EXCRETION OF AMINO ACIDS IN THE BOUND FORM IN THE URINE OF PATIENTS SUFFERING FROM KWASHIORKOR

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Some years ago Westall[1] directed attention to the occurrence of small peptides in normal urine which he believed might be significant in intermediary protein metabolism. Prompted by Westall's observations and our own interest in disturbances in protein metabolism,[2,3] we believed that examination of the urinary peptides of protein-depleted kwashiorkor patients might provide important information about the condition. In particular, we wished to answer the following question:

Is there a pattern of urinary peptides characteristic of the protein-depleted state, changing to a recognizably normal one on clinical recovery?

Included in our enquiry is a search for evidence of partial blocks in synthesis or breakdown of body proteins

caused by lack of dietary amino acids, especially essential ones, or of enzymes or co-enzymes.

A recent publication by Edozien and Phillips[4] described a comparative study of the partition of nitrogen in spot urinary specimens of subjects on normal, low-protein and kwashiorkor diets. Their paper did not attempt to explore the issues with which we are concerned, but the relevant findings will be discussed later.

MATERIALS AND METHODS

The patients studied were diagnosed clinically as having severe kwashiorkor and requiring hospitalization. Only males have been studied, owing to the difficulties of 24-hour urine collections in females. Six non-European males have been investigated up to the present time.

The clinical material consisted of:

Case 1, H.L. African male aged 1 year 3 months. Weight 16 lb. 8 oz. Admitted with florid kwashiorkor. Hb. 14 G./100 ml., albumin 2·09 G./100 ml., globulin 2·62 G./100 ml.

Case 2, A.O. Coloured male aged 1 year 9 months. Weight 14 lb. 8 oz. Moderately severe kwashiorkor. Hb. 12 G./100 ml.

Case 3, J.C. Coloured male aged 2 years 4 months. Weight 20 lb. 4 oz. Mild kwashiorkor.

Case 4, F.B. Coloured male aged 4 years. Weight 23 lb. 14 oz. Severe kwashiorkor. Hb. 10 G./100 ml., albumin 1·74 G./100 ml., globulin 1·88 G./100 ml.

Case 5, T.I. Coloured male aged 1 year 9 months. Weight 20 lb. Moderately severe kwashiorkor. Hb. 9·5 G./100 ml., albumin 1·38 G./100 ml., globulin 2·56 G./100 ml.

Case 6, P.J. Aged 1 year. Weight 13 lb. 8 oz. Admitted with kwashiorkor complicated by severe dehydration from gastro-enteritis. Hb. 7 G./100 ml., serum sodium 165 mEq./l., serum chloride 146 mEq./l., blood urea 49 mg./100 ml., serum calcium 6·6 mg./100 ml. Only the initial 24-hour urinary collection was made since the patient became extremely ill and died 1 week after admission.

Cases 1 – 5 were given clear feeds on the day of admission (day 1). On days 2 and 3 skim milk was offered which was changed to half-cream and full-cream milk on days 4 and 6 respectively.

During the collection of the 24-hour urine specimens, the patients were on metabolic beds. No preservatives were added to the specimens, which were kept at −10°C. as soon as collection was completed.

Total nitrogen was determined by the micro-Kjeldahl procedure. Urinary ammonia was measured by alkaline distillation, and urinary urea by the carbamido-diacetyl reaction using the technicon auto-analyser.[5] All amino-acid assays were made on urine which had been deproteinized with tungstic acid by addition of 1·0 ml. of 0·67N H_2SO_4 and 1·0 ml. of 10% sodium tungstate to 10·0 ml. of urine. After standing overnight at 4°C., the mixture was filtered and the resulting filtrate was stored at −10°C. Urinary free and total α-amino nitrogen was measured by the colorimetric ninhydrin method.[6]

Acid hydrolysis was performed in sealed tubes containing equal volumes of urine and concentrated HCl in a thermostatic oven at 120°C. for 18 hours. The hydrolysate was filtered to remove humin.

Analysis of individual amino acids was performed by ion-exchange chromatography on Amberlite CG 120 and the effluent analysed for ninhydrin-positive material by the automatic technique of Spackman *et al.*[7] Single-channel recording of the effluent was used, with a 2·9 mm. flow cell at a wave-

TABLE I. 24-HR. EXCRETION OF URINARY NITROGENOUS COMPOUNDS

Case No.	Day	24-hr. volume (ml.)	Total α-NH_2 N (mg./24 hrs.)	Free α-NH_2 N (mg./24 hrs.)	Bound α-NH_2 N (mg./24 hrs.)	Ratio of bound/free α-NH_2 N	NH_3 N (mg./24 hrs.)	Urea N (mg./24 hrs.)	Total N (mg./24 hrs.)
1 (H.L.)	1	23·5	13·3	5·95	7·4	1·24	96	57	241
	3	239	71·7	47·6	24·1	0·52	378	336	1,410
	5	887	265	113	152	1·34	434	1,812	4,200
	15	557	108	55·7	42·3	0·76	986	2,060	3,403
	17	370	47·3	24·0	23·3	0·97	1,095	1,280	2,586
	19	438	90·7	23·1	67·6	2·92	2,203	628	3,066
2 (A.O.)	2	190	53·7	14·4	29·3	2·04	181	222	551
	4	846	158	64·0	94·0	1·47	255	614	1,286
	14	460	39·5	13·6	25·9	1·90	160	496	1,359
	15	281	45·5	29·0	16·5	0·57	123	894	1,206
	16	236	21·5	10·0	11·5	1·15	1,412	520	2,556
3 (J.C.)	1	292	71·5	35·0	36·5	1·04	169	678	972
	3	153	37·9	20·6	17·3	0·84	174	1,028	1,637
	5	35	10·0	5·0	5·0	1·00	45	284	364
	13	470	40·8	21·2	19·6	0·92	158	1,120	1,936
	15	448	64·2	32·6	20·6	0·63	390	3,440	4,880
	17	408	39·2	17·7	21·5	1·22	178	1,520	2,340
4 (F.B.)	1	785	91·0	18·4	72·6	4·00	390	1,580	2,410
	3	340	64·0	26·7	37·3	1·40	290	604	1,357
	5	998	117·8	73·1	44·7	0·61	303	1,893	2,409
5 (T.I.)	2	217	84·8	34·8	50·0	1·44	—	1,420	—
	4	261	49·1	22·4	26·7	1·19	—	1,520	—
	6	245	77·8	16·7	61·1	3·66	—	1,160	—
	22	467	58·8	30·0	28·8	0·96	—	2,690	—
	24	463	37·1	18·0	19·1	1·06	—	1,480	—
	26	424	45·8	19·5	26·3	1·40	—	1,630	—
6 (P.J.)	1	105	27·1	20·0	7·1	0·35	239	361	925

TABLE II. URINARY EXCRETION OF AMINO ACIDS IN μ MOLES PER 24 HOURS

	Day							
	1 (23·5)*		*3* (239)		*5* (887)		*15* (557)	
	Free	*Bound*	*Free*	*Bound*	*Free*	*Bound*	*Free*	*Bound*
Amino nitrogen (mg./24 hrs.)	6	7·4	47·6	24·1	113	152	55·7	42·3
Taurine	58	[<0·3]‡‡	167	24	163	38	1	29
Hydroxyproline	[<1·4]	79	[14–42]	141	[<53]	635	[<33]	225
Aspartic acid	6	35	32	170	[<5·3]	462	8	326
Threonine	—‡	19	—‡	119††	—‡	391††	13	123
Serine	—‡	30	—‡	253††	—‡	682††	3	187
Asparagine + glutamine**	4	—	481	—	869	—	45	—
Sarcosine	1	[<0·3]	[<1·4]	[3–8·5]	[<5·3]	71	31	20
Proline	[<1·4]	51	[<14]	292	[<53]	238	[<33]	82
Glutamic acid	2	104	35	504	89	2,321	43	776
Citrulline	[0·1–0·4]	0·5	[<1·4]	[3–8·5]	[5·3–16]	—	[3·3–10]	[<6·5]
Glycine ***	54	110	656	223	1,438	772	236	1,152
Alanine ***			126	54	686	443	57	333
α—Amino-adipic acid ***	0·6	[<0·3]	3·2	16	80	[<10·6]	4	11
α—Amino-n-butyric acid ***								
Valine	[0·1–0·4]	7·5	[1·4–4·2]	39	[5·3–16]	100	[<3·3]	60
Cystine	[<0·1]	23	[1·4–4·2]	51	46	92	[<3·3]	152
Cystathioneine	0·6	1·0	21	[<3]	[5·3–16]	14	[<3·3]	[6·5–20]
Methionine†	1·7	3·3	29	[3–8·5]	[5·3–16]	11	7	35
Isoleucine	1·9	3·0	24	[3–8·5]	[5·3–16]	85	[3·3–10]	44
Leucine	0·5	12·5	11	58	[5·3–16]	226	[3·3–10]	111
Tyrosine	[<0·1]	5·6	3·7	47	[5·3–16]	146	[3·3–10]	131
Phenylalanine	[<0·1]	6·0	4·2	43	[5·3–16]	139	[3·3–10]	145
β-Alanine	[<0·1]	3·4	[1·4–4·2]	[<3]	[<5·3]	38	[3·3–10]	100
β-Aminoisobutyric acid	11	12	12	28	[5·3–16]	87	[3·3–10]	[<6·5]
Hydroxy lysine	[0·1–0·4]	3	5	10	—	—	8	[<6·5]
δ-Amino-n-butyric acid	2	[<0·3]	17	[<3]	83	80	21	[<6·5]
Lysine	—	—	2·3	[<3]	—	—	[<3·3]	26
3-Methyl histidine	2	[<0·3]	—	—	—	—	304	45
Histidine	15	[<0·3]	602	169	1,030	[<10·6]	217	10

* Figures in parenthesis thus (represent the 24-hour urinary volume for that day in ml.
‡‡ Values enclosed within brackets thus [ ] could not be determined more precisely, for technical reasons.
** Asparagine and glutamine are eluted together as a single peak. The results are expressed as glutamine.
*** In high concentrations, glycine and alanine are not completely resolved. α—Amino-adipic and α—amino-n-butyric acid are eluted as a single peak.
† Includes methionine sulphoxides.
‡ Threonine and serine are obscured in these unhydrolysed specimens by an unidentified component Fig. 2).
†† These are total excretions, i.e. free plus bound forms.

length of 570 mμ. At this wavelength, hydroxyproline and proline gave colour constants of 1·93 and 4·20 respectively, compared with 37·1 for leucine. 98% confidence limits for these 2 substances in the 1 μ mole range is estimated at ± 20%. The 30° - 50° C. system was used. For the 150 cm. neutral and acidic column, temperature and buffer change was made at 11·5 hours (345 ml. of effluent) and the buffer breakthrough occurred after cystine. The temperature change for the 50 cm. column was made at 14 hours (420 ml. of effluent). A 2·00 ml. aliquot of deproteinized urine (equivalent to 1·67 ml. of original urine) was first dried over KOH in a vacuum desiccator, and then dissolved in 2 ml. of citrate buffer (0·2 M : pH 2·2) before being applied to the column. For the basic amino-acid analysis on the 50 cm. column, the aliquot was brought to pH 10 - 11 with 2N KOH to remove ammonia before desiccation.

The bound amino acids were analysed in a 2·00 ml. aliquot of hydrolysate (equivalent to 0·835 ml. of original urine). Here also ammonia was removed at pH 10 - 11 before the basic amino-acid analysis. The least quantity of proline and of hydroxyproline which could be detected in the applied sample was 0·100 μ moles, whereas the corresponding value for leucine and other amino acids of high colour yield was 0·01 μ moles.

Results were calculated as μ moles of amino acid excreted per 24 hours.

RESULTS

Twenty-four-hour excretions of free and bound α-amino nitrogen were estimated in all 6 cases. In 4 of these, collections were available during the acute phase and on recovery just before discharge from hospital. In 1 case urines were not available during the phase of recovery, while Case 6 died 7 days after admission.

The results of data on α-amino nitrogen excretion, together with 24-hour urinary volumes and total nitrogen content are shown in Table I. Differences between total and free α-amino nitrogen are reported as bound α-amino nitrogen. The ratio of bound to free forms has been calculated.

Case No. 1 was further analysed for urinary excretion of individual free and bound amino acids by ion-exchange column chromatography during the acute phase of the illness on the 1st, 3rd and 5th days after admission. In

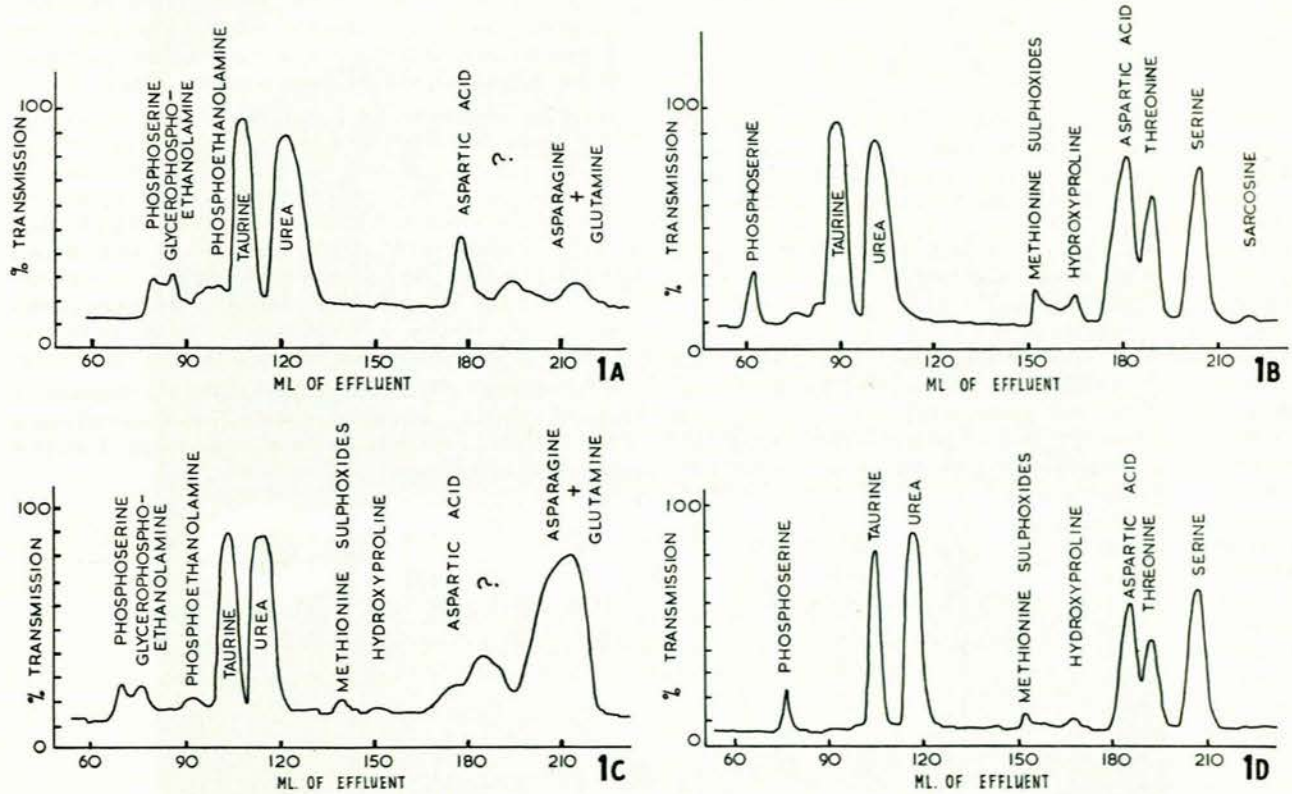

Fig. 1. Effluent pattern from 150 cm. column (30° - 50°C. system). The figures along the ordinates indicate percentage transmission through the flow cells, while those on the abscissae represent millilitres of effluent. Labelling of the peaks is derived from the site of the peaks of corresponding pure standard compounds on the effluent curve. Owing to the increased amounts of ninhydrin-positive material released after acid hydrolysis, exactly half the unhydrolysed sample volume has been used for analysis of the hydrolysed specimen to avoid overloading the system. The patterns are those from Case 1 (H.L.).

A. Day 1. 24-hour volume 23·5 ml. Unhydrolysed specimen. Sample 1·67 ml.
B. Day 1. Hydrolysed specimen. Sample 0·835 ml.
C. Day 3. 24-hour volume 239 ml. Unhydrolysed specimen. Sample 1·67 ml.
D. Day 3. Hydrolysed specimen. Sample 0·835 ml.

Acid-labile material is noted in 2 distinct areas of the effluent pattern. The first, before taurine and the second between aspartic acid and asparagine/glutamine. The peak marked '?' is broad and unlike the sharp peak of threonine seen after hydrolysis. Peptides from dipeptides to octapeptides can be expected to yield broad peaks. The tripeptide, reduced glutathione, in tissue extracts, is found in this position, but has never been demonstrated in urine.

addition the excretion on the 15th day was assayed as an index of the recovery phase. The results are shown in Table II.

Two groups of acid-labile materials which reacted with ninhydrin were noted. The first occurs in the area of the effluent curve immediately preceding taurine and urea at an effluent volume of 60 – 100 ml. at the site of expected emergence of phosphoserine, phosphoglyceraldehyde and phosphoglyceroethanolamine. Maximum concentrations of these compounds were noted on day 1, the urine of which was the most concentrated. At least 3 acid-labile components have been noted (Figs. 1 A and B). Smaller amounts of these components were noted on days 3, 5 and 15 and the concentrations are roughly inversely proportional to the 24-hour urinary volume.

A second complex of acid-labile compounds was noted in the site of elution usually occupied by aspartic acid, threonine and serine. At least 2 broad peaks were identified which differed markedly from the sharp peaks and 'clean' symmetrical pattern of the free amino acids after acid hydrolysis (Figs. 1 C and D). The maximum concentration of this complex was found on the 3rd day.

DISCUSSION

When we examine our data on the urinary excretion of ammonia and urea in this small series of kwashiorkor patients, it is clear that there is a small rate of breakdown of protein at the time of admission of the kwashiorkor subject to hospital. This is in accordance with recent determinations which show a very slow turnover rate of such patients' serum albumin[8] and a quickening of the catabolic rate during protein repletion.

The average daily excretion of free amino acids covering the first week of observation was higher than corresponding normals[9] and higher than those of the subsequent convalescent stages.

Similar relationships held also for bound forms; the maximum daily excretion of both free and bound amino acids was attained around the 5th day of treatment, which coincides in fact with the greatest rate of change of serum-protein pattern.[2] Although in several cases great fluctuations of urinary volume appear to dictate the excretion of free and bound amino acids, the lack of correlation between the ratios of bound to free forms and urinary volume (Fig. 2) implies an even greater influence of other factors. Since peptides are rapidly cleared from the circulation, this ratio may furnish an indirect index of impaired protein metabolism at the peptide level.

As regards the composition of the bound forms, certain observations appear worthy of special mention. Essential amino acids are being lost in the bound form from the body even in the grossly protein-depleted state. In normal adults essential amino acids are excreted mainly in the bound form.[10] The only comparable values available for children[9] show a similar pattern. The ratios of bound to free essential amino acids which we have found are higher than their quoted controls. The one exception found is histidine, which is excreted mainly in the free form. Proline and hydroxyproline are excreted in grossly increased amounts in the first week. The fact that the maximum excretion of proline occurred on the 3rd day while hydroxyproline is excreted maximally on the 5th day, is presumptive evidence of at least 2 compounds containing these amino acids in bound form in the urine. It is significant that 2 peptides containing both hydroxyproline and proline have been isolated from the urine of patients suffering from rheumatoid arthritis.[11] Our findings provide incontrovertible evidence of continued collagen degradation in the protein-depleted state.

With the enrichment of the diet with both dispensible and non-dispensible amino acids, it is important to note that the excreted peptides become relatively richer in essential amino acids such as valine, phenylalanine, and leucine as compared with glycine and alanine. This finding is also consistent with the appearance of such bound forms as abortive peptide fragments arising as intermediates in protein turnover. The content of the basic amino acids, lysine, arginine, and histidine in bound form was uniformly low although anserine was readily detectable.

It is proposed to make a more detailed examination of the peptides which contribute to the bound-amino nitrogen of the urine, as a contribution to the knowledge of protein metabolism in kwashiorkor.

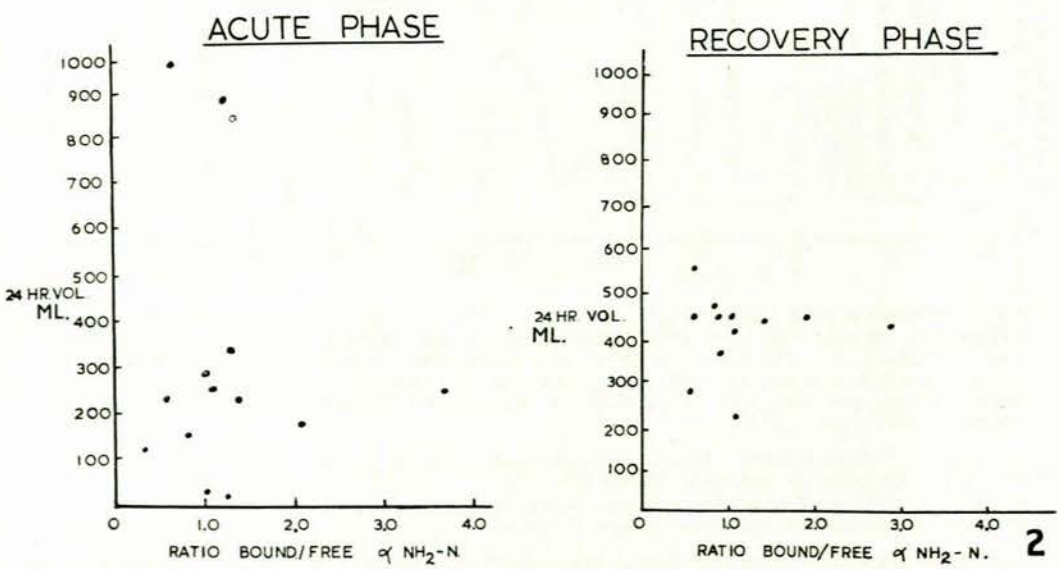

Fig. 2. Lack of correlation between the ratio of bound to free α-NH_2 nitrogen and the 24-hour urinary volume is shown in the acute and recovery phases. Both parameters show a wider scatter during the acute phase.

SUMMARY

The urinary excretion of free and bound forms of amino acids has been investigated in 6 patients with kwashiorkor.

During the acute phase of the disease there is a continued loss of free and bound amino acids and there is an absolute increase in the losses of bound forms in the acute, as compared with the recovery phase. The ratio of free to bound forms remains relatively constant.

In the single patient investigated in more detail, essential amino acids were found in the urine in the acute stage and were present mainly as bound forms.

There is evidence of increased breakdown of collagen in the protein-depleted state.

These findings are discussed with regard to a possible block at the peptide level of protein metabolism in protein-depleted states caused by lack of essential amino acids.

We wish to thank Dr. P. M. Smythe, under whose care the patients were admitted to hospital, for his cooperation in this work and for the clinical data, and Miss M. Hines for technical assistance.

We are also grateful to Dr. J. W. Mostert, Superintendent of the Red Cross War Memorial Childrens' Hospital, for permission to use the records.

This work was supported by a research grant from the Council for Scientific and Industrial Research.

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