AMINO-ACID TOLERANCE CURVES AND AMINO-ACIDURIA IN COOLEY'S AND SICKLE-CELL ANAEMIAS

BY

C. CHOREMIS, K. KIOSSOGLOU, F. MAOUNIS, AND B. BASTI

From the Paediatric Clinic, Athens University

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This study was prompted by the previous finding of amino-aciduria (Choremis, Zannos, and Basti, 1957) and qualitative and quantitative changes of serum proteins in thalassaemia and sickle-cell anaemia (Allamanis, 1955; Afentaki, 1949; Minnich, Na-Nakorn, Chongchareonsuk, and Kochaseni, 1954). The electrophoretic pattern shows a fall in albumin, a moderate fall in the α_1 , α_2 , and β globulin fractions and an increase in the γ globulin fraction (Allamanis, 1955). In some cases liver function tests (thymol turbidity and cephalin-cholesterol flocculation tests) are positive (Choremis and Kyriakidou, 1954; Minnich *et al.*, 1954). This abnormal electrophoretic pattern, and especially the increase in γ globulin, does not necessarily indicate liver damage, although this may occur.

In order to elucidate further the amino-acid metabolism in Cooley's and sickle-cell anaemia total plasma amino-acids and urine paper chromatography studies for amino-acids were undertaken after oral administration of protein and protein hydrolysate, as well as after intravenous injection of amino-acids. In this way the proteolytic activity of the gastro-intestinal tract, the absorption of amino-acids, their intermediate metabolism and their rate of disappearance from the blood stream, and especially their excretion through the kidneys, were studied.

There are no references in the available literature to any study on the metabolism of amino-acids in thalassaemia and sickle-cell anaemia.

In a previous paper, the above observations stimulated an investigation of another aspect of protein metabolism in these anaemias, namely, the amino-acids in blood serum and their excretion in the urine (Choremis *et al.*, 1957). A few reports have appeared, however, dealing with amino-aciduria in other types of anaemia. Souchon and

Grunau (1952) described one case of congenital spherocytic anaemia and cholelithiasis with amino-aciduria which was considered to be due both to liver damage and to faulty reabsorption by the tubules. Weaver and Neill (1954) reported five cases of pernicious anaemia with abnormal excretion of taurine and some overexcretion of lysine, leucine, and cystine. This type of amino-aciduria was attributed to lack of vitamin B₁₂ which is known to be involved in methyl synthesis and transmethylation of certain amino-acids. The same authors also reported five cases of other varieties of anaemia without any pathological findings. Pare and Sandler (1954) described 12 cases of March haemoglobinuria with amino-aciduria. In the above cases the renal tubular defect was considered to be the cause of the abnormal amino-aciduria (overexcretion of cystine in all cases and of β -amino-isobutyric acid in 11 of the 12 cases).

Material

Ten patients with Cooley's anaemia, aged 6 months to 4 years, with haemoglobin levels between 3.4 and 9.3 g.% and red blood cell counts of 1,260,000 to 3,100,000 per c.mm. were examined. Blood urea was normal in all cases, ranging from 16 to 32 mg./100 ml. Thymol turbidity and cephalin-cholesterol flocculation tests were negative in eight of the 10 cases. Seven of the patients had recently had blood transfusions. Two had undergone splenectomy more than two years previously.

The second group was of 10 cases of sickle-cell anaemia, aged 3 to 12 years, with haemoglobin levels between 3 and 8.6 g.% and red cell counts of 860,000 to 2,500,000 per c.mm. The blood urea level was normal in all cases, ranging from 19 to 35 mg. per 100 ml. Thymol turbidity and cephalin-cholesterol flocculation tests were negative in most cases. Five of the patients had recently had blood transfusions. Three had undergone splenectomy more than one year before.

Ten normal children, aged 6 months to 8 years, were used as controls.

No glycosuria was found in any of the three groups.

Method

The plasma amino-nitrogen was determined by the chromatographic method of Frame, Russell, and Wilhelmi (1943), as modified by Fister (1948). The accuracy of the method was assessed by doing 200 determinations on four samples of known amino-N content (4, 8, 12, and 16 mg.).

For the study of urinary amino-acids paper chromatography as described by Consden, Gordon, and Martin (1944) and Dent (1947, 1948) with a slight modification was used.

Whatman No. 3 filter paper was used for the urine chromatograms and a volume of urine containing 500 μ g. of nitrogen. The urine was first treated with ammonium molybdate and nitrogen peroxide on the paper, after which two-dimensional ascending chromatography was carried out employing phenol-water as the first solvent and pyridine-*amyl*-alcohol-water as the second. Sodium cyanide and ammonium hydroxide were added to the phenol box and diethylamine to the pyridine box. Subsequently, the papers were dried, sprayed with a 0.1% ninhydrin solution and dried at room temperature for 24 hours. The amino-acid spots thus developed were identified according to their position on the paper. Their colour intensity was compared with the test spots of pure taurine.

Although two-dimensional paper chromatography is very useful for qualitative determination, it does not otherwise provide an accurate quantitative basis for the estimation of amino-acids due to the varying sensitivity of the latter to ninhydrin. More accurate quantitative estimations of amino-acids by microbiological assays or chemical methods (Van Slyke) were not undertaken in the present study.

Plasma amino-N determination and urine paper chromatography studies for amino-acids were run for all three groups after administering (a) calcium caseinate ("casec") orally, (b) protein hydrolysate ("nesmida") orally, and (c) casein hydrolysate ("amigen") by intravenous injection.

The experiment was carried out as follows:

(a) *Oral Calcium Caseinate*.—The children were starved for 12 hours, after which the first sample of blood was taken. Then a protein meal was given by gavage, consisting of calcium caseinate, which contains 88% protein. The amount given was 1.72 g. (=1.5 g. of protein=235 mg. of nitrogen) per kilogram of body weight diluted in 30 ml. of water and 5% sugar added. Subsequent samples of blood were taken 30, 75, 150, and 240 minutes after the completion of gavage.

Urine was collected before the gavage of the test meal as well as between 0 and 75, 75 and 150, and 150 and 240 minutes after the test meal.

(b) *Oral Protein Hydrolysate*.—The experiment was carried out as previously described, the only difference being the use of powder protein hydrolysate, which contains 88% protein. The amount given was 2 g. (=1.5 g. of protein=230 mg. of nitrogen) per kilogram of body weight.

(c) *Intravenous Casein Hydrolysate*.—Amigen, 3 ml. per kg. body weight, was injected. The subsequent samples were taken from a different vein five, 15, 30, and 90 minutes after the injection had been completed.

Urine was collected before the injection as well as between 0 and 15, 15 and 30, and 30 and 90 minutes after it.

During the above period no food was given to the children except water, and any vomiting or diarrhoea, even of the slightest, cancelled the test for that day.

Children with fever or diarrhoea, or who had been given sulphonamide drugs for the last three days preceding the experiment, were excluded.

Results

The mean amino-nitrogen curves after the ingestion of calcium caseinate are shown in Fig. 1.

As can be seen in all tested children, there was an increase of the plasma amino-N levels of approximately 2 mg. per 100 ml. above the fasting level in at least one post-prandial sample. No difference has been noted in the plasma amino-N levels 75 minutes after the ingestion of calcium caseinate between the healthy controls and patients with Cooley's or sickle-cell anaemias. In most cases the highest level of the plasma amino-N was 75 minutes after the test meal. The plasma amino-N level declines gradually after 75 to 150 minutes in healthy controls, whereas in Cooley's and sickle-cell anaemias the fall is abrupt.

It was finally noted that 240 minutes after the protein meal the mean plasma amino-N value in healthy controls did not approach the original fasting level; this might be due to continuation of hydrolysis of the intact protein from some time after the four-hour test period. On the other hand, in Cooley's and sickle-cell anaemias, the mean amino-N value did approach the original fasting level 240 minutes post-prandially.

Fig. 2 is a histogram of the elimination of urinary amino-acids after the ingestion of calcium caseinate.

The greatest elimination of amino-acids is observed, as a rule, between 75 and 150 minutes after the ingestion of calcium caseinate; in normal children (13–15 spots of amino-acids, with colour intensity ranging between that given by 10 and 80 g. of taurine), and in those suffering from Cooley's or sickle-cell anaemias (13–18 spots of amino-acids, with colour intensity ranging from just above normal to considerably over the intensity

FIG. 1.—Mean amino-nitrogen curves after the ingestion of casein.

given by 100 μ g. of taurine), corresponding to the fall in the plasma amino-N curve. Amino-aciduria appears to be more marked in Cooley's and sickle-cell anaemias.

From 150 to 240 minutes after the ingestion of the test meal the amino-aciduria decreases in normal controls (12–14 spots of amino-acids with colour intensity ranging between that given by 10 and 60 μ g. of taurine), whereas patients with Cooley's and sickle-cell anaemias continue to excrete increased amounts of amino-acids (13–18 spots with colour intensity ranging between that given by 10 and 100 μ g. of taurine).

Fig. 3 demonstrates the mean amino-nitrogen curves after the ingestion of protein hydrolysate.

As can be seen, all children showed a sharp rise in the plasma amino-N level 30 minutes after the test meal. In most cases the fasting level is exceeded by 100%. This elevation is consistent and more pronounced in the second post-prandial sample. No difference between the three groups was noted in the plasma amino-N levels 75 minutes after the ingestion of protein hydrolysate. The peak of the curve was found 75 minutes after the test meal.

The plasma amino-N level declines gradually after 75 to 150 minutes in healthy controls (still exceeding twice the fasting level), whereas in

Cooley's or sickle-cell anaemias a sharp fall is noted.

It was finally noted that 240 minutes after the test meal the original fasting level is again approached in all the three groups. Values below the fasting level are designated as negative quantities.

Fig. 4 is a histogram of urinary amino-acid elimination after the ingestion of protein hydrolysate.

In healthy controls the most striking increase in urinary amino-acids occurs from 75 to 150 minutes from the test meal (13–17 spots of amino-acids, with colour intensity ranging between that given by 10 and 80 μ g. of taurine); amino-aciduria continues beyond the 150 minutes, but to a lesser degree. On the other hand, in cases of Cooley's or sickle-cell anaemias, the greatest urinary output of amino-acids is between 0 and 75 minutes from the ingestion of the test meal (15–17 spots of amino-acids with colour intensity ranging from just above normal for considerably over the intensity given by 100 μ g. of taurine); amino-aciduria continues beyond the 75 minutes and remains constantly increased, even after four hours.

Fig. 5 gives the mean amino-nitrogen curves after the intravenous administration of casein hydrolysate.

FIG. 2.—Histogram of elimination of amino-acids after the ingestion of protein (calcium caseinate).

FIG. 3.—Mean amino-acid curves after the ingestion of casein hydrolysate.

As can be seen in all tested children, the amino-N level reaches its highest value at the completion of the injection of casein hydrolysate and then starts declining, so that in the five-minute sample the increase is 75% above the initial level. The decline continues progressively in healthy controls, whereas in patients with Cooley's or sickle-cell anaemias a sharp fall in the plasma amino-N level is noted.

Finally a return of the amino-acidaemia to fasting levels is noted in all the three groups after 90 minutes, in some instances to substantially lower than the fasting level, but this is not statistically significant. It is possible that this latter effect is referable to a synthetic process which requires some of the original amino-acid supply of the body (Harper, 1949).

Fig. 6 is a histogram of the elimination of urinary amino-acids after the injection of casein hydrolysate.

In healthy controls the highest degree of elimination was noted 15 minutes after the intravenous injection of casein hydrolysate (16–18 spots of amino-acids, with colour intensity ranging between that given by 10 and 90 μ g. of taurine); amino-aciduria still continues even 90 minutes from injection but to a lesser degree.

In Cooley's and sickle-cell anaemia the highest degree of amino-acid elimination was sooner than that noted in the cases of normal children, occurring within 15 minutes from the injection of casein hydrolysate (15–18 spots of amino-acids, with colour intensity ranging from just above normal for considerably over the intensity given by 100 μ g. of taurine). Amino-aciduria has already returned to the pathologically increased fasting levels in 90 minutes.

Discussion

Choremis and his associates (1957) showed that amino-aciduria without hyperamino-acidaemia is a constant finding in Cooley's and sickle-cell anaemia; it was also speculated that the amino-aciduria was due to a defective reabsorption of amino-acids in the renal tubules because of the possible damaging effect of haemosiderin.

The distinction between the metabolic and renal mechanism of amino-acidurias is very difficult. The most satisfactory way to prove the nature of an amino-aciduria is, according to Dent (1954), by estimating the individual amino-acid clearance. Since such a procedure could not be undertaken owing to technical difficulties, it was thought that the probable defective reabsorption of amino-

FIG. 4.—Histogram of the elimination of urinary amino-acids after the ingestion of casein hydrolysate.

FIG. 5.—Mean amino-nitrogen curves after the intravenous administration of casein hydrolysate.

acids in the renal tubules could be studied as described above. By the same procedure, incidentally, it was possible to study both the proteolytic activity of the gastro-intestinal tract and the absorption of amino-acids. It was clearly shown that the latter functions were perfectly normal in patients with Cooley's and sickle-cell anaemia.

It is well known that when hyperaminoacidaemia occurs, especially after the rapid intravenous administration of amino-acids (Harper, 1949), some amino-acids escape the reabsorbing capacity of the renal tubules. After loading the patients of all three groups with oral calcium caseinate and protein hydrolysate and intravenous casein hydrolysate, amino-acids were excreted as expected; however, in the patients with Cooley's and sickle-cell anaemia the amino-aciduria was far more excessive (both quantitatively and qualitatively) than in the controls, indicating a possible defective reabsorption in the renal tubules. This excessive amino-aciduria was also reflected in the total plasma amino-N concentrations, which declined faster and more abruptly in the patients than in the controls, following both the oral and intravenous loading with protein and protein hydrolysate.

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The "renal" versus the "overflow" nature of the amino-aciduria in such patients seems to be fairly certain; it is also quite probable that the increased glomerular filtrate (Papamatheaki, 1954) in patients with congenital haemolytic anaemias contributes little if anything to the degree of amino-aciduria, which is mainly due to a defective reabsorbing capacity of the renal tubules.

However, the exact mechanism of the amino-aciduria is not known; nevertheless it may be speculated that, as in most cases with congenital defects of the renal tubules, an enzymic dysfunction might be responsible for the amino-aciduria (Bickel, 1956; Fanconi, 1954, 1956; Jackson and Linder, 1953; Payne, 1956).

Conclusion

The increased elimination of amino-acids coupled with the abrupt fall of the plasma amino-N curve indicates that in Cooley's and sickle-cell anaemias the main error in handling amino-acids lies in their decreased kidney reabsorption. An enzymic dysfunction might be responsible.

FIG. 9.—Histogram of the elimination of urinary amino-acids after the intravenous injection of casein hydrolysate.

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