ARGININOSUCCINIC ACIDURIA

AN INBORN ERROR OF AMINO ACID METABOLISM

BY

B. LEVIN, HELEN M. M. MACKAY and V. G. OBERHOLZER

From Queen Elizabeth Hospital for Children, London

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It is probable that many, if not all, of the inherited ('inborn') metabolic disturbances result from a specific enzyme defect, arising from a single abnormal gene. Examples are now known of a number of such inherited defects, some involving amino acids, which result in a block of a metabolic pathway. For example, the normal pathway for the metabolism of phenylalanine and tyrosine is by a series of conversions, each mediated by a specific enzyme. The lack of any one of these enzymes will lead to a specific disease and at least four such diseases have been identified, the most common of which is phenylketonuria.

Similar enzyme deficiencies involving the metabolism of amino acids are, however, still very uncommon. A new instance has been recorded by Allan, Cusworth, Dent and Wilson (1958). They have described two sibs, suffering from mental retardation, who excreted in the urine large amounts of an amino acid not normally present. Later Westall (1960a, b) identified this substance as argininosuccinic acid, a known intermediate in the bio-synthesis of urea. The condition must be rare, since Allan *et al.* (1958) stated that 1,500 mentally deficient patients were screened and no further examples discovered.

This paper records an infant with an identical abnormality, detected within a month of birth in December 1958, and the only other example of this type so far described. A metabolic disorder was suspected because clinical examination and routine pathological investigations had revealed no obvious cause for a sudden onset of severe illness in the first week of life. Paper chromatography of the urine showed a gross amino aciduria due to the presence of large amounts of an amino acid and some progress had been made towards identifying the amino acid before the article by Allan *et al.* (1958) was noted. The similarity between the properties of the amino acids in their cases and ours led us to suspect that they were identical, and this was later proved by

comparison with a specimen of argininosuccinic acid (ASA) isolated from the published cases and kindly provided by Dr. Westall.

The detrimental effect on this infant of his metabolic disorder, and the history of the two earlier cases, indicated the desirability of early investigations which might provide a pointer for rational dietetic therapy such as that now in use in certain other inherited metabolic disturbances. Some such investigations are described in this paper, but so far they have not paved the way for treatment.

Case Reports

Two Cases Previously Described. The two cases described by Allan *et al.* (1958) were sibs, a girl, M., and a boy, K., coming under observation at the ages of 3 years and 5 months and 6 years and 3 months, respectively. The parents were not consanguineous. Of their five children, the first and third were normal; the fifth apparently had kernikterus as a baby and had had an exchange transfusion for Rh (D) incompatibility; he died of bronchopneumonia at 4½ months of age.

Both the sibs, M. and K., with amino-aciduria, were mentally defective when first examined, but in neither case did the neonatal history suggest the likelihood of kernikterus. M.'s Gesell rating was 32 and K.'s rating on the Terman-Merrill scale was 50. M. had a moderate amount of vomiting in her early months, and after the age of 2 suffered from convulsions, for which she was admitted to hospital at the age of 3 years and 5 months. After these convulsions, there was a period of severe incoordination when she was unable to feed herself or stand. K. had never had convulsions, but electroencephalography 'showed definite evidence of epilepsy'. The two children had a similar facial appearance and a 'sad and wistful' expression. Both had similar hair, dry and friable and giving a matted appearance. M.'s skin was slightly rough on the arms and dorsum of the hands. K.'s skin texture was normal. Both children had systolic murmurs, possibly due to an interventricular septal defect.

According to their mother, whose intelligence was 'above average', both children appeared normal during

TABLE 1
BIOCHEMICAL FINDINGS IN PLASMA OR SERUM

	First Month (1)	9 Months (2)	10 Months (Acutely Ill) (3)	13 Months (4)
Sodium (mEq per litre)	142	138	131	133
Potassium (mEq per litre)	5.4	3.6	4.6	4.5
Carbon dioxide capacity (mEq per litre)	22	—	22	—
Chloride (mEq per litre)	105	105	100	105
Urea (mg. per 100 ml.)	44	13	27	14
Non-protein nitrogen (mg. per 100 ml.)	56	27	66	26
Total protein (g. per 100 ml.)	5.5	5.24	4.4	6.89
Albumin (g. per 100 ml.)	3.6	3.45	—	4.25
Globulin (g. per 100 ml.)	1.9	1.79	—	2.64
Serum bilirubin (mg. per 100 ml.)	—	—	2.5	0.5
Thymol turbidity (units)	2.5	3	1	2
Thymol flocculation	Negative	Negative	Negative	Negative
Zinc sulphate turbidity (units)	—	—	2.5	1.5
γ-globulin turbidity (units)	5	4	5	5
Calcium (mg. per 100 ml.)	10.1	9.9	7.0	10.2
Phosphate (mg. per 100 ml.)	7.2	2.3	1.2	6.3
Phosphatase (K.A. units)	18.1	22.7	27.2	21.4
S.G.O.T. (units)	—	46	196	78
S.G.P.T. (units)	—	46	132	74
Blood sugar (mg. per 100 ml.)	54	—	126	—
Cholesterol (mg. per 100 ml.)	202	70	110	—
Fatty acid esters (mg. per 100 ml.)	925	—	—	—

the first year of life. M. was a 'lovely, bonny, happy baby' with normal hair. K. thrived well and until 15 months old had 'beautiful curly hair'. M. sat up at 8 months and walked at 11 months. K. sat up at 8 months and walked at 13 months.

No mention of liver enlargement or abdominal distension was made in either child. The level of alkaline phosphatase was moderately high, but apart from this, liver function, so far as assessed, was not disturbed. Both sibs excreted large amounts of ASA in the urine. This substance was present in higher concentration in the cerebrospinal fluid than in the plasma. This disorder of amino acid metabolism was not present in any other member of the family.

Present Case. The case investigated by us differed in various respects from the two already described.

First Three Months of Life. J., a boy, was born in hospital on December 24, 1958, after a normal pregnancy and delivery, birth weight 8 lb. 11 oz. The parents were not consanguineous and J. was the first child. He was noted to have been 'cyanosed for 2 minutes after birth'. He was breast fed and appeared normal until 5 days old, when he sucked badly. The next day his abdomen rapidly became distended, though the stools were normal. He was apathetic; his weight then was 8 lb., his rectal temperature 94° F. There was some nasal obstruction, his respirations were rapid (probably due to his distension) and there were some signs at the base of the lungs. He was admitted that evening to the Queen Elizabeth Hospital for Children. Radiographs showed gaseous distension of the bowel, but revealed no pulmonary lesion. Next day, at 7 days of age, he was unconscious with occasional convulsive movements of the right arm. There was oedema of the ankles; he vomited five times and the vomit contained altered blood. The liver was felt and was possibly enlarged, but abdominal distension made palpation difficult. The white blood cell count

was 26,200 per c.mm. (polymorphs 44%, eosinophils 1%, lymphocytes 53%, monocytes 2%). Plasma electrolyte levels were within normal limits (Table 1, column 1), and the cerebrospinal fluid appeared normal on routine examination. He was given a glucose electrolyte mixture by tube as he could not suck. The vomiting diminished and at 11 days he was able to take a half-cream dried milk mixture with sugar. At 3 weeks the liver was enlarged to the level of the umbilicus. Liver function tests were, however, normal. Chromatography of the urine showed that an unknown amino acid was being excreted in large amounts and one with similar R_f value was present in the cerebrospinal fluid. This amino acid was later proved to be argininosuccinic acid (see below). Thereafter, liver enlargement and abdominal distension were always present, although in varying degree. At the age of 3 months the liver edge was firm and hard.

There was intermittent vomiting until he was over 2 months of age. His weight at 4 weeks was about average for his birth weight according to our assessment (Levin, Mackay, Neill, Oberholzer and Whitehead, 1959), but thereafter his gains were irregular and inadequate. He was inactive, often fretful and he suffered from recurrent otorrhoea. There was occasional slight oedema of the feet and ankles and the skin tended to be patchily rough and dry. The degree of his alertness varied considerably, probably inversely with his malaise, but he was late in reaching every milestone.

Dietetic Changes

(a) PROTEIN-FREE DIET FOR ONE WEEK. It seemed reasonable to postulate that the child's illness and retardation were due to a toxic effect of argininosuccinic acid and that by analogy with the treatment of phenylketonuria, improvement would occur if the production of the amino acid could be reduced. Therefore, as a preliminary investigation towards this end, the effect

TABLE 2
URINARY EXCRETION OF ARGININOSUCCINIC ACID AND UREA

Diet	Protein (or Equivalent) Intake (g./day)	Total Nitrogen (mg.)	Urea Nitrogen (mg.)	ASA Nitrogen (mg.)	ASA Nitrogen As % of Total Nitrogen	ASA Nitrogen As % of Urea Nitrogen	ASA Excretion (g./day)
Half-cream national dried milk	36	3,445	2,500	571	16.6	22.8	3.0
No protein		262	138	112	43	81	0.6
Synthetic amino acids	10	860	394	326	39.2	81	1.65
Casein hydrolysate with low arginine content	15	1,261	659	364	29	55	1.9*
As above + 2 g. arginine	17	1,235	613	381	31	62	2.0*
As above + 3 g. arginine	18	1,867	848	598	32	72	3.1

* Mean of three days.

of removal of protein from the diet on the formation of argininosuccinic acid, as assessed by its excretion in the urine, was investigated. Hence, at 3½ months of age, the infant's half-cream dried milk mixture was replaced by a protein-free mixture providing an equivalent number of calories and composed of gluten-free flour, sugar and arachis oil, together with salt and adequate amounts of vitamins A, B, C and D. Although just after the change in diet there was a recurrence of otorrhoea, within five days the abdominal distension was strikingly diminished, the liver became smaller, and the excretion of argininosuccinic acid fell by 80% (Table 2). On the other hand, the gums had become red, appetite failed and by the seventh day there were skin changes, widespread and extensive patchy dry erythematous areas, with intertrigo and angular stomatitis. The infant was fretful and had lost 16 oz. in seven days. The dried milk feeds were then restarted, the skin began to improve within 24 hours and soon the appetite was good; eight days later the abdominal distension was again gross and the liver reached nearly to umbilical level. The changes in the baby's condition seemed directly related in time to the changes in diet.

(b) DRIED MILK DIET. At 4 months of age, 11 days after half-cream dried milk was restarted, the infant was sucking and much more contented. He weighed 12½ lb. and had more than regained his loss in weight on the protein-free diet. He was, however, only about 3½ lb. over birthweight and over 3 lb. below our expected average weight for infants weighing 8 to 9 lb. at birth (Levin *et al.*, 1959). His diet was changed to full-cream dried milk and sugar, and in the next few days he relapsed, with loss in weight, redness of gums and angular stomatitis and worsening of the skin condition—only to improve again, this time without change in the diet.

At 5 months the infant weighed only 12 lb. 5 oz., was retarded in all his attainments and was thought to be of subnormal intelligence. An assessment was attempted by Dr. Agatha Bowley when the infant was 4 months and 11 days, and she considered his reactions were those of a 2-month baby.

(c) ARGinine-FREE DIET, FOLLOWED BY DRIED MILK DIET. At 5½ months of age, an attempt was made to assess the effect of complete elimination of arginine from the diet. For this, a synthetic amino acid mixture, containing the eight essential amino acids together with

additional glycine to make up nitrogen requirement, was utilized. To provide adequate calorie intake, cane sugar, lactose and gluten-free flour were also given. Although he lost some weight and his liver enlarged a little, he was otherwise unchanged, but a week after the commencement of the new diet, he became fretful and miserable and gradually became unconscious. He was tube fed with a glucose electrolyte mixture and improved within 24 hours and a normal diet was gradually reintroduced. He began to gain weight again, his liver diminished in size but he developed a number of indolent pustules on the scalp.

During the next two months the infant made better progress. He gained weight and the scalp condition was greatly improved, although the ulcers did not heal completely. By 7½ months he weighed about 17½ lb. and had cut two teeth and a month later a further two.

(d) DIETS VARYING IN ARGinine CONTENT. Despite the unsatisfactory response to the synthetic amino acid mixture, a further endeavour was made to assess the effect of low and high arginine diets. Accordingly, when the infant was 9 months old, and weighed 18 lb. 1½ oz., he was taken off ordinary feeds and given a casein hydrolysate preparation, from which about half the arginine had been removed. There was little change in his condition during the next fortnight, except that an upper respiratory tract infection developed, his skin condition tended to deteriorate and he lost a little weight. As it was thought that there might be a relative deficiency of lysine and tryptophan in his diet, these were now added to his feeds. After 12 days on the casein hydrolysate preparation, arginine was added for a period of 17 days, giving at first 2 g. and, for the last four days, 3 g. of arginine per day. The liver altered little in size, but the scalp condition improved. The effect of the alteration in arginine intake on ASA excretion is discussed below. Three days later, whilst he was still being fed on the casein hydrolysate, but without added arginine, an episode of serious illness began with fluctuating severity, necessitating many rapid changes in treatment. It commenced with a urinary infection (*Esch. coli*) treated with chloramphenicol and neomycin. Eight days later, he began to refuse feeds and to vomit, the liver became larger and he had occasional jerking movements of the elbows and there was a tendency for the head to deviate to the right. By next day he was worse

and, since vomiting increased in severity, glucose-saline was given by the intravenous route. Liver function tests (Table 1, column 3) showed only a moderate degree of impairment. His condition worsened, he became spastic on the right side and began to have Jacksonian convulsions, affecting the same side, for which he was treated with paraldehyde. On the same day large amounts of blood were aspirated from the stomach and he was given a blood transfusion. At times he could barely be roused. However, he improved and began to take fluids by mouth, but marked oliguria developed. Because of this and a persistent oedema, he was given only arachis oil and glucose by mouth and a restricted fluid intake. This treatment was continued for 11 days, with the gradual substitution during the last six days of unsweetened half-cream dried milk, with sugar added. Since oedema persisted, he was given Edosol (low sodium dried milk) and continued on this for 10 days, after which the ordinary milk feeds were restored, with the addition of cereal and apple puree. The oedema gradually diminished. A month after the commencement of this episode of acute illness, he returned to his usual state of health. His weight was somewhat less than it had been one month earlier. His liver was still enlarged to the level of the umbilicus and his skin was still scaly, dry and 'spotty'. When he was nearly 10 months, Dr. Bowley considered him to have the developmental picture of a 7- to 8-month-old baby.

He began to gain weight more satisfactorily and remained relatively well apart from a slight upper respiratory infection. When he was discharged from hospital on February 24, 1960, age 14 months, his weight was 20 lb. 4½ oz.

Second Admission. He was readmitted on May 31, at the age of 17 months, having had seven convulsions on the previous days, with twitching of the arms and legs. These episodes lasted for only one minute, without loss of consciousness and without vomiting. The enlargement of the liver and the condition of his skin were unchanged. The fits continued in diminished number after admission, and were controlled with phenobarbitone and epanutin. Examination of the cerebrospinal fluid obtained 16 days after admission, when the fits had ceased, revealed an increased cell count (102 lymphocytes per c.mm.). The complement fixation tests for lymphocytic choriomeningitis and mumps virus, and the leptospiral agglutination tests, were negative. His stay in hospital was prolonged by a sequence of chest and skin infections for which he was treated with antibiotics. He was discharged on September 6, 1960, aged 20 months, weighing 22 lb. 12 oz., having lost 1 lb. 5 oz. during his 14 weeks' stay in hospital. At 15 months he was able to pull himself up, and stand with support, and at 21 months he began to walk with support. When he was 18 months Dr. Bowley considered him to have a developmental age of about 12 months. Encephalography was also carried out at this time and Dr. B. Gordon reported as follows:

'The dominant activity in the parieto-occipital region is regular and symmetrical at 5-6 c/s. No

other significant activity is seen either when the child is awake or during a period of sleep. The E.E.G. is normal for the age.'

Third Admission. Two months after discharge, the patient was admitted for a third time on November 7, 1960, having had five convulsions of short duration during the previous 12 hours. The convulsions consisted of generalized twitchings with cyanosis. His tonsils were enlarged and inflamed, he had a temperature of 103·7° F. with raised pulse and respiratory rates. One further convulsion occurred after admission. Treatment with penicillin was followed by an erythematous rash. This disappeared when the antibiotic was withdrawn and phenegan given. The infection finally yielded to terramycin. In view of the fits, long-term anticonvulsant therapy with phenobarbitone was prescribed. He was discharged on November 24, 1960. There had been little or no change in the size of the liver nor in the skin condition.

Present State. The child is now (March 1961) 2½ years of age, weighs 25 lb. 4 oz. and is mentally much retarded. He can only stand if supported and understands no words. The gross liver enlargement and the roughness of the skin persist. The hair still has a brittle character, but the finger-nails and toe-nails are now apparently normal.

Family History. There was no history of fits, mental defect or other relevant disease in the parents, grandparents and other relatives of the mother, nor in the only two relatives of the father who are known to him.

Hair. A specimen of hair was kindly examined by Dr. A. Jarrett, who reports as follows:

'The hair shows breaks of the trichorrhexis nodosa type involving mainly the fine type of hair. The colour fluorescence of the breaks with acridine orange is red and this indicates a metabolic abnormality of the hair keratin.'

Comparison of the Present Case with the Two Previously Described. The three patients, for the sake of clarity, are indicated below by the following letters: present case, J.; previously described cases, girl sib, M., her brother, K.

All three patients were excreting large amounts of ASA and had higher concentrations of this amino acid in the cerebrospinal fluid than in the plasma. All three appeared normal at birth. J. showed some symptoms at 6 days of age, whereas M. and K. were apparently symptom-free during their first year. All are now mentally retarded; J. and M. suffer from fits and K. has an electroencephalogram indicating epilepsy. All three appear to have a facial resemblance to one another (Fig. 1), and have brittle hair. J. suffered from extensive lesions of the skin and buccal mucous membrane and had brittle toe-nails and finger-nails. M. had some localized roughness of the skin; K. has normal skin texture.

FIG. 1.—Appearance of child at 17 months.

Striking features in J.'s condition are gross enlargement of the liver, abdominal distension and poor physical progress, with periods of apathy and fretfulness. He has also had periods of unexplained unconsciousness. None of these figure in the history of the two sibs. On the other hand, he has no abnormal cardiac findings, and M. and K. have systolic murmurs, presumably indicating a cardiac lesion.

A raised alkaline phosphatase level has been found in all three patients, and J. has had definite evidence of liver dysfunction. It may be that the raised alkaline phosphatase level found in M. and K. indicate liver dysfunction in the sibs also.

Biochemical Findings and Liver Function Tests. Some relevant biochemical findings are summarized in Table 1. The findings, including the liver function tests, were normal shortly after admission (column 1), i.e. in the first month of life, though during this period the alkaline phosphatase fluctuated between 18.1 and 32.7 King-Armstrong units per 100 ml. Column 2 shows the findings at 9 months of age whilst the infant was having casein hydrolysate from which half of the arginine had been removed, and just before the severe episode of illness already described. Liver function tests were still within normal limits despite the grossly enlarged liver. The only abnormality found was a very low serum phosphorus, due to the low phosphorus content of the casein hydrolysate preparation. Column 3 gives the findings 24 days later at the worst phase of this episode of illness, when the infant was 10 months old; the serum transaminases by then indicated some impairment of liver function, and in addition both alkaline phosphatase and serum bilirubin levels were raised. Again, as a result of the low dietary phosphorus, serum phosphorus and calcium levels were low. Column 4 gives the findings at about 13 months of age, after recovering from the acute symptoms of this period; the serum transaminases were still slightly raised, but all other biochemical findings had returned to normal.

Laboratory Investigations

Paper Chromatography. Urine (0.005 ml.) was applied to the paper, undiluted and untreated, using butanol-acetic acid-water and phenol-ammonia as solvent systems. The amino acids were detected with a cadmium acetate-ninhydrin reagent (Heilmann, Barrolier and Watzke, 1957) prepared by dissolving 100 mg. cadmium acetate in a mixture of 10 ml. water and 5 ml. glacial acetic acid and making up to 100 ml. with acetone. To this solution 1 g. ninhydrin was added before use. The paper chromatogram was dried, dipped into the reagent, and allowed to remain in the dark for 24 hours in an ammonia-free atmosphere.

Two-way chromatography (Fig. 2) using the above solvent systems, as well as one-way chromatography of fresh urine using butanol-acetic acid-water, usually revealed only one intense ninhydrin-positive band (R_F value 0.11 in butanol-acetic acid-water) due to the presence of argininosuccinic acid, in addition to the amino acids normally seen in urine. Sometimes, however, a small band of R_F 0.06 due to an anhydride of the acid, was detected close to the main ASA band. Two bands were also detected with phenol-ammonia-water, the main one being the ASA fraction of R_F value 0.27 and the other, a much smaller one of R_F value 0.49 corresponding to an anhydride (Ratner, Petrack and Rochovansky, 1953); occasionally, if the urine had been standing at room temperature for some time, a considerable degree of conversion into the anhydride occurred and two fractions of equal intensity were obtained.

Westall (1960b) has shown, however, that ASA can be converted into two anhydrides (denoted by B and C), a six-membered ring form, and a five-membered ring form, which can be separated by two-way chromatography, using phenol-ammonia-water and lutidine-water as the solvent systems. He has provisionally assigned the five-membered ring structure to that anhydride (Form C) which has an R_F value of 0.49 in phenol-ammonia-water. We have also been able to demonstrate three separate spots by using butanol-acetic acid-water and phenol-ammonia-water as the two solvent systems in two-way chromatography and have by this means shown that the second band R_F 0.06 obtained by one-way chromatography using the former system, is anhydride B, and that anhydride C fails to separate from ASA in this solvent system.

Cerebrospinal fluid was also used undiluted and usually untreated, but sometimes treated as for plasma. The amino acids are extracted from plasma by passing 0.2-0.5 ml. through Amberlite 120 (H) in a column 7-8 cm. long containing approximately 0.4 g. resin. After washing well with water, the amino acids are eluted with 10 ml. 5 M. ammonium hydroxide. The eluate is taken to dryness *in vacuo* and the residue redissolved in 0.05 ml. water. Usually, 0.03 ml. was taken for chromatography. Two-way chromatograms of serum and spinal fluid are shown in Figs. 3 and 4 respectively.

Isolation of Barium Arginino-succinate from Urine (R. G. Westall, personal communication). The procedure

FIG. 2.—Two-way chromatogram of urine. The intense spot is the argininosuccinic acid (ASA). The two anhydrides (B and C) are indicated.

followed was similar to that used by Ratner *et al.* (1953) for isolating the acid after synthesis by an enzymatic method. Barium chloride solution (30 g. %, 9 ml.) was added to 100 ml. urine, followed by barium hydroxide (saturated solution, 20 ml.), and well mixed. The resulting mixture should be strongly alkaline. After standing at 4° C. overnight, the precipitate was separated either by filtration, using a Buchner funnel and Whatman No. 42 filter paper, or by centrifuging. To the clear filtrate was added three times its volume of absolute alcohol and the mixture was allowed to stand at 4° C. for 24 hours.

After decanting most of the supernatant and then centrifuging, the precipitate was redissolved in about 20 ml. water, centrifuged and reprecipitated with three times its volume of absolute alcohol. The final precipitate was washed first with 75% and then with 87% and finally with absolute alcohol, and then dried *in vacuo*.

For use as a standard in chromatography, an aqueous solution of the potassium salt was used. This was obtained by dissolving 0.25 g. of the pale yellow solid barium salt in 2.0 ml. warm water, adding 1.0 ml. of 1 M. potassium sulphate solution and finally centrifuging to remove precipitated barium sulphate and other insoluble material. The concentration was checked by estimation of the total and amino-nitrogen.

Estimation of Argininosuccinic Acid in Urine, Cerebrospinal Fluid and Plasma. Quantitative determinations

of the amino acid were usually performed on one-way rather than two-way chromatograms, using butanol-acetic acid-water or phenol-ammonia as the solvent systems. The paper chromatogram was stained with the cadmium acetate-ninhydrin reagent, as described above. The argininosuccinic acid segment was cut out, covered with 2 ml. methyl alcohol in a test-tube and allowed to stand for two to three hours. The alcohol was removed and the paper washed three times with 1 ml. amounts of methanol. The total eluate was centrifuged to remove debris and the final solution read in a cuvette at 509 m μ against a blank on the same paper. A normal solution of free argininosuccinic acid was used as a standard.

Glutamine was estimated in a similar way, using glutamine as a standard for comparison.

Proof of Identity of the Amino Acid. The unknown amino acid was identified first by a process of elimination and then by comparison with a specimen of known argininosuccinic acid. Known amino acids with similar R_F values in the same solvent systems were excluded by specific spot tests where applicable, e.g. cysteine and cystathionine. Phospho-ethanolamine was excluded by the failure to detect phosphorus in the eluate of the unknown spot. The only test on the paper giving a positive result was the Jaffé test, but the colour developed was weak compared with what might have been expected from the ninhydrin reaction. Proof of

identity with argininosuccinic acid rested also upon the following:

(a) The R_F value in phenol-ammonia-water was identical with that of argininosuccinic acid, and a mixture of the unknown with known acid gave only one spot after chromatography.

(b) Barium argininosuccinate was obtained in large yield from the patient's urine by the procedure described above.

(c) The solution of free argininosuccinic acid obtained by heating the aqueous solution of the barium salt with acid showed, on one-way chromatography, two ninhydrin positive bands, with R_F values corresponding to argininosuccinic acid and to its anhydride (Ratner *et al.*, 1953) and on two-way chromatography three bands, corresponding to the acid and its two anhydrides (Westall, 1960b).

Argininosuccinic Acid Levels in Urine, Plasma and Cerebrospinal Fluid. The method used suffered from the drawback that it was not always possible to isolate the required fraction completely. However, owing to the preponderant amount of the amino acid present, especially in urine, this did not constitute a serious error. In plasma, the error was minimized by using a normal plasma as blank, i.e. taking as blank value the ninhydrin fraction, having in normal plasma the same R_F value as argininosuccinic acid. No such difficulty arose with cerebrospinal fluid where the glutamine and argininosuccinic acid were the only preponderant spots, and the two have very different R_F values.

A number of 24-hour specimens of the urine were directly analysed for argininosuccinic acid, using known acid as standard. At other times a close approximation could be obtained by deducting urea nitrogen from total urinary nitrogen, allowing a further 10% of the total nitrogen for all other nitrogen-containing compounds usually present in urine and converting the residual nitrogen into weight of argininosuccinic acid. The range of excretion varied from 1.5 g. to 3.0 g. per 24 hours, according to diet. Although no other urinary amino acid was determined quantitatively, visual assessment of the stained chromatogram showed that there was no increase in the excretion of the other amino acids normally found.

The plasma level of argininosuccinic acid was estimated on several occasions, a typical value found being 4.4 mg. per 100 ml. The renal clearance calculated on a 24-hour specimen of the urine with a concentration of 0.28 g. per 100 ml. when the plasma level was 4.4 mg. per 100 ml. was 100 ml. per minute per 1.73 sq. metre, a value in good agreement with that found by Cusworth and Dent (1960). On the same occasion, the cerebrospinal fluid was found to be 9.5 mg. per 100 ml., a value more than twice that of the plasma, again agreeing well with those found by Cusworth and Dent (1960) in the original cases. It is also of interest to note that the level of glutamine in the cerebrospinal fluid was 8 mg. per 100 ml., i.e. within normal limits. Visual assessment showed that the plasma amino acid levels, other than argininosuccinic acid, were also within normal limits.

Effect of Protein and Amino Acid Intake on Argininosuccinic Acid Excretion

It has been shown (Ratner *et al.*, 1953) that argininosuccinic acid is an intermediate in the ornithine cycle, which it is generally believed is responsible for most, if not all, the urea synthesized in the body, with the liver as the main site of its production. It is reasonable to assume that in our patient argininosuccinic acid is implicated in the mental retardation. By analogy with phenylketonuria, rational therapy, as already stated, would involve a decrease in the formation of argininosuccinic acid and for this reason the effect of alteration in protein intake on its production as measured by excretion in the urine was investigated. The results are shown in Table 2.

A diet completely free from protein, but adequate in calories, resulted in a fall of argininosuccinic acid output from an initial level of 3.0 g. per day to about 0.6 g. per day (20% of initial level) by the seventh day. During the same period, urea excretion fell from 5.4 g. to 0.3 g. per day, i.e. 6% of initial level, a much greater fall than that of argininosuccinic acid. Whereas, on his normal diet, argininosuccinic acid nitrogen constituted less than 17% of the total urinary nitrogen, on the protein-free diet it constituted as much as 43%.

A comparable effect was observed when, in attempting to assess the effect of elimination of arginine from the diet, his normal feeds were replaced by a synthetic amino acid mixture, containing only the eight essential amino acids. The protein intake was only about 10 g. per day, very much less than on his normal feeds. Again, the daily excretion of argininosuccinic acid was considerably diminished compared with that on an ample protein intake and the ratio of argininosuccinic acid nitrogen to urea nitrogen and total nitrogen excreted were both greatly increased and were similar to those found when protein was completely eliminated from the diet.

When he was fed casein hydrolysate from which about half the arginine had been removed, again the protein intake (15 g. per day) was less than on his normal feeds. The total argininosuccinic acid excretion was a little higher than on his synthetic amino acid diet, although less than on his normal diet. The proportion of argininosuccinic acid nitrogen excretion to that of urea nitrogen or total nitrogen was less than on his synthetic amino acid feeds, and greater than that found when on normal diet. These results may be due to a combination of two effects, one due to the lowered protein intake, tending to raise the proportion of total nitrogen excreted as argininosuccinic acid and the other due to the lowered arginine intake, tending to lower the proportion of total nitrogen excreted as argininosuccinic acid.

The addition of, at first, 2 g. and afterwards 3 g. of arginine to his casein hydrolysate feeds, resulted in an increase of argininosuccinic acid excretion and in the proportion of argininosuccinic acid nitrogen to total nitrogen excretion (Table 2).

Discussion

In the ornithine cycle as modified by Ratner *et al.* (1953) and Ratner and Pappas (1949), citrulline

combines with aspartic acid by means of a condensing enzyme to form argininosuccinic acid and the cleavage of this substance to arginine and fumaric acid is reversibly catalysed by a splitting enzyme, argininosuccinase. The latter is present in mammalian liver, kidney and heart (Ratner *et al.*, 1953), and other organs, e.g. spleen, etc. (Walker, 1958), but the acid has not yet been found in plasma, cerebrospinal fluid or urine of man (Tomlinson and Westall, 1960), although presumably it must occur, if only transiently, in tissue cells. The presence in this patient of argininosuccinic acid in relatively high amounts suggests that the defect lies in an absence of the splitting enzyme, argininosuccinase.

That the deficiency is an inherited genetic disorder is suggested by the fact that in our case the anomaly was present at least 23 days after birth and also that the two previously reported cases (Allan *et al.*, 1958) were sibs. The level of urea in the blood was within normal limits and varied with protein intake, so that the capacity to synthesize urea was present. The amount of urea excreted was too great to be accounted for by its derivation solely from the arginine of the dietary or endogenous protein. For example, on one day during which the infant was on casein hydrolysate feeds, he excreted about 1.5 g. urea which, if it were all derived from arginine, would mean an intake of 4.3 g. arginine; he was actually receiving 0.39 g. arginine per day. It must be concluded, therefore, that most of the urea formed is derived from a urea cycle, presumably in the liver.

Allan *et al.* (1958) have suggested, on the basis of the higher levels of argininosuccinic acid in the cerebrospinal fluid compared with those in the plasma—a result which we also found in our case—that this metabolite is formed in the brain and diffuses into the cerebrospinal fluid; this suggestion received support from the recent work of Sporn, Dingman, Defalco and Davies (1959a, b and c) who showed that urea synthesis occurred in rat brain *in vitro*, contrary to the previous belief that urea was formed solely in the liver. Further, argininosuccinase has been found by Walker (1958) in the brain of the dog and by Ratner (private communication quoted by Tomlinson and Westall, 1960) in the brain of rats, steers and monkeys. This has also been demonstrated by Tomlinson and Westall (1960) who found evidence of enzyme activity in rat brain and other organs.

If the suggestion by Allan *et al.* (1958) is correct, it leads to the conclusion that there is an inherited enzyme deficiency present in the cells of one organ in the body, but not in another. It has been postulated (Landing, 1960), however, that in here-

ditary metabolic diseases the gene abnormality must be present in all cells in the body from birth and it is difficult to see how this could be reconciled with the foregoing conclusion. One possibility may be that the biosynthesis of urea in the liver in these cases is accomplished not by the ornithine cycle, but by another, normally little used. For example, Bach (1939) has presented some evidence that glutamic acid could take up ammonia to form glutamine which can combine with a further molecule of ammonia and carbon dioxide to yield glutamic acid and urea. Whether this is correct or not, it is not impossible that other cycles for urea synthesis exist in the body. On this supposition, argininosuccinic acid would be produced in our patient wherever the ornithine cycle should normally function, e.g. in the liver and in the brain. The lower argininosuccinic acid level in the blood compared with that in the cerebrospinal fluid would then be due to the rapid clearance of this substance by the kidney.

Another possibility is suggested by the fact that the cleavage of argininosuccinic acid is a reversible reaction and the reverse step may be necessary to provide argininosuccinic acid for other metabolic pathways (Ratner *et al.*, 1953) and it is the alternative pathway for argininosuccinic acid which is blocked, allowing the acid to accumulate.

The effect of variation in protein intake is interesting. As might be expected, when a protein-free diet was given, the excretion of argininosuccinic acid was considerably reduced, but not completely abolished and, in fact, it formed a considerably higher proportion of the total nitrogen excretion than when the patient was on a normal diet. That is, in protein deprivation, relatively more nitrogen is deviated to the synthesis of argininosuccinic acid than to the synthesis of urea, the amount of argininosuccinic acid nitrogen excreted falling not far short of that of urea nitrogen. If this is occurring in the brain, it suggests that this is a more essential cycle than that forming urea in the liver. It is interesting to note that during this time the liver diminished almost to normal size. This may have been due more to the lack of protein in the diet than to a restoration of the condition of the liver to normal.

Since ornithine and citrulline are not present in casein or in a normal diet, it was logical to attempt to reduce the formation of argininosuccinic acid by a reduction of arginine in the feeds, despite the fact that this acid is not the immediate precursor of ASA in the ornithine cycle. This was done as described above by oral feeds of a solution containing in suitable proportions the eight essential amino acids with additional glycine. Although

there was some reduction in the total amount of argininosuccinic acid excreted per day, this may have been largely due to the fact that the total protein intake was much less than on his normal feeds, similar to the effect found then on a protein-free diet. The liver this time was not reduced in size, in fact it became larger, suggesting that the reduction of ASA formation and excretion was not directly connected with the size of the liver. Again the child's condition deteriorated and did not improve until the amino acid feeds were withdrawn and normal feeding resumed.

Later, a casein hydrolysate mixture from which much of the arginine had been removed became available. On these feeds, daily ASA excretion started to rise above that on the mixture of synthetic amino acids, although less than when on a normal high protein diet. When arginine was added to the casein hydrolysate, ASA excretion was further increased, as was the proportion of ASA nitrogen excretion to total nitrogen excretion. Although Westall (1960b) concluded from his feeding experiments that restriction of arginine intake would not be of much value, our results point to a different conclusion. The different result in our patient is probably due to the relatively greater amount of arginine, 3 g. daily, added to his feeds which contained only 0.39 g. per day, whereas in Westall's patient, an 8-year-old boy, 2 g. arginine were added to a basic protein intake of 30 g. daily, containing about 1.2 g. arginine. However, some of our results are based on the analysis of the single day's excretion and must be assessed with caution as the differences are relatively small and there are, in any case, appreciable daily variations in the amounts of ASA excreted.

During this period of feeding, the child's liver altered little in size, again suggesting that there is little relation between ASA formation and the size of the liver. As on both previous occasions when he had been taken off his normal diet, on this occasion also, he became ill towards the end of the feeding experiment. The fall in plasma phosphorus level from 4.8 to 1.7 mg. per 100 ml. was almost certainly due to the continued low intake of phosphorus from the casein hydrolysate.

Microscopic examination of the hair revealed trichorrhexis nodosa, but whereas the breaks in the hair due to the more usual form of this condition fluoresce green with acridine orange, in our patient the fluorescence was red. An identical finding was present in the two original children with argininosuccinic aciduria (Allan *et al.*, 1958; Jarrett and Dent, personal communication). It is now obvious that the hair anomaly forms part of the condition

and is connected with the metabolic abnormality. Since arginine forms an important constituent of the hair keratin, it seems possible that the failure to form arginine from ASA leads to a deficiency of arginine with the formation of an abnormal hair keratin.

Summary

An infant who had, in the first week of life, a sudden onset of severe illness with abdominal distension, gross liver enlargement, blood-stained vomiting and a period of unconsciousness, was found to be excreting large amounts of argininosuccinic acid (ASA), an intermediate compound in the biosynthesis of urea. His subsequent history has been of mental and physical retardation, persistent liver enlargement, skin lesions and episodes of convulsions, or loss of consciousness. The clinical features of the present case are compared with those of the two previously reported cases in one family (Allan *et al.*, 1958). An identical hair anomaly was found in all three cases.

The level of ASA in the cerebrospinal fluid was higher than that in the plasma, whilst the blood urea was normal. Reduction of protein intake apparently resulted in the reduction of ASA formation, and addition of arginine to the feeds gave increased ASA excretion. Although the accumulation of ASA in the cerebrospinal fluid and a blood urea within the normal range might be explained by a genetic deficiency of argininosuccinase in the urea cycle in the brain, this postulate would necessitate a genetic defect in the cells of only one organ, other cells being normal. It is therefore suggested that in these cases urea is synthesized in the liver by a cycle other than the normal one involving ASA or, alternatively, ASA accumulates because of a defect in metabolic pathways other than the biosynthesis of urea.

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REFERENCES

- Allan, J. D., Cusworth, D. C., Dent, C. E. and Wilson, V. K. (1958). A disease, probably hereditary, characterised by severe mental deficiency and a constant gross abnormality of aminoacid metabolism. *Lancet*, **1**, 182.
- Bach, S. J. (1939). Experiments on the metabolism of glycine. *Biochem. J.*, **33**, 90.

- Cusworth, D. C. and Dent, C. E. (1960). Renal clearance of amino acids in normal adults and in patients with aminoaciduria. *Ibid.*, 74, 550.
- Heilmann, J., Barrolier, J. and Watzke, E. (1957). Beitrag zur Amino Säurebestimmung auf Papierchromatogrammen. *Hoppe-Seyl. Z.*, 309, 219.
- Landing, B. H. (1960). Hereditary metabolic disease. *Metabolism*, 9, 198.
- Levin, B., Mackay, Helen M. M., Neill, Catherine A., Oberholzer, V. G. and Whitehead, T. P. (1959). Weight gains, serum protein levels and health of breast fed and artificially fed infants, full term and premature. *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 296. H.M.S.O., London.
- Ratner, S. and Pappas, A. (1949). Biosynthesis of urea: Part I—Enzymatic mechanism of arginine synthesis from citrulline. *J. biol. Chem.*, 179, 1183.
- , Petrack, B. and Rochovansky, O. (1953). Biosynthesis of urea: Part V—Isolation and properties of arginino-succinic acid. *Ibid.*, 204, 95.
- Sporn, M. B., Dingman, W., Defalco, A. and Davies, R. K. (1959a). A method for studying metabolic pathways in the brain of the intact animal. *J. Neurochem.*, 4, 141.
- , —, —, — (1959b). The synthesis of urea in the living rat brain. *Ibid.*, 5, 62.
- , —, —, — (1959c). Formation of urea from arginine in the brain of the living rat. *Nature (Lond.)*, 183, 1520.
- Tomlinson, S. and Westall, R. G. (1960). Argininosuccinase activity in brain tissue. *Ibid.*, 188, 235.
- Walker, J. B. (1958). Role for pancreas in biosynthesis of creatine. *Proc. Soc. exp. Biol. (N.Y.)*, 98, 7.
- Westall, R. G. (1960a). Argininosuccinic aciduria. Identification of the metabolic defect—A newly described form of mental deficiency. *Proceedings 4th Int. Cong. Biochem. Vienna, 1958*, 15, Abstracts No. 13-34, 168. Pergamon, London.
- (1960b). Argininosuccinic aciduria—Identification and reactions of the abnormal metabolite in a newly described form of mental disease, with some preliminary metabolic studies. *Biochem. J.*, 77, 135.