacts which have no place in the metabolism of ASA. Ratner *et al.* (1953) found that the anhydride form C was inert in various enzyme systems. Although B has so far not been tested, it is potentially the more interesting substance since, as Ratner (1954) has observed, it can be regarded as a substituted dihydro-orotic acid, a substance which has been shown to be on the pathway to pyrimidine synthesis (Wright *et al.* 1951).

In addition to the chemical problems, believed to concern cyclization of ASA as mentioned above, there still remains the problem of the unidentified substances designated Y and Z. It is now established that Z is not a chemical degradation product of ASA, as was thought earlier, but is derived from Y, which was present in the crude barium argininosuccinate preparation. The possibility still remained that Y might be an artifact formed by procedures employed for the isolation of ASA. However, it was detected on electrophoresis diagrams (by the use of the alkaline nitroprusside-ferricyanide reagent) of fresh urine specimens from the patient excreting large amounts of ASA. It was not present in a number of normal urines, including those from the unaffected members of the patient's own family. Hence it would seem reasonable to assume that Y is a naturally occurring metabolite that, like ASA itself, is normally an intracellular substance which is only rarely excreted in the urine. With this assumption and

with the chemical evidence that Y must have a structure somewhat similar to that of ASA (since it gives the same final degradation products, namely, aspartic acid and ornithine), it can be speculated that Y may be another intermediate in the series of reactions which make up the Krebs-Henseleit urea cycle. On the other hand, Y may represent an attempt by the patient to conjugate some of the excess of ASA formed in the tissues and to produce a derivative which is less harmful and better tolerated. It is hoped that when both Y and Z are fully characterized it will be possible to explain their function.

In considering the pathogenesis of the clinical syndrome in the two affected children we are in some difficulty because of inadequate data. At the moment no other similar patients have been described and enough family data is therefore lacking. Nevertheless, it seems probable that the disease is hereditary and probably due to some enzyme defect, similar to those already found in other metabolic diseases, e.g. phenylketonuria, galactosaemia, etc. On considering the urea cycle, Ratner *et al.* (1953) have shown that an enzyme (argininosuccinase) occurs in the liver and catalyses the breakdown of ASA with the formation of arginine and fumaric acid. One might expect therefore that the genetic defect might be a deficiency in this enzyme. If our current ideas are correct, this should lead to an inability to form urea whereas Allan *et al.* (1958) showed that in the disease the ability to form urea seemed unimpaired. Hence it is difficult to believe that the splitting enzyme is absent in the liver, which is regarded as the seat of urea production.

The second unusual feature is that the concentration of ASA is nearly three times as high in the cerebrospinal fluid as in the systemic blood plasma (Allan *et al.* 1958). This was a very striking finding since most of the amino acids found in cerebrospinal fluid are present in much higher concentration in plasma. This makes it most unlikely that the cerebrospinal fluid derives its ASA from the plasma, and in any case ASA has poor permeability properties (Walker & Myers, 1953) and could hardly be expected to penetrate the blood-brain barrier. Thus at present there is no better suggestion than that already discussed by Allen *et al.* (1958) in which they speculate that the unknown metabolite (ASA) may be formed in the brain, or in some other structure closely associated with the cerebrospinal fluid, and leaks from there to the blood, from which it is rapidly cleared by the kidney. This suggestion is not as bizarre as it might seem. For years it has been assumed that urea is formed only in the liver and that its presence in the cerebrospinal fluid was due to infiltration from the blood plasma. Recent work by Sporn, Dingman,

Defalco & Davies (1959) has shown that rat brain is capable of urea synthesis *in vivo* and Walker (1958) has shown that argininosuccinase (splitting enzyme), previously found in mammals only in the liver and kidney (Ratner, 1954), is also present in various other organs of the dog, including the brain.

The feeding experiments were undertaken to see how far the excretion of ASA could be influenced by the diet. In this way it was hoped that some indication might be given which would lead to a rational therapy which would lessen the synthesis of ASA and lower its concentration in the body fluids. If, for instance, arginine had increased the excretion of ASA, then it would be reasonable to attempt to lower the intake of arginine. However, the only compounds found to increase ASA output were ornithine and citrulline, substances not found as such in appreciable quantities in the normal diet. At the moment therefore it is difficult to see what dietary therapy can be usefully tried.

The increased output of ASA after taking additional ornithine and citrulline serves as additional evidence for its identification, and for its close relationship, metabolically with these compounds. The effect of a higher protein diet was not tried since the child seemed disinclined to eat more protein than was given in the 30 g./day regimen.

EXPERIMENTAL

Isolation of barium argininosuccinate. Urine (25 l.) from case 2 (Allan *et al.* 1958) was decreased in volume to 4 l. in a rotating evaporator (Van Heyningen, 1949). A hot saturated solution containing 350 g. of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ was added with stirring and the mixture was allowed to stand overnight. The precipitate was removed by filtration and to the filtrate (5.4 l.) 3 vol. of ethanol and 50 g. of solid $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ were added. After standing for 4 hr. the precipitate was separated by centrifuging, transferred to a large Büchner funnel and washed with 75% ethanol. After a final washing with 95% ethanol the solid cake was broken up and allowed to dry at room temperature. The powder was finally dried in a desiccator over H_2SO_4 . Yield of crude barium argininosuccinate was 160 g. Two-way paper-chromatographic analysis for amino acids with phenol- NH_3 and lutidine-water (2.2:1, v/v) as solvents and 25 μg . of the sample showed a large spot, due to ASA, and weak spots, due to traces of aspartic acid and glutamic acid. The crude barium salt (60 g.) was dissolved in 200 ml. of water and an insoluble residue was discarded. Ethanol (600 ml.) was added to the clear solution and the reprecipitated barium salt was filtered and dried. The yield was 47 g. Paper-chromatographic analysis showed ASA as the only ninhydrin-reacting constituent.

Preparation of free argininosuccinic acid (1). The purified barium salt of ASA (5 g.) was dissolved in 25 ml. of water and the small insoluble residue was discarded. The clear solution was chilled (4°) and $\text{n-H}_2\text{SO}_4$ (19 ml.), also cooled, was added to bring the pH of the mixture to 3.4. At the exact end point no precipitate was obtained when either more acid or weak $\text{Ba}(\text{OH})_2$ solution was added to a centri-

fuged portion of the mixture. The BaSO_4 was removed by centrifuging and the clear solution was freeze-dried. A fluffy pale-cream-coloured powder was obtained. Yield was 2.4 g. (Found: N, 18.9. Theory: N, 19.3%).

Preparation of free argininosuccinic acid (2). A glass tube 15 cm. long and 2.4 cm. in diameter constricted to capillary bore at the lower end and plugged with glass wool was filled with Dowex-50 cation-exchange resin (4% cross-linked and 100–200 mesh; 7.5 g. dry wt.). The resin was packed as a slurry after pretreatment with 2N-HCl and washing with water by decantation. Aq. n-NH_3 soln. was run down the column until the effluent was strongly alkaline. The column was then washed with water to remove the excess of NH_3 and kept at 4° until required. The purified barium salt of ASA (5 g.) was dissolved in 40 ml. of water and a small insoluble residue discarded. The solution was chilled and then passed through the prepared resin column. The effluent containing the soluble ammonium salt of ASA was collected and freeze-dried. Final drying was carried out in a Fischer drier at 80°. Yield was 2.1 g. (Found: N, 19.1. Theory: N, 19.3%).

Preparation of argininosuccinic acid anhydride (substance C). Free ASA (1.5 g.) was dissolved in 10 ml. of water and was allowed to stand at room temperature for 2 days. Ethanol (10 ml.) was added and as soon as crystals appeared a further 20 ml. of ethanol was added gradually. After standing at 4° overnight the crop of crystals was filtered off and dried. Yield 0.7 g. [Found: C, 44.1; H, 6.10; N, 20.4 (Dumas). $\text{C}_{10}\text{H}_{16}\text{O}_5\text{N}_4$ requires C, 44.1; H, 5.92; N, 20.6%].

Isolation of substances derived from partial degradation of crude argininosuccinic acid with alkali

The crude barium salt of ASA (10 g.) was dissolved in 100 ml. of water and 2N- H_2SO_4 was added cautiously until the mixture was adjusted to pH 3.4. BaSO_4 was removed by centrifuging and the supernatant was mixed with an equal volume of n-NaOH and refluxed for 4 hr. The hydrolysate was cooled and diluted to 1 l. with water and then run through a resin-column system (Westall, 1952), consisting of two columns coupled one beneath the other, filled with the anion-exchange resin Dowex-2 (8% cross-linked and 100–200 mesh). The upper column was made from a filtration tube 1.7 cm. in diameter and having a resin bed 12 cm. long (containing 10 g. of dry resin). The second column had a tube 1.2 cm. in diameter with a resin bed 8 cm. high (3.5 g. of dry resin). The tubes were packed with a slurry of resin in water and then coupled together with capillary glass tubing. The resin was converted into the OH form by passing 2N- NaOH down the columns followed by washing with water until the pH of the effluent was between 7 and 8. After the diluted hydrolysate was passed down the columns a further 100 ml. of water was run on and this was followed by the displacing agent 0.1N-HCl. As soon as a positive ninhydrin reaction was obtained on testing the effluent, fractions (8–10 ml.) were collected until the effluent became strongly acid. Samples (5 μl .) from the fractions were spotted on paper strips and the strips were analysed by electrophoresis. The amino acid composition of the fractions is illustrated in Fig. 3.

Isolation of ornithine hydrochloride. Fractions 1–11 were mixed, adjusted to pH 6 with n-HCl and evaporated at reduced pressure to 5 ml. Crystals were obtained after the gradual addition of ethanol to give a final volume of 20 ml.

The crystals were collected and recrystallized from water and ethanol. Yield was 0.82 g. (Found: C, 35.8; H, 7.1; N, 16.7. $C_5H_{13}O_2N_2Cl$ requires C, 35.6; H, 7.7; N, 16.6%).

Isolation of citrulline. Fractions 13–16 were combined and evaporated under reduced pressure to 2 ml. Crystals were obtained after the addition of ethanol. Yield after recrystallization was 0.21 g. (Found: C, 41.6; H, 7.7; N, 23.8. $C_6H_{13}O_2N_3$ requires C, 41.1; H, 7.5; N, 24.0%).

Isolation of substance B. Fractions 17–19 were mixed and evaporated under reduced pressure to 2 ml. A crystalline product was obtained by the addition of ethanol. Yield was 0.14 g. This material gave a lower figure for its N content than was expected and it was ultimately discovered that it was contaminated with an unknown, non-ninhydrin-reacting ampholyte which had a lower N content. A pure sample of B was obtained later by repeated recrystallization, from aqueous ethanol, of a sample obtained from a larger-scale fractionation carried out in a similar manner. Yield was 1.7 g. [Found: C, 43.9; H, 6.1; N, 20.8 (Dumas). $C_{10}H_{16}O_4N_4$ requires C, 44.1; H, 5.9; N, 20.6%].

Isolation of substance Z. Substance Z formed a white deposit shortly after the fractions were collected. This white powder was filtered off from fractions 22–26 and the fractions were decreased in volume to 10 ml. and a further crop was obtained. This powder was very slightly soluble in water and was washed well with cold water before drying. Yield was 0.32 g. (Found: C, 44.61; H, 5.46; N, 15.3%).

Isolation of aspartic acid. Small amounts of aspartic acid crystallized spontaneously in fraction tubes 28–32. This material was filtered off and a second crop was obtained by decreasing the volume of the mother liquor to 5 ml. Both crops were combined and recrystallized from hot water. Yield was 0.21 g. (Found: C, 36.37; H, 5.33; N, 10.70. $C_4H_7O_4N$ requires C, 36.1; H, 5.3; N, 10.52%).

SUMMARY

1. The unknown metabolite previously described as the predominant amino acid in the urine of two mentally defective sibs has been isolated and has proved to be argininosuccinic acid.

2. The behaviour of argininosuccinic acid in aqueous solution has been studied. It apparently forms an equilibrium mixture with two other modified forms which are probably cyclized anhydrides.

3. The complicated behaviour of argininosuccinic acid on resin columns can be explained on the basis of its known reactivity. It is, nevertheless, possible to assay it by the method of Moore & Stein (1954). Each patient with argininosuccinic aciduria excreted about 3 g. of argininosuccinic acid per 24 hr. in his urine.

4. Argininosuccinic acid and its two anhydride forms yield ornithine and aspartic acid after long

hydrolysis with strong alkali, but with weaker alkali for a shorter time a number of intermediates are formed which include arginine and citrulline.

5. An unknown substance has been found in the urine of one patient with argininosuccinic aciduria which after treatment with strong alkali breaks down to yield aspartic acid and ornithine.

6. The gross excretion of argininosuccinic acid seemed little affected by lowering the protein content of the diet over a short period or by adding arginine, but it was increased by feeding ornithine and citrulline.

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