The Amino-aciduria in Fanconi Syndrome. A Study Making Extensive Use of Techniques Based on Paper Partition Chromatography

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It has long been known, and confirmed by direct isolation experiments (Honda, 1922, 1923; Wada 1930), that small quantities of many amino-acids are normally excreted in the urine, but that their excretion in large amounts occurs only in rare pathological conditions. The term amino-aciduria is reserved for such cases. It was first described in acute yellow atrophy of the liver, where its onset coincides with a large rise in the blood level of amino nitrogen and is presumably due to a simple overflow from the blood into the urine.

The reported occurrence of amino-aciduria in the Fanconi syndrome (Fanconi, 1936; McCune, Mason & Clarke, 1943) is of distinct interest because in this syndrome any possible relationship to acute yellow atrophy can be excluded. The mechanism of the amino-aciduria was therefore likely to be entirely different, and although it was suggested that a low kidney threshold for amino-acids may be the causative factor, no systematic work was carried out. The blood level of amino nitrogen was not determined and moreover the earlier workers seem to have thought that the amino-acids in the urine could be titrated quantitatively by the method for organic acids of Van Slyke & Palmer (1920).

The literature concerning the syndrome is surveyed by McCune *et al.* (1943). The syndrome is of unknown etiology and usually presents as typical rickets in a child of 2–5 years, although cases in adults presenting as osteomalacia have also been reported. The bony lesions are not cured by normal doses of vitamin D. In addition the patients have renal glycosuria and the constituents of the blood show changes which clearly distinguish the disease from renal rickets (renal hyperparathyroidism), namely, low inorganic phosphate and a normal blood urea and non-protein nitrogen. The alkaline phosphatase is usually raised and the alkali reserve reduced. The urine has a high ammonia coefficient. Cirrhosis of the liver and generalized cystinosis may be found at post-mortem. The close resemblance to the syndrome described by Milkman (1930, 1934) should be noted.

It was thought profitable to study further the amino-aciduria in Fanconi syndrome. The introduction by Consden, Gordon & Martin (1944) of a comparatively simple technique for the detection of amino-acids has provided a most effective weapon for the purpose. It has already been shown to be applicable to pathological specimens of urine and other body fluids (Dent, 1946*a*). The chronic loss of amino-acids in the urine might be the cause of some of the symptoms, or if it were merely the result of a low renal threshold in the presence of normal amino-acid metabolism then such a patient would enable information to be obtained with comparative simplicity as to the normal amino-acids present in blood under various conditions. At the onset of the investigation certain specific points were set out for study:

(1) What is the nature and extent of the amino-aciduria?

(2) What is the mechanism of excretion?

(3) Is there any relation between the amino-aciduria and the renal glycosuria?

(4) Is the amino-aciduria the primary cause of the disease?

(5) Is there any disturbance in sulphur metabolism such as might account for the cirrhosis of the liver or the cystinosis?

Except for (4), these questions have been answered fairly satisfactorily. A preliminary report of some of the results described here has already appeared (Dent, 1946*b*).

EXPERIMENTAL

Patients studied

Full clinical details of the patients E.C., V.R. and E.B. will be reported elsewhere (Stowers & Dent, 1947; Hunter, 1947). All showed the typical features of the disease. E.C. and V.R. who came to post-mortem showed marked cirrhosis of the liver with recent primary carcinoma. V.R. in addition had multiple metastases in the lungs and many other organs. E.C. had had repeated haematemeses from oesophageal varices and died in liver coma. E.B. has signs of increasing portal obstruction but is otherwise fairly well.

Patient E.C. This patient (age 34) has provided nearly all the material for this study. Three 24 hr. specimens of urine had been collected from him

during the previous Oct., Nov. and early Jan. when he was attending as an outpatient. He was given, on admission in late Jan. a high protein diet with extra milk. However, frequent attacks of vomiting interfered drastically with his intake. It was only possible to estimate his daily protein intake for one week in Jan. Urines were collected from midnight to midnight by the nursing staff till 4 Feb. 1946, when the more reliable method of collecting the urine directly by means of a funnel connected to a Winchester quart bottle under the bed was instituted. Thymol was used as a preservative till 25 Jan. 1946 when the frequent occurrence of bacterial infection led to 50 ml. of approx. 1·5 N-HCl being used. The acid was placed in the bottle and the urine added as passed. The completed specimens were kept at 4°. According to Van Slyke, MacFadyen & Hamilton (1943), no loss of amino nitrogen occurs during 5 months at 4° with only thymol as a preservative.

The amino-acids were given by mouth, suitably flavoured and suspended or dissolved in water, at 4 a.m. after the patient had emptied his bladder. The urine was then collected 3 and 6 hr. afterwards. The rest of the day's urine was collected separately with the small amount, if any, of the urine voided at 4 a.m. added to it.

Patient V.R. The urine that could be collected from this patient during the last 48 hr. was added as passed to 100 ml. approx. 1·5 N-HCl in the stock bottle. Owing to the small volume passed (730 ml.) the final acid concentration was much higher than originally intended. For various reasons the urine was allowed to stand at room temperature for a considerable time. The amino nitrogen was determined a few days after, but the chromatogram was not done till a month later.

Patient E.B. Four samples of urine passed on 7, 10, 11 and 13 Mar. 1946 were obtained. None contained albumin or bilirubin; all contained normal traces of cystine and urobilin. Reducing substances (Benedict's reagent) were found only in the urine of 13 March and this was the only urine containing an excess of amino-acids detectable by the one-dimensional chromatogram. The further investigations were therefore confined to this specimen of urine.

Methods

Chemical determinations

Amino nitrogen was determined in the urines by the formol titration method (Van Slyke & Kirk, 1933). The vacuum distillation with baryta was carried out for 30 min. and continued longer if the distillate was still alkaline, as it often was.

Amino nitrogen was determined (by Miss E. Richardson) in the serum by the ninhydrin CO_2 method.

Nitrogen was determined throughout by the micro-Kjeldahl method.

Inorganic and ethereal sulphate were determined by the standard gravimetric methods (Peters & Van Slyke, 1932), and neutral sulphur by Benedict's method, except that after 2 Feb. 1946, nitric and perchloric acids were used as the oxidizing mixture (Pirie, 1932), because this is more reliable for determining all the sulphur in methionine.

Methionine was determined by the hydrogen peroxide oxidation method of Toennies & Callan (1939) as applied to urine by Albanese, Frankston & Irby (1944).

Cystine was regularly tested for by the qualitative cyanide-nitroprusside reaction (Brand, Harris & Biloon, 1930). Usually the test gave faint colours; occasionally definitely stronger than normal but never in any way comparable to the reaction obtained from known cystinurics. No quantitative methods were therefore applied.

Calcium was also tested for qualitatively by Sulkowitch's reagent. No excessive outputs were detected.

Inorganic phosphate was determined by uranium titration (Peters & Van Slyke, 1932) using Tinct. Cocci as internal indicator.

Glucose was determined by Benedict's volumetric method.

Partition chromatograms for detecting amino-acids

The method of Consden *et al.* (1944) as applied to urines by Dent (1946*a*) was used throughout, 25 μl. of urine being taken each time. Phenol was always used as the first solvent, 1 ml. of conc. ammonia (sp.gr. 0·880) being added to the cabinet. Collidine was used as the second solvent. The leading edge of the phenol always carried forward a great deal of dirty brown material, partly from the paper, partly presumably from decomposition of the phenol. This was always cut off with scissors before running the collidine in the other direction, as otherwise it interfered with the flow of the collidine. The strength of the ninhydrin colours was compared against an arbitrary colour standard, as soon as developed, owing to the marked fading which occurred in a few days. The scale used was divided into 10 units, 1 being a very faint colour, 10 a deep purple. The spots of rather different shade could only be roughly compared.

The identification of ninhydrin reacting substances on the chromatograms was attempted in the first place by comparing the positions of the various spots (R_F values) against a synthetic mixture of pure amino-acids run on another chromatogram simultaneously in the same cabinet, very good agreement being usually obtained (Dent, 1946*a*). In more doubtful cases the pure amino-acids were added to the urine and their superposition with the unknown on the developed chromatogram confirmed or otherwise. This was always necessary if only a few spots appeared, as in normal urines. The stability of the spots after hydrolysis of the urine had also to be confirmed. Confusion with a peptide is the only serious source of error, there being an almost infinite number of possible peptides while the number of pure amino-acids, i.e. spots persisting after hydrolysis, is strictly limited. The R_F values found here differ slightly from those reported by Consden *et al.* (1944), and are reported here together with many new values in Table 6.

The above methods were applied to the identification of the following amino-acids in the urine of E.C.: cysteic acid, aspartic acid, glutamic acid, serine, glycine, threonine, alanine, α-amino-*n*-butyric acid, histidine, citrulline, arginine, valine, leucine (and/or isoleucine), phenylalanine, tyrosine, proline, hydroxyproline. Unfortunately the

technique as here described does not distinguish between eucine and *iso*leucine, and further investigation is necessary before it is known which of these is present. Methionine travels to a position so close to that of the leucines that a confusion is possible unless both are present in sufficient strength as to be separately visible, as in the urines of E.C. after methionine feeding. The identification of these amino-acids was confirmed by treating the urine on the paper, before starting the chromatogram, with 10μl. of 30% H_2O_2 (perhydrol) which was then allowed to dry. This converted the methionine quantitatively into the sulphone which then appeared in a much less ambiguous position near valine. The leucines were unchanged and remained in their original position. The validity of this procedure was readily confirmed by using synthetic mixtures and by treating similarly the urine of E.C. after methionine feeding. The H_2O_2 treatment also served to show cystine, which it quantitatively converted into cysteic acid. Cystine usually decomposed completely during the chromatography; cysteic acid however is quite stable once formed. Possibly the H_2O_2 pre-treatment will eventually become the standard method for showing up on the one chromatogram all the amino-acids present in a mixture. Its use was avoided here in routine testing owing to the doubt as to the possible reactivity of H_2O_2 under these conditions with other amino-acids, especially with tryptophane (cf. Toennies & Homiller, 1942). Unfortunately, no α-amino-*iso*butyric acid was at hand for comparison; it is believed however that it would be distinguishable from the *n*-butyric derivative. The matching with the urine was done particularly carefully in this case.

The presence in the urine of histidine and tyrosine, both of which give weak reactions with ninhydrin, was readily confirmed in the present study by means of the one dimensional chromatogram run with collidine. Under these conditions histidine has an R_F value of 0·10–0·14 and tyrosine of 0·44. They are thus well separated on the paper strip and are readily developed by stroking the paper first with diazotized sulphanilic acid and then with sodium carbonate solution (Pauly, 1904) each from a small micro-pipette. It makes no difference if the strip has first been treated with ninhydrin. The diazo reaction is more sensitive than the ninhydrin method for these two amino-acids, histidine gives a bright red colour with 5μg. and tyrosine a golden brown with 20μg. (after running on the collidine strip). Normal urines show only a trace of histidine and no tyrosine; the urines of E.C. gave a much stronger histidine colour and a definite reaction for tyrosine. When a normal urine is concentrated to one-tenth vol. *in vacuo* it usually shows tyrosine by this method as well as another substance reacting to give a chocolate brown colour with the Pauly reagent, and having an R_F value of 0·64. This interesting substance has also been seen in much larger amounts in a pathological urine (liver disease) and would seem to repay further investigation. Another substance giving a bright red colour like histidine but with an R_F value of 0·30 has also been found in similar urines. Rough matching showed that this may be histamine. The collidine strip method is the most simple and specific general method known to the writer for the micro detection of histidine and tyrosine in the presence of each other.

The presence of methionine sulphoxide in the urine of E.C. was shown only after some difficulty and it is still not possible to say whether any of it is present as such in the fresh urine, or whether it has all been formed as an artifact by oxidation of methionine during storage of the urine or preparation of the chromatogram. The histidine spot often appeared to be double, the slower moving part in the phenol being greenish (like the colour given by histidine) and the faster part being purple. Careful matching experiments have shown that the faster part of the spot was due to methionine sulphoxide, which is not therefore clearly distinguishable on the chromatogram from histidine. The diazo reaction is also of value in this differentiation as, of course, only histidine gives a positive result. On the samples of urine from E.C. after methionine feeding it was thus possible to show that the large increase in size in the histidine + methionine sulphoxide spot was due entirely to the increased excretion of the latter compound, the histidine excretion remaining constant. At other times however the spot was chiefly contributed by histidine.

It was not possible to identify the substance called 'over-glycine' (Fig. 1). It was always very weak and so its appearance or otherwise in a given urine was within the

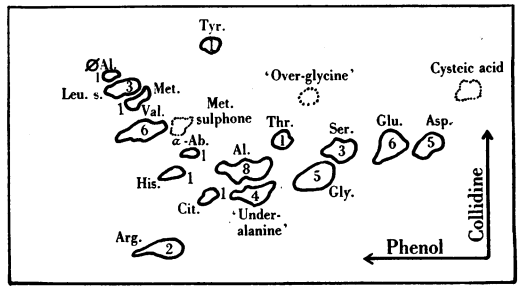


Fig. 1. Tracing showing the positions of the amino-acids in a typical urine of E.C. The figures inset represent relative colour strengths on an arbitrary scale. 'Over-glycine' is usually very weak. Methionine sulphone and cysteic acid are regularly seen if the urine is previously treated with H_2O_2, but the former is very weak except after methionine feeding. Methionine sulphoxide occurs just to the left of histidine. øAl. = phenylalanine. α-Ab. = α-amino-butyric acid.

range of experimental error. A very similar if not identical substance occurs in greater amounts in other pathological urines, and has been shown to be stable to hydrolysis. It is therefore believed to be due to an unknown amino-acid.

The rough quantitative estimation of amino-acids was done by matching the size and strength of the ninhydrin colours by trial and error against synthetic mixtures. The urine of E.C. passed on 7 Jan. 1946 was taken for this purpose. It is not claimed that the accuracy of this method is greater than ±30%. Only weak colours should be compared, the urine being diluted if necessary. Marked daily variations of the outputs of some amino-acids occurred.

For chromatography the serum or plasma (Fig. 2) was treated with 10 times its volume of 95% ethanol, filtered and finally concentrated to one-tenth of the original volume before taking 25μl. as usual for the chromatogram. This method was chosen in order to avoid more reactive protein precipitants which might hydrolyze peptides.

Many other substances previously reported to react with ninhydrin (e.g. ammonium salts, reducing agents) have

been run on the chromatograms but (except for β-alanine and glucosamine, see Table 6) no reaction resembling that obtained with α-amino-acids and peptides has been encountered.

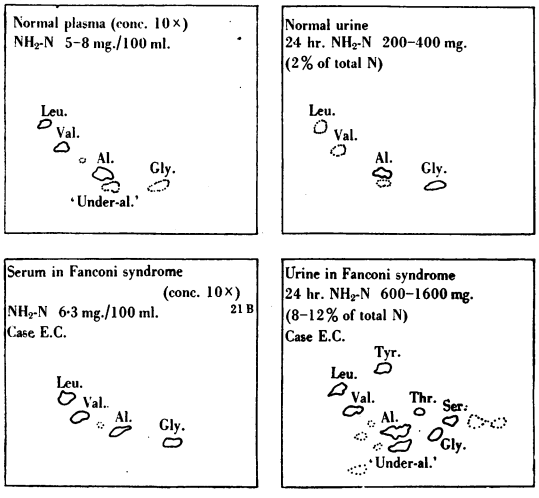


Fig. 2. Four tracings showing main amino-acids in plasma (or serum) and in the urine of normals and in Fanconi syndrome. The outline of the weaker spots is dotted. α-Amino-*n*-butyric acid is seen between alanine and valine.

It may be added here that the use of small squares of filter paper (12 × 12 cm.) shows promise as a rough method. Only 3 μl. of urine is required and they take only 2–3 hr. to run with each solvent.

Other urinary constituents on the chromatograms

To determine the movement of these on the chromatograms synthetic mixtures were made up of all the main constituents of normal urine, in the strength in which they usually occur (see Fig. 3) as well as of glucose in the concentration occurring in the urine of E.C. Each solution (25 μl) was then run for a distance of 25 cm. on the one dimensional chromatogram with phenol and again on another strip with collidine. The position, if any, taken up by the substance in question was determined by the use of specific developers. Sodium hippurate however could not be developed at the normally occurring strength so the concentration had to be increased to 1% (w/v). The developers are tabulated in the previous column. The results are shown on Fig. 3. In addition a separate set of phenol chromatograms of all the above constituents was run and the strips sprayed with ninhydrin and heated at 100°. No coloured bands of any kind appeared.

Substance	Method of development	Result
All chlorides	Dip into 0·1 N-$AgNO_3$, wash with distilled water, expose to light	Black band
Sulphate and phosphate	Dip into saturated basic lead acetate solution, wash, expose to H_2S	Brown band
Uric acid	Dip quickly into 1% ammoniacal $AgNO_3$	Black band
Glucose	Dip quickly in 1% ammoniacal $AgNO_3$, hang up in dark to dry, then heat to 100° for 10 min.	Black band
Creatinine	Picric acid, NaOH (Jaffé reagent)	Red band
Urea	Apply streaks of 10% $Hg(NO_3)_2$ to the paper, followed by streaks of 2 N-NaOH	Colourless band on grey background
Na hippurate	Observe in ultra-violet light	Yellowish white fluorescence

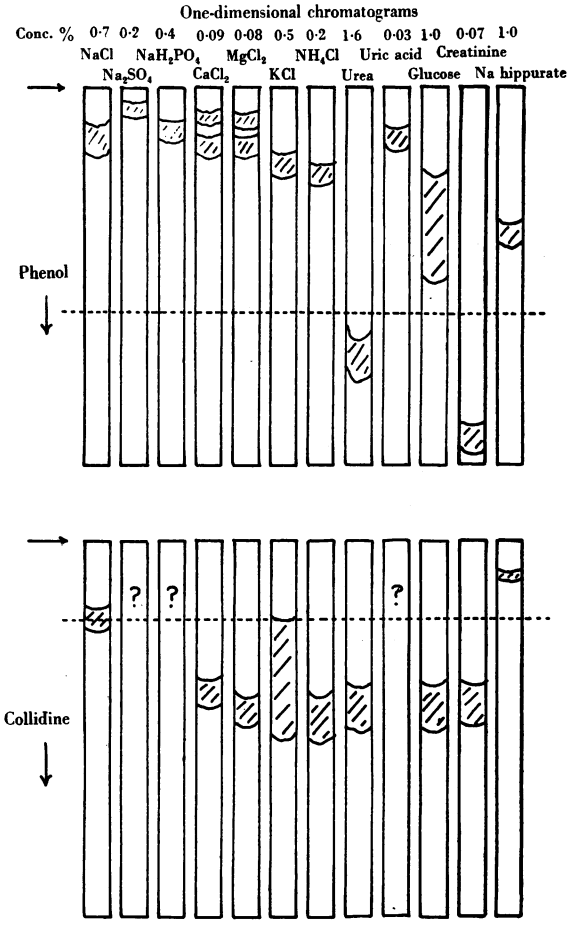
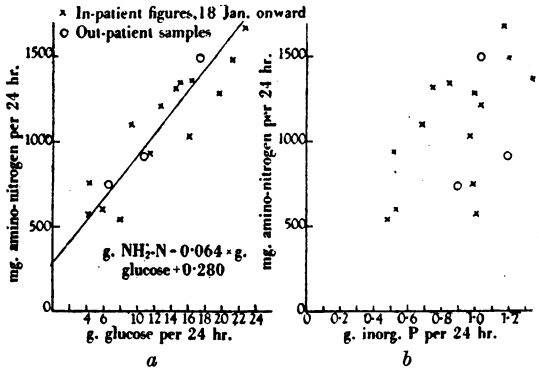


Fig. 3. These drawings are to scale with respect to the position and size of the bands. The substance in question has been placed at the top end of the strip and the solvent has moved to the extreme end of it. The three strips marked '?' gave no bands in collidine. The salts with divalent kations (Ca, Mg) gave double bands in phenol. The slower band in each case was the stronger and sharper. The theoretical position of 'under-alanine' in both chromatograms is marked with a dotted line.

It is interesting to remark here that many other substances in pathological urines have also been shown to move characteristically on the chromatograms, e.g. homogentisic acid, melanogen, a substance giving a red ultra-violet fluorescence resembling that of a porphyrin, and lactose.



Figs. 4*a* and *b*. Case E.C. Urine analyses.

Hydrolysis of urine

The urine was mixed with an equal volume of conc. HCl and refluxed for 24 hr. It was then evaporated to dryness *in vacuo* and again evaporated 3 times after addition of 10 ml. of water. The residue was made up to the original urine volume and 25 μl. taken for the chromatograms as usual. The coloured impurities present did not interfere in any way. More than the merest trace of protein was never present. No attempt was made to remove this first.

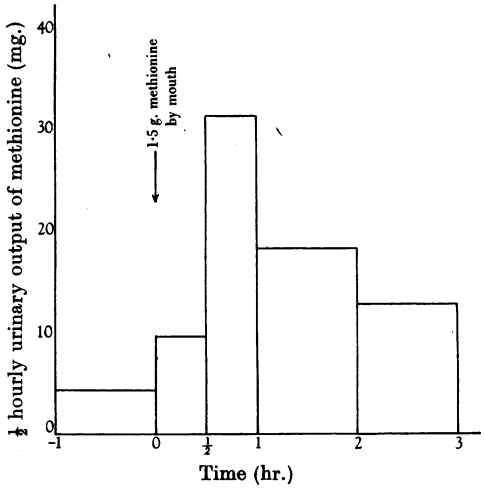


Fig. 5. Normal methionine saturation curve given by E.C.

Isolation and reactions of 'under-alanine'

Attempts to match this spot (see Fig. 1) with any known amino-acid or other substance which might react with ninhydrin to give a colour all failed. The substance gave a ninhydrin reaction slowly at room temperature. The colour closely matched the slightly reddish purple given by glycine. The possibility that the spot might be due to an artifact was considered since it usually has very much the same size and shape as the alanine spot just above it. However it always appeared when the chromatogram was repeated and whatever the order of the solvents used. Running the chromatogram in the presence of vapour of HCl or NH_3 had but little effect on the R_F values so the substance was not likely to be basic or acidic. Hydrolysis of the urine, however, caused its complete disappearance while all the other spots remained (Table 4). It must therefore be a peptide. From Fig. 1 it can be seen that its isolation would not be possible by a one-dimensional method using either of those solvents. From Fig. 3 it can be seen that none of the common urinary constituents move at the same speed in both solvents. They would not therefore contaminate the 'under-alanine' spot on the paper. It was decided to attempt the separation of this component by a two-dimensional method rather than experiment with unfamiliar solvents which would entail re-determination of all the R_F values in question. The following method was used:

On two large squares (22 × 18 in.) of No. 1 Whatman filter paper along a line 6 cm. from one long edge 28 spots, each from 25 μl. of the urine of E.C. passed on 7 Jan. 1946, were placed at regular intervals (see Fig. 6, A). The sheets

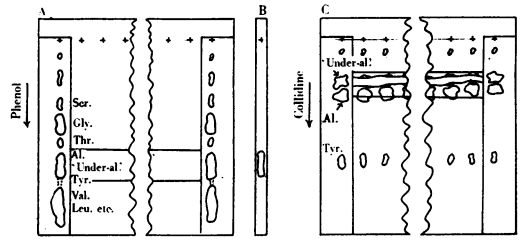


Fig. 6. The crosses in A represent places where 25 μl. of urine were placed, in B and C where the solution containing alanine, 'under-alanine' and tyrosine was placed after washing from the appropriate piece of A. The spots outlined represent amino-acids developed with ninhydrin to serve as markers. A and B were run with phenol, B serving to confirm lack of contamination with adjacent amino-acids. In C which was run with collidine the final isolation of pure 'under-alanine' was performed (see text).

were run with phenol in the chromatogram cabinet in the usual way. On one sheet three, and on the other four, strips of paper each corresponding to the run of a drop of urine were cut out, developed with ninhydrin and then reinserted into the sheets in their original positions, so as to serve as markers. The pieces of paper on the rest of the sheet corresponding to the developed positions of alanine, 'under-alanine' and tyrosine on the markers, could readily be marked out and cut off. The papers were washed by soaking water along them in the apparatus shown in Fig. 7 which was very convenient for the purpose. After some hours the washing was stopped and the paper dried and developed with ninhydrin to confirm removal of all the amino-acids. This always occurred readily and it is believed that these amino-acids moved forward almost as fast as the edge of advancing water. The combined wash waters were evaporated to dryness and made up to 525 μl. with distilled

water, i.e. to the volume of 21 spots of 25μl. of original urine. Of this 25μl. was run on a phenol strip (Fig. 6, B). It gave when developed with ninhydrin a single spot corresponding in position and intensity to the amino-acids cut out.

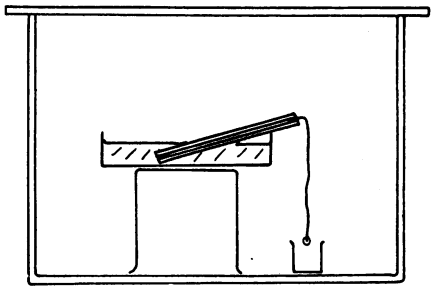


Fig. 7. The piece of filter paper containing the required amino-acids is pinched as shown between two pieces of glass resting in a Petri dish. The water in the dish rises by capillary attraction between the plates and is thus supplied to the filter paper. The wash liquor eventually drops off the end of the paper into the little beaker. Evaporation must be prevented by the larger outer vessel.

Two further sheets of paper (Fig. 6, C) were taken for the collidine run. The remaining 20 lots each of 25μl. of solution were placed parallel to the shorter edge of the paper so as to have a maximum length of run. After running with collidine to the full length of the paper it was removed and dried. The two lateral strips of each sheet were cut away, developed with ninhydrin and reinserted. This time the 'under-alanine' could not be cut away as before owing to the very small space, about 4 mm., between it and the alanine spot, this space often being curved. It was isolated as shown by cautiously cutting away strips of paper and developing with ninhydrin until the entire alanine spot and the two extreme edges of 'under-alanine' could be accounted for. This was the only stage at which any definite loss of material is believed to have taken place. The pure 'under-alanine' was washed off the paper as before. Evaporation of the solution left a minute residue of small stumpy needles not quickly soluble when they were made up to a volume of 400μl. (i.e. $16 \times 25\mu$l.) with water. This was the stock solution for the experiments described in the following paragraphs.

Check for purity. 25μl. of solution were run on the two-dimensional chromatogram. A spot corresponding exactly to 'under-alanine', but weaker than from 25μl. of original urine, was obtained together with the merest visible trace of glycine. Glycine is not likely to have been present from an error in cutting the chromatogram as it is some inches away. It is more likely to have appeared from decomposition of 'under-alanine'. The absence of any alanine as impurity was particularly gratifying.

Hydrolysis. 25μl. of solution were sealed in a capillary glass tube with 25μl. of conc. HCl. After 24 hr. in boiling water the liquid was transferred to a watch glass and evaporated *in vacuo*. This was repeated after addition to the residue of a drop of water. The crystalline residue was readily soluble in water. It was transferred to the appropriate position on a large square of filter paper and a two-dimensional chromatogram made. After development it showed two strong spots in the positions of serine and of glycine. The strengths of the spots corresponded to about 4–6μg. of serine and 5–9μg. of glycine. From these quantities found in 25μl. of original urine, the concentration of 'under-alanine' in the urine must be at least 400 mg./l. The molecular ratios are very close to 1 serine : 2 glycine.

Final identification of hydrolysis products. The above experiment was repeated with 4μg. of serine and 3μg. of glycine added to the mixture on the chromatogram. After development the sheet showed only serine and glycine, the markers having coincided exactly with the hydrolysis products.

End-group determination. This was done in the following three ways:

(*a*) 25μl. of solution were mixed with 5μl. of 0·1 N-$NaNO_2$ and 25μl. of conc. HCl, the latter being added very slowly. After 15 min. at room temperature, a small crystal of urea was added to destroy the remaining HNO_2 and the whole transferred to a sealed tube and heated at 100° for 16 hr. After removal of the acid as above it was run on the chromatogram. It showed one strong spot corresponding to about 5–9μg. of glycine.

(*b*) 25μl. of solution were mixed with 5μl. of 1% $NaHCO_3$ and 5μl. of 4% fluorodinitrobenzene in ethanol (Sanger, 1945) and sealed in a capillary tube. Most of the reagent precipitated at once as oily drops. The capillary tube was mounted obliquely in a test tube and the whole turned 3 hr. on a roller mill, this giving good stirring up of the oily drops. The capillary was then opened, the contents transferred to a watch glass and evaporated to dryness *in vacuo*. The residue was washed with ether till no oil remained, and the yellow crystals remaining were taken up in 50μl. of 6 N-HCl, sealed in a capillary tube and heated for 2 hr. at 100° (dinitrophenylglycine, if present, is hydrolyzed appreciably on longer heating under these conditions). After removal of the acid as usual, it was run on the chromatogram. A fast moving yellow spot presumably of the dinitrophenyl derivative of the end amino-acid was seen during the phenol run. About 4–7μg. of glycine only were found on the final chromatogram.

(*c*) 25μl. of solution were mixed with 5μl. of 10% $NaHCO_3$ and 20μl. of 1% KIO_4. After 30 min. at room temperature 20μl. of 1% tartaric acid were added to decompose the excess KIO_4. After another 10 min., 10μl. of 8·25% KI and 40μl. of 2 N-HCl were added. The copious precipitate of I_2 was taken up by cautious addition of an excess of Na_2SO_3 solution (it would have been better to allow most of the I_2 to evaporate first). An equal volume of conc. HCl was added and the whole heated at 100° in a capillary tube for 16 hr. After removal of the acid as usual the residue was transferred to the chromatogram without any attempt to remove the remaining inorganic substances. About 5–9μg. of glycine only was found.

Check on the technique. Pure *l*-seryl-glycine (from Dr J. S. Fruton) had R_F values in phenol and collidine quite different from those of 'under-alanine' (Table 6). It was hydrolyzed as above and run on the chromatogram. The spots were roughly matched and found to contain about 3μg. of serine and 2·2μg. of glycine which gives a molar ratio of 1 : 1. This was unexpectedly close as the accuracy of the matching is not good. However, this experiment shows that the method is of some value and that the relative proportions of serine and glycine are not appreciably

changed by decomposition under the conditions of the hydrolysis. It must be added, however, that the overall yield of serine and glycine from the pure seryl-glycine used was only about 50%.

Reactions of reducing substance in the urine. The urine of 7 Jan. 1946 (case E.C.) was taken for these tests. It gave a red precipitate on boiling in the usual manner with Benedict's qualitative reagent. It also gave a typical glucosazone (m.p. 207°) and a positive fermentation test for glucose performed as described by Harrison (1937). Selivanoff's test was negative.

RESULTS

These are given in the appropriate Tables and Figs.

Other analyses (Case E.C.)

These will be described in detail for the blood and urine by Stowers & Dent (1947). A brief summary of typical findings in the case E.C. is given for completeness. Urobilin and bilirubin were tested for repeatedly in the urine throughout the illness. Urobilin was present in slight excess throughout, bilirubin was never found (Fouchet test), this in spite of the slightly yellow appearance of the sclera of the patient in the last few days of life.

Date	
29. iv. 45	Glucose tolerance curve normal with sugar in all specimens of urine
7. viii. 45	Alkaline phosphatase 63 units (King & Armstrong). Alkali reserve 56 vol. %
16. vii. 45	Ammonia coefficient of urine 32%
18. ix. 45	Plasma inorganic phosphorus 1·8 mg./100 ml.
18. ix. 45	Serum calcium 11·3 mg./100 ml.
19. i. 46	Blood urea 39 mg./100 ml.
23. i. 46	Serum amino nitrogen 6·3 mg./100 ml.

X-rays of the skeleton showed marked generalized osteomalacia, with numerous pseudo-fractures without callus formation, tri-radiate pelvis, 'fish disc' lumbar vertebrae.

The results of the rough quantitative estimation of separate amino-acids are given only in the summary. They refer to the 24 hr. urine of 7 Jan. 1946.

The amino-aciduria was first tested for and found in July 1945. It occurred in every sample of urine tested subsequently until the death of the patient in Feb. 1946.

DISCUSSION

The individual points set out for study are reviewed under the following headings.

The nature and extent of the amino-aciduria. The normal variation in daily output of amino nitrogen in the urine is considerable. The question is well reviewed by Kirk (1936) who also adds the results of many further investigations of his own upon both normal subjects and patients with liver disease. The best method of determination is the modified formol titration (Van Slyke & Kirk, 1933) and 24 hr. outputs determined this way usually vary from 100 to 400 mg. which is 1–2% of the total nitrogen. There is no significant difference in cirrhosis of the liver, infective hepatitis and obstructive jaundice.

Albanese & Irby (1944), using a different and simpler technique depending on the volumetric determination of copper in the soluble amino-acid copper salts, find consistently higher results. In 15 normal men the amino nitrogen output was 200–700 mg./24 hr. which was 3–4% of the total nitrogen. This method was avoided here as it has not yet been compared adequately with the formol method and moreover it has limitations due to the comparative insolubility of certain amino-acid copper salts.

The daily excretion of an average of *c.* 1050 mg. of amino nitrogen in the case E.C. (see Table 1) can only be considered as grossly abnormal. The daily variation was considerable, from 491 to 1655 mg., the concentration varied from 44 to 116 mg./100 ml. and the ratio NH_2-N/total N from 3·1 to 13·0. The latter figures can be compared with those of 4–16 found by Stadie & Van Slyke (1920) in a case of acute yellow atrophy. Table 1 shows that this ratio is the most constant and therefore probably the most significant of the various methods of expressing the amino nitrogen output, especially if the low figures for the Oct. and Nov. samples are set aside. It is suggested that it is this figure which should always be determined when any case of excessive amino-aciduria is under consideration. Probably it is safe to say that any value greater than 3 is abnormal when determined by the formol titration. It is noticed in the case E.C. that although there was probably a tendency for the amino-acid output to rise from Oct. to Jan. yet there was no sudden rise in the last days before death nor indeed in the last month of life. Whatever the significance of the amino-aciduria it was not closely linked to the process causing death. The contrast with the terminal rise in urinary amino-acid output in the last few days of fatal acute yellow atrophy of the liver could not be more marked.

Unfortunately it was possible to determine the protein intake of E.C. over a period of only 7 days. There was then but little correspondence with amino nitrogen output in the urine (Table 1). During the last days of life, hardly any food was taken but the total nitrogen and amino nitrogen outputs both rose.

The large day to day variations in total amino nitrogen in the urine still remain very puzzling. Promoting a diuresis by drinking water did not significantly alter the total amount excreted (22nd Jan.). When however a spontaneous diuresis occurred so that fluid output was greater than fluid intake, the total excretion of amino nitrogen rose

Table 1. *Case E.C.* 24 *hr. output of urinary constituents*

Date	Urine (ml.)	Total N (g.)	Total NH_2-N (mg.)	NH_2-N (mg./ 100 ml.)	(NH_2-N/ total N) ×100	Total S (mg.)	Total N/total S	Glucose (g.)	Inorg. P (g.)	Total N/total P	Protein eaten (g.)	Remarks
16 Oct.	1585	12·40	740	46	3·8	717	17·3	6·4	0·90	13·8	—	
18 Nov.	2080	14·35	910	44	3·1	—	—	10·7	1·20	12·0	—	
7 Jan.	1320	13·75	1480	112	8·1	—	—	17·2	1·03	13·3	—	
18 Jan.	940	8·66	1090	116	12·6	610	14·2	9·1	0·69	12·6	—	
19 Jan.	2000	12·15	1524	76	12·5	749	16·2	—	—	—	—	3 pints of blood transfused
20 Jan.	2220	9·88	1123	51	11·4	543	18·2	—	—	—	—	
21 Jan.	1935	10·81	1365	71	12·6	676	16·0	—	—	—	—	
22 Jan.	1035	5·50	491	47	8·9	334	16·4	—	—	—	—	Water excretion test, 1 l. of water drunk
23 Jan.	1910	10·72*	749*	39	7·0	782	13·4	4·2	0·99	10·8	75	
24 Jan.	1950	13·18*	1646*	84	12·5	1665	8·0	—	—	—	84	10 g. of *dl*-methionine by mouth
25 Jan.	1060	12·91	560	53	4·3	1086	11·9	4·1	1·01	12·8	30	(Urines preserved with HCl from now on). Haematemesis, 400 ml.
26 Jan.	1370	8·67	826	601	9·5	591	14·7	—	—	—	53	
27 Jan.	1610	10·30	947	59	9·2	731	14·1	—	—	—	74	
28 Jan.	828	5·72	499	60	8·7	380	15·0	—	—	—	60	5 g. of *dl*-serine by mouth
29 Jan.	815	5·25	592	73	11·3	319	16·4	5·8	0·51	10·2	54	
30 Jan.	850	4·89	531	62	10·9	296	16·5	7·9	0·49	10·0	—	
31 Jan.	1250	7·53*	892*	71	11·9	740	10·2	—	—	—	—	8·05 g. of *l*-cystine by mouth
1 Feb.	1400	11·12	1305	93	11·7	1980	5·6	14·4	0·75	14·8	—	Haematemesis
2 Feb.	1670	14·03	1200	72	8·5	1160	12·1	12·3	1·04	13·5	—	
3 Feb.	2150	15·10	1655	77	11·0	1000	15·1	22·6	1·17	12·9	—	
4 Feb.	1950	19·01	1350	69	7·1	1280	14·8	16·2	1·34	14·2	—	
6 Feb.	1600	15·70	1275	80	8·1	1055	14·9	19·5	0·99	15·9	—	Noon to noon urine collection started
7 Feb.	1530	14·59	1021	67	6·8	1050	13·9	16·0	0·97	15·0	—	
8 Feb.	1315	10·24	1335	104	13·0	1710	6·0	14·9	0·85	12·1	—	10 g. of *dl*-methionine by mouth
9 Feb.	2300	14·79	1475	63	10·0	1820	8·1	21·0	1·20	12·3	—	Haematemesis, 200 ml. 2 pints of blood transfused
10 Feb.	1285	8·45	929	72	11·0	778	10·9	11·4	0·52	16·2	—	In coma
11 Feb.					Incontinent, no samples							
12 Feb.	(100)	(1·19%)	—	103	8·7	(0·054%)	21·8	—	—	—	—	Catheter specimen of urine
13 Feb.	(90)	(1·14%)	—	83	7·3	(0·055%)	20·7	—	—	—	—	Died 4 a.m. Urine from bladder at post-mortem

* Duplicate determinations.

Table 2. *Case E.C. Output of urinary constituents after amino-acids by mouth*

Date	Time (hr.)	Urine (ml.)	Total N (g.)	Total NH_2-N (mg.)	NH_2-N (mg./ 100 ml.)	(NH_2-N/ total N) ×100	Total S (mg.)	Total N/total S	SO_4-S (mg.)	(SO_4-S/ total S) ×100	Inorg. P (g.)	Total N/total P	Amino-acid given
24 Jan.	0– 3	190	1·31	291	153	22·1	229	5·7	83	36·4	—	—	10 g. *dl*-methionine
	3– 6	210	1·42	222	106	15·6	226	6·3	128	56·6	—	—	
	6–24	1550	10·45	1133	73	10·8	1210	8·6	880	72·7	—	—	
25 Jan.	0–24	1060	12·91	560	53	4·3	1086	11·9	843	77·6	—	—	
28 Jan.	0– 3	128	0·99	185	144	18·7	61	16·2	—	—	0·074	13·4	5 g. *dl*-serine
	0– 6	125	0·94	124	99	13·3	56	16·8	—	—	0·057	16·5	
	6–24	575	3·79	190	32	5·0	263	14·4	—	—	0·340	11·1	
29 Jan.	0–24	815	5·25	592	73	11·3	319	16·4	—	—	0·515	10·2	
31 Jan.	0– 3	290	1·73	177	61	10·4	110	15·6	80	72·0	—	—	8·05 g. *l*-cystine
	3– 6	200	1·28	154	77	12·0	128	10·0	97	76·1	—	—	
	6–18	760	4·52	561	74	12·4	501	9·0	382	76·3	—	—	
1 Feb.	0–24	1400	1·11	1305	93	11·7	1980	5·6	1640	82·8	—	—	
2 Feb.	0–24	1670	1·40	1200	72	8·5	1160	12·1	813	70·1	—	—	

N.B. Only 24 hr. urines were collected after the second 10 g. of methionine on 8 Feb. 1946.

(Fig. 8). Such diureses frequently occurred in this patient whose ascites and oedema varied from time to time. The tentative suggestion follows that the spontaneous diureses represent excretions of excesses of tissue fluids in which the amino nitrogen and total non-protein nitrogen exist in the proportions found in the urine.

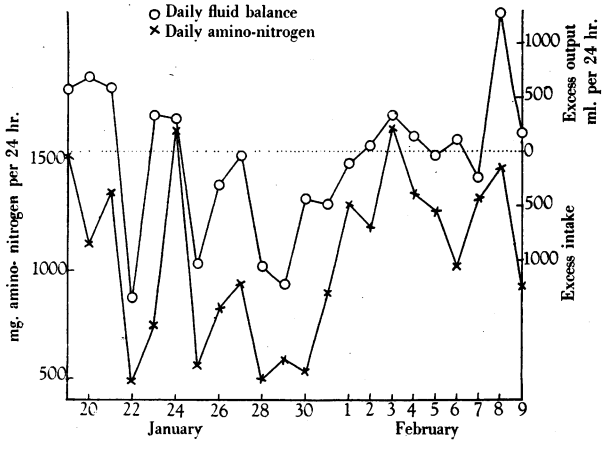


Fig. 8. Curve to show relationship between fluid balance and 24 hr. output of amino nitrogen (case E.C.). Fluid balance was measured by the nursing staff as the difference in volume between total fluid intake and urine output.

The other cases of Fanconi syndrome have shown lower outputs of amino nitrogen (Table 3). However, the output is considered abnormal in both, largely on the basis of the raised NH_2-N/total N ratios of 3·6 and 4·8 in V.R. and E.B. respectively.

Table 3. *Urine in other cases of Fanconi syndrome*

Date	N (g./100 ml.)	NH_2-N (mg./100 ml.)	(NH_2-N/total N) × 100
	Case V.R.		
2 Oct. 1945	1·09	39	3·6
	Case E.B.		
13 Mar. 1946	0·92	44	4·8

In the former, terminal renal failure with nitrogen retention may have affected the output. (Compare the case of acute yellow atrophy with grossly raised blood amino nitrogen and urinary retention; Rabinowitch, 1928.) In E.B. who is still fairly healthy, the amino-aciduria was intermittent and was only found in one of 4 samples submitted. It does appear from this and from the lower ratios of the Oct. and Nov. samples of the case E.C. that, taken over a period of some months, there is a suggestion that the amino-aciduria increases as the disease progresses. It must be pointed out that all three cases had cirrhosis of the liver, E.C. and V.R. also with primary carcinoma. Cirrhosis in itself, however, does not produce an amino-aciduria (Kirk, 1936) and this has recently been confirmed by the author, using chromatograms, for a small series of cirrhoses and secondary carcinomata of the liver.

With regard to the individual amino-acids found in the urine, it is seen that all those commonly found in protein hydrolysates except lysine and tryptophan have been detected in the urine of E.C. at some time or another, and for technical reasons it is not possible to say whether both leucine and isoleucine, or only one of these, are present. Proline has been seen only once, in the urine of 9 Feb. 1946. In addition citrulline and α-amino-*n*-butyric acid have been identified as occurring in small but fairly constant amounts. Methionine sulphoxide and cysteic acid were found occasionally, but could have been produced by oxidation under the conditions of storage. Methionine sulphoxide is a particularly interesting substance from its potential importance in connexion with oxidation-reduction potential. It oxidizes cysteine to cystine (Toennies & Kolb, 1939). It will be important if it can be shown that it occurs naturally for it could then act as an oxygen carrier. One strong spot on the chromatogram, 'under-alanine', is believed to be due to a considerable excretion of a peptide which liberates serine and glycine on hydrolysis and is probably seryl-glycyl-glycine, though this requires confirmation by synthesis. Another weak but fairly constant spot, 'over-glycine', is believed to be due to a new amino-acid. From its position on the chromatogram it can be surmised that it is probably of novel composition. A higher homologue of glutamic acid would be expected to occupy such a position. It is a pity that the occurrence or otherwise of asparagine and glutamine cannot be definitely inferred by partition chromatography in the presence of glycine and alanine, with both of which they overlap. This is likely to be a permanent defect in the method as it is to be expected that the partition coefficients in any other solvents would be similar, the contribution to them of the —CO . NH_2 group being similar to that of the hydrogen atom (McMeekin, Cohn & Weare, 1936). It can be said that there are unknown substances present which set free large amounts of aspartic and glutamic acids on hydrolysis. No otherwise suggestive evidence of the presence of asparagine and glutamine has been obtained.

The naming of new spots by the prefixes 'under' and 'over' with respect to the position of known amino-acids has been found very convenient as the regular positions of the common amino-acids soon become very familiar to anyone working with the chromatograms. Spots to the right or left are named with the prefixes 'slow' or 'fast' respectively,

this referring to their speeds in the phenol solvent; e.g. 'fast-glycine' is a new spot found in hydrolyzed urines which is still undergoing investigation. It occurs just to the left of the glycine spot on the chromatograms as drawn in this paper.

A glance at the chromatogram of the urine gives at once an accurate qualitative and rough quantitative estimate of amino-acid outputs (Table 4). However, as the standard amount of 25 μl. of urine is taken each time, it is the concentration and not the total 24 hr. output of amino-acids that is being estimated. The amino-acid pattern was remarkably constant over long periods. Only aspartic and glutamic acids varied considerably from day to day (see Table 4, case E.C.), if the days following ingestion of methionine and serine are excluded. When a lower concentration of amino nitrogen was passed by E.C. the reduction chiefly affected the amino-acids of higher molecular weight (valine and leucine especially), in fact the chromatogram began to resemble that of V.R. and E.B. At first 'under-alanine' was found to be a constant constituent but later on it was occasionally absent. It is believed more likely that it was originally present in all the urines of E.C. and that during the wait of some months in an imperfect refrigerator partial hydrolysis to glycine and seryl-glycine occurred. The latter would not show as a separate spot on the chromatogram owing to its relatively poor strength of colour with ninhydrin, and is itself stable enough to resist further mild hydrolysis. The additional glycine would not be very conspicuous on an already strong spot. It can be noted that in those urines in which no 'under-alanine' was found the aspartic and glutamic acid contents were invariably high, these also being liberated by hydrolysis. The two chromatograms made on the urine of 13 Feb. also confirm this (Table 4). It will be necessary in future to guard against such instability by greater precautions in storage. The urine of E.B. gave a chromatogram not far removed from those characteristic for the weaker ones of E.C. That of V.R. was similar except that 'under-alanine' was absent and serine much stronger than usual. Here it is believed that complete hydrolysis of the 'under-alanine' to serine and glycine had taken place under the conditions of preservation in dilute hydrochloric acid at room temperature.

Nearly all the time E.C. was an in-patient he was on a diet of milk as sole source of protein. Casein, hydrolyzed with acid and run on the chromatogram in comparable amount, gave a distinct picture quite unlike that given by the urine before or after hydrolysis. E.C. was on a mixed diet as an out-patient but the urinary amino-acid distribution was unchanged. There therefore is no suggestion of the urinary amino-acids representing a simple overflow of the amino-acids from ingested protein, such as might have escaped the attention of the liver owing to the portal obstruction and consequent anastomoses with the systemic circulation. The excretion was selective.

The question of the abnormal distribution of the amino-acids in the urine of E.C. has now to be considered. Many normal urines have been investigated by chromatography and at the normal concentrations of 10–30 mg. of amino nitrogen/100 ml. the main amino-acids seen on the chromatogram are glycine and alanine. Traces of glutamic acid, valine and 'under-alanine' sometimes appear. Concentration of the urine leads to difficulties owing to the large amounts of other substances present which then begin to interfere with the development of the chromatogram, and the methods of removing these so far tried (ion exchange, solvent extraction, etc.) tend to alter the relative amounts of the amino-acids present. Leucine, histidine, tyrosine, citrulline, serine, 'under-alanine' and α-amino-n-butyric acid have however been demonstrated at one stage or another in normal urine, but it is impossible to say definitely whether the relative amounts of the various amino-acids present are similar to those found in Fanconi syndrome. The general impression is that in the latter there is a relative increase in the amounts of dicarboxylic acids liberated on hydrolysis, and of hydroxyamino and higher molecular weight monoaminomonocarboxylic acids. This is well shown if a normal urine is compared against a urine from Fanconi syndrome which has been diluted to a comparable concentration of amino nitrogen, but this procedure does not allow the strengths of the weaker spots to be compared.

The mechanism of the amino-aciduria. Some factors already considered above have suggested that a simple overflow of amino-acids from the blood into the urine as in acute yellow atrophy is not a likely mechanism. This is finally confirmed by the finding of a normal value for serum amino nitrogen (6·3 mg./100 ml.). There must therefore be some form of lowered renal threshold, presumably a defective reabsorption of amino-acids in the tubules from the glomerular filtrate. Fig. 2 attempts to show diagrammatically that although the serum amino-acid distribution in the case E.C. was very similar to the normal serum and to a normal urine (which therefore resembles a simple plasma filtrate), yet the urinary distribution of E.C. was quite distinct, thus suggesting that the tubular defect is of different degree for different amino-acids. It is interesting to note that both 'under-alanine' and α-amino-n-butyric acids have been definitely identified in normal blood and urine as well as in the Fanconi syndrome.

The relation between the amino-aciduria and the glycosuria. Fanconi (1936), who found that the glycosuria in his cases was renal in type, was the first

Table 4. *Case E.C. Two-dimensional chromatograms of the urine*

(Day to day strengths of amino-acid ninhydrin reactions on an arbitrary scale of 10 units. 1 unit = faint colour, 10 = intense colour. 25μl. of urine taken in each case.

Date	Aspartic acid	Glutamic acid	Serine	Glycine	Threonine	Alanine	'Under-alanine'	Citrulline	Histidine + methionine sulphoxide	α-Aminobutyric acid	Hydroxyproline	Valine	Leucine and/or isoleucine	Methionine	Phenylalanine	Tyrosine	'Over-glycine'	Arginine	Remarks
16 Oct.	1	1	5	7	1	7	7	1	1	—	—	2	1	—	—	<1	1	<1	
18 Nov.	—	—	2	6	2	6	5	—	2	1	—	2	2	—	—	<1	<1	—	
7 Jan.	—	1	4	6	2	8	7	1	2	2	<1	5	4	—	<1	2	—	1	
	7	8	>10	8	3	10	—	<1	<1	1	2	5	4	—	1	2	—	<1	Urine hydrolyzed 2 hr. at 100° with 6N-HCl
18 Jan.	4	4	2	5	1	8	—	1	<1	2	—	3	3	—	—	2	1	1	
23 Jan.	2	6	2	5	<1	10	—	<1	<1	<1	—	1	1	—	—	<1	—	—	
24 Jan.	3	6	3	6	1	8	—	1	6	3	2	4	3	5	—	<1	—	—	0–3 hr. after methionine
	2	7	4	6	1	7	—	2	6	3	—	6	3	8	1	2	<1	1	3–6 hr. after methionine
	6	7	3	5	1	10	—	1	4	2	—	5	1	2	—	1	<1	1	6–18 hr. after methionine
25 Jan.	—	3	2	6	<1	6	1	—	1	1	—	1	1	—	—	<1	<1	—	
28 Jan.	—	—	⩾10	7	2	7	6	1	1	2	—	3	2	—	—	1	—	—	0–3 hr. after serine
	3	7	7	7	1	7	—	1	1	1	—	4	2	—	1	2	<1	1	3–6 hr. after serine
29 Jan.	—	3	5	6	1	7	3	2	<1	1	—	4	2	—	1	1	1	1	
31 Jan.	1	4	3	6	1	6	2	1	2	<1	—	3	2	—	—	<1	<1	1	0–3 hr. after cystine
	—	1	3	5	1	6	4	1	1	1	—	3	1	—	—	2	—	<1	6–18 hr. after cystine
1 Feb.	3	—	2	5	1	7	—	1	1	1	—	2	2	—	—	1	—	—	
2 Feb.	3	4	3	4	1	7	—	1	1	1	—	3	2	—	—	1	<1	<1	
3 Feb.	5	7	4	6	2	6	3	2	1	2	—	4	3	—	—	—	1	<1	
7 Feb.	—	—	2	5	1	5	4	1	1	1	—	3	1	—	—	1	—	—	
8 Feb.	—	—	3	6	<1	7	4	1	4	2	—	3	1	2	—	1	1	—	0–24 hr. after methionine
9 Feb.	—	<1	4	7	2	8	4	2	3	1	—	5	2	—	—	1	3	1	
12 Feb.	—	—	4	5	2	7	6	1	1	2	—	6	4	—	2	3	<1	1	Catheter specimen
13 Feb.	—	—	3	6	1	7	5				Spoilt					2	—	—	Post-mortem specimen. Fresh
	5*	7	3	6	1	6	—	?*	1	2	—	4	2	—	1	3	<1	1	Post-mortem specimen. After 4 months in refrigerator

* N.B. The second specimen for 13 Feb. was developed some months after the first. Pure citrulline was added to it to serve as a marker so the strength of the original spot cannot be ascertained.

Case V.R. 20 Oct.	<1	2	6	5	1	4	—	—	2	—	—	—	—	—	—	—	—	—	Urine taken during last 2 days of life. Some renal failure developing
Case E.B. 13 Mar.	—	—	2	5	<1	3	2	—	4	<1	—	—	—	—	—	—	<1	—	

N.B. The strength of the ninhydrin reaction does not give a direct measure of amino nitrogen, unless only the same amino-acid is being considered. The above figures can be compared vertically but not horizontally.

to suggest a similar mechanism for the amino-aciduria. The case E.C. also showed a renal glycosuric blood-sugar curve and it was very early noticed on qualitative testing of the Oct., Nov. and early Jan. urine specimens that the glycosuria increased with the increasing amino-aciduria. Quantitative determinations of glucose output were later done on all the available specimens of urine and the results plotted against the corresponding amino nitrogen output are shown in Fig. 4A. There appears to be a simple straight line relationship between the two which can be expressed by the formula:

$$\text{g. } NH_2\text{-N} = 0{\cdot}064 \times \text{g. glucose} + 0{\cdot}280.$$

The formula agrees well over a fivefold range of glucose outputs. The inference cannot be avoided that there is some close connexion, previously unsuspected, between the actual chemical mechanisms of glucose and amino-acid reabsorptions in the tubules. The urines of five cases of typical renal glycosurics, as well as from a normal man who had been given a massive glycosuria by the subcutaneous injection of 20 mg. of phloridzin were examined. In no case was the amino nitrogen output increased.

Table 5. *Sulphur partition of urine of case E.C.*

Date	Total S (mg.)	SO_4-S (mg.)	Ethereal S (mg.)	Neutral S (mg.)	Methionine (mg.)
16 Oct.	717	577 (80%)	62 (9%)	78 (11%)	340

The percentages of the total sulphur are shown in brackets. The methionine contains 73 mg. of sulphur which is 94% of the neutral sulphur. All these figures are within normal limits. All determinations in duplicate.

In the case E.B., of the 4 specimens of urine passed on different days the only one which contained glucose (0·11 g./100 ml.) was the only one with a raised concentration of amino nitrogen (43·7 mg./100 ml.). The trace of glucose in the urine of V.R. was not estimated quantitatively. It is interesting to recall here the intermittent glycosuria found in the original case of Milkman's syndrome (Milkman, 1934).

Was the amino-aciduria the primary cause of the disease? It must first be pointed out that the total output of amino-acids in E.C. is about 10 g./day and therefore corresponds to only a small fraction of his protein intake. There is therefore no question of a general protein deficiency being brought about by the urinary losses. As far as concerns individual amino-acids there is likewise no conspicuous excretion of any particular essential amino-acid, all of which are taken in the diet in much larger quantities than they are being excreted. The only point that will be made is that of the disproportionate loss of hydroxyamino-acids. Serine, in particular, is lost both in the free form and as the peptide 'under-alanine'. Probably 1–2 g./day were put out in the case E.C. Serine, however, is not an essential amino-acid and can presumably be synthesized by the normal body as fast as it may be required. It is interesting, however, to recall the key position which the amino-acid occupies. It can provide the carbon chain for the synthesis of cystine from methionine (du Vigneaud, Kilmer, Rachele & Cohn, 1944). It is a precursor of ethanolamine and hence of choline (Stetten, 1941). It can occur naturally as a phosphoric ester (Lipmann & Levene, 1932; Posternak & Pollaczek, 1940). There is a strong presumption that any of these mechanisms may be defective in the Fanconi syndrome. In addition,

Table 6. *R_F values of amino-acids and other ninhydrin reacting substances on the two-dimensional chromatograms*

($P = R_F$ in phenol. $C = R_F$ value in collidine. All chromatograms run first with phenol then with collidine. The colours are compared against the purple given by valine.)

Substance	P	C	Colour
Aspartic acid	0·17	0·27	Blue
Glutamic acid	0·28	0·29	Purple
Serine	0·35	0·28	Brownish purple
Glycine	0·42	0·22	Reddish purple
Threonine	0·50	0·30	Purple
Alanine	0·62	0·25	Slightly bluish purple
'Under-alanine'	0·59	0·21	Reddish purple
α-Amino-*n*-butyric acid	0·71	0·30	Purple
Histidine	0·77	0·24	Greenish purple
Citrulline	0·66	0·22	Purple
Arginine	0·87	0·13	Purple
Lysine	0·80	0·11	Purple
Valine	0·80	0·34	Purple
Methionine	0·82	0·42	Purple
Leucines	0·85	0·43	Purple
Phenylalanine	0·86	0·44	Blue
Tyrosine	0·63	0·53	Dull brownish purple
Tryptophane	0·77	0·47	Purple
Proline	0·86	0·24	Bright yellow
Hydroxyproline	0·77	0·31	Yellowish brown
'Over-glycine'	0·42	0·35	Purple
Cystine	0·21*	0·11*	Purple
Cysteic acid	0·08	0·35	Blue
Methionine sulphoxide	0·79	0·24	Purple
Methionine sulphone	0·69	0·35	Bluish purple
Dihydroxyphenyl-alanine	0·33*	0·44*	Yellow
Asparagine	0·43	0·21	Olive brown
Glutamine	0·57	0·23	Purple
β-Alanine	0·68	0·21	Purple
Glucosamine	0·20	0·53	Blue
Seryl-glycine	0·53	0·31	Dull yellow turning purple later
Glutathione	0·35*	0·10*	Purple

* These values refer only to one-dimensional chromatograms in the given solvent. It is not usual to see these compounds on two-dimensional chromatograms owing to decomposition.

serine injury to rats (Fishman & Artom, 1945) produces similar urinary changes (proteinuria, glycosuria, occasional amino-aciduria). An attempt to reproduce this on one rat, using chromatographic examination of urines, showed that only serine was present in excessive amounts in the urine.

Sulphur metabolism. Attempts to disclose an error of sulphur metabolism have all met with failure. No cystine deposits were found in the organs of E.C. and V.R. Methionine may be the only amino-acid which is excreted in normal quantities in the urine. The cystine output is at most only slightly raised. Cystine and methionine given in large quantities by mouth were normally metabolized. Methionine sulphoxide has also been found in the urine after the administration of methionine to normal subjects. The methionine saturation curve (Fig. 5) was also within normal limits (Albanese *et al.* 1944).

A very interesting finding after the methionine feeding was the distinct increase in the output of α-amino-*n*-butyric acid. When the serial chromatograms were compared side by side the fact was more convincing than Table 4 would indicate. It has also been seen in a normal adult after methionine had been given by mouth. It appears that a new possible mechanism in the metabolism of methionine has been brought to light, namely, direct desulphurization to α-amino-*n*-butyric acid, such as can be carried out with Raney nickel (author's unpublished work, see also Mozingo, Wolf, Harris & Folkers, 1943). The synthetic *dl*-methionine administered was shown to be chromatographically pure.

Finally it should be noted that when serine had been fed there was no change in sulphur output, nor in the gross distribution of the other amino-acids. A fall in sulphur output might have been expected if the additional serine had been partly diverted to the production of much-needed cystine from methionine.

In conclusion, it is still not possible to attribute the Fanconi syndrome to any single cause. A new form of renal damage involving low thresholds for amino-acids, glucose and phosphorus is proved and will account for all the findings except the cirrhosis of the liver. The possible role of chronic acidosis, especially favoured by Fanconi and by McCune *et al.*, has not been considered here as it was not proved to be present in the case E.C. who was most thoroughly investigated, although the high ammonia coefficient of the urine was suggestive.

SUMMARY

1. The amino-aciduria in three cases of Fanconi syndrome has been investigated with paper chromatograms. One case was more thoroughly investigated from other points of view over a period of 4 months.

2. The amino-aciduria was renal in type, i.e. due to a low kidney threshold for amino-acids. The total output of amino-nitrogen in the urine was related to the output of glucose according to a straight line relationship, although both varied considerably from day to day. It also appeared to be related directly to the fluid balance of the patient.

3. The following free amino-acids were found in the urine of one case (where the 24 hr. output of amino-acid was roughly estimated it is given in parenthesis): cystine, aspartic acid, glutamic acid, serine (0·25 g.), glycine (0·8 g.), threonine (0·5 g.), alanine (1·0 g.), valine (0·5 g.), leucine and/or *iso*-leucine (0·4 g.), methionine (0·34 g.), phenylalanine, tyrosine (0·5 g.), arginine, citrulline, histidine, proline, hydroxyproline and α-amino-*n*-butyric acid. Cysteic acid and methionine sulphoxide were sometimes present but may have been produced by secondary decomposition of cystine and methionine.

4. A ninhydrin-reacting substance 'over-glycine' was found. It is suggested that this is a new amino-acid and its behaviour on the chromatogram suggests that it is of novel composition.

5. A peptide 'under-alanine' containing serine and glycine was also found in large amounts. In one case 0·75 g. at least was excreted daily in the urine. It is believed to be serylglycylglycine, but this has yet to be confirmed by synthesis. Complexes liberating large amounts of aspartic and glutamic acids on hydrolysis, were also present.

6. No error in metabolism of sulphur amino-acids could be shown in one case of Fanconi syndrome specially investigated. After methionine was given by mouth the output of α-amino-*n*-butyric acid increased as if some direct desulphurization had taken place. This is apparently a normal mechanism. There was also a large increase in the output of methionine sulphoxide. This has also been found in normal subjects. Its possible formation in the urine from methionine by secondary oxidation in storage has not been adequately excluded.

7. It has not been possible to prove that the chronic loss of amino-acids in the urine is responsible for the other features of the syndrome.

8. α-Amino-*n*-butyric acid and 'under-alanine' have been found in normal plasma and urine.

9. Developments in the technique of paper chromatography have been devised to enable: (*a*) small quantities of substances in biological fluids to be isolated for further study of their chemical properties; (*b*) the simultaneous detection of histidine, histamine and tyrosine in urines; (*c*) the detection of methionine and cystine without loss from decomposition and without confusion with other amino-acids.

10. In the course of this work it has been shown that most of the common organic and inorganic substances present in urine, and many others

present in pathological conditions, have characteristic movements on the paper chromatograms which are sufficiently specific to constitute a criterion for their recognition. Further, by means of this technique the existence of new compounds may be disclosed.

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Porphyrinuria in Rats Fed Oxidized-Casein: a Preliminary Communication

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The work described in this paper arose out of attempts by one of us (C.E.D.) to devise a diet sufficiently low in cystine and methionine to produce acute hepatic necrosis in rats (for review see Glynn, Himsworth & Neuberger, 1945) with more success than was possible with natural proteins with a low content of these amino-acids.

Oxidized-casein (Toennies, 1942), produced by the action of hydrogen peroxide on casein dissolved in formic acid, appeared to be eminently suitable for this purpose since Bennett & Toennies (1942) have shown that, when supplemented with tryptophan and methionine, it is nutritionally adequate for the young rat although growth is not quite as good as on natural casein. Food consumption was about 20% lower on the supplemented oxidized-casein diet than on the control diet. This would suggest that no essential amino-acids other than methionine and tryptophan are destroyed by the oxidation procedure. Comparative analyses (Toennies, 1942) of casein and oxidized-casein for threonine, serine, cystine, methionine and tryptophan show that from the latter protein, methionine and tryptophan are completely lacking, cystine is reduced from 0·36% to about 0·16% whilst serine and threonine have not been affected. It was considered that methionine