5. Myosin adenosine triphosphatase is strongly inhibited by stilboestrol and related compounds. This inhibition is probably of the non-competitive type.

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The Excretion of Amino Acids by the Human

A QUANTITATIVE STUDY WITH ION-EXCHANGE CHROMATOGRAPHY

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Application of the simple, highly specific method of paper chromatography (Consden, Gordon & Martin, 1944) to urine analysis (Dent, 1946) confirmed that certain amino acids were excreted in small amounts in the urine of healthy subjects. Definite patterns of excretion have been later demonstrated, characteristic of the individual and varying but little with exogenous changes (Dent & Harris, 1951; Dent, 1952). Stein (1953), using chromatography on columns of ion-exchange resins, has now reported accurate values for twenty-one urinary amino acids.

In the present study an excessive excretion of some or many of the amino acids in amounts much greater than normal is taken as a definition of amino aciduria. Certain patients show amino aciduria together with characteristic, but apparently unrelated, symptoms and biochemical changes. Such disorders of amino acid output are thought to occur mainly as a result of two distinct mechanisms, the 'renal' mechanism, when the plasma levels are normal and the 'overflow' mechanism when a rise in plasma level is the immediate cause of the increased excretion (Dent, 1954).

Much of the evidence for this hypothesis rests on qualitative experiments, using chiefly paper chromatography. The more quantitative data have been obtained by methods of an insufficiently specific kind, such as α-amino nitrogen determinations, and have given rise to controversy. Thus in the Fanconi syndrome conflicting opinions are held: Dent (1947, 1954) has reported normal plasma levels, but Bickel & Smellie (1952) and Woolf (1951) have reported levels raised 50–100% above the normal range, so that only Dent considers this amino aciduria to be renal.

Of the general methods available for the quantitative determination of amino acids, paper chromatography is subject to large errors with biological fluids. Microbiological assay is limited by several important criteria. For example, only an amino acid for which a known organism has a specific growth requirement can be assayed, the values obtained being liable to error if other compounds can be substituted for the test substance. On the other hand, the quantitative method of ion-exchange chromatography (Moore & Stein, 1951; Stein, 1953) is accurate within the limits of experimental error, can assay all the amino acids present, is unaffected by combined forms or homologues of the amino acids and facilitates the identification of previously unknown constituents. The experimental conditions used do not permit separation of optical isomers, if present, but this is probably unimportant except in feeding experiments with unnatural isomers, which have higher renal clearances than the natural isomers (Bonetti & Dent, 1954). The ion-exchange method, although time-consuming, permits a re-examination of the

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theories of amino aciduria by providing accurate determinations of the amino acids in both urine and plasma. From these figures plasma clearances can be calculated for each individual amino acid.

The present study has been confined to the healthy human and to patients with the Fanconi syndrome (both in the child and the adult), the 'Hartnup syndrome' (defined below), cystinuria, phenylketonuria and a new syndrome in which excessive glycine excretion occurs together with severe osteomalacia. A variant of the normal, involving excretion of large amounts of β-amino*iso*butyric acid in the urine (Crumpler, Dent, Harris & Westall, 1951), has also been studied.

EXPERIMENTAL

Patients studied

Healthy individuals. Nos. 1, 2 and 3 were males aged 27, 45 and 40 years respectively. No. 15 was a female aged 22 years and no. 8 a 41-year-old healthy male showing an excessive excretion of β-amino*iso*butyric acid.

Phenylketonuria. The patient studied (no. 6) was a girl of 2½ years, of apparently unrelated parents, but the elder sibling also was a phenylketonuric. On a constant milk diet (equivalent to about 1·5 g. of phenylalanine/day) she excreted large amounts of phenylpyruvic acid as shown by the strong blue-green colour reaction on addition of $FeCl_3$ solution to the urine. A random urine sample, collected about 3 hr. after food, was analysed and the daily output calculated from the total nitrogen when 24-hourly collections were available subsequently on the same diet.

Child Fanconi syndrome. The patient was a 4-year-old child showing the typical clinical and biochemical signs, including the skeletal lesions, cystine crystals in the cornea and a marked proteinuria. The urine was analysed while she was maintained on a citrate-bicarbonate mixture with large doses of vitamin D_2, and while she was making excellent clinical recovery (Fig. 3 of Dent & Hodson, 1954).

Adult Fanconi syndrome. Subjects 4 and 5 (female); 10 and 11 (male). The four individuals were siblings of unrelated parents, no. 4 being the proposita (see Dent & Harris, 1951). She had a gross amino aciduria as shown by paper chromatography, and severe osteomalacia which had been treated by alkalis and vitamin D. The urine specimen from this patient was infected with bacteria due to inadequate preservation. The analytical values are not reported here but were very similar to those for urine from the patient's sister. Specimen U 5 had been frozen at $-10°$, after addition of toluene, for over a year since the death of the patient. None of the other subjects originally showed any clinical symptoms, although all were short in stature and all showed the usual changes in plasma and urine electrolytes. Since chromatography showed them to have the typical amino aciduria of the Fanconi syndrome (Dent, 1947), they were thought to be in the course of developing the disease. There was also a further sibling normal in every respect. No. 10 has since developed osteomalacia and is being treated with alkalis and vitamin D, but no. 11, the youngest, is well and untreated.

The Hartnup syndrome. An account of this new form of hereditary amino aciduria is in the course of being written up by the many investigators concerned. Brief mention of it has been made by Dent (1952) under the name then in current use, 'Harts syndrome'. The proposita (no. 9) was a female of 22 years. She was almost symptom-free at the time. Her brother (no. 12), aged 14, was studied after a febrile attack of unknown origin. He was on an ordinary diet for one day, before the test period, after being on a low protein diet.

Cystinuria. No. 16 was a man of 44 years diagnosed as cystinuria with stone formation and essential hypertension; X-rays revealed stones in the pelves of both kidneys. He was being treated with sodium bicarbonate and a high water intake.

Osteomalacia with excessive glycine excretion. Certain patients showing vitamin D-resistant rickets or osteomalacia exhibit a high glycine excretion (Astrup & Dent, unpublished observation). Two patients with this 'superglycine' pattern were studied. The first, a 21-year-old woman, had a markedly short stature, gross osteomalacia on X-ray and phosphaturia with a low renal threshold for phosphate. Her plasma phosphate level was lower than normal. The second patient, a 36-year-old man, was clinically similar but had been satisfactorily treated for many years with large doses of vitamin D_2. His urine was analysed when the level of plasma phosphate had been raised by therapy and when he was somewhat overdosed with vitamin D_2.

Collection, treatment and analysis of specimens

Except for the sample from the phenylketonuric child, only samples from 24-hourly collections of urine, preserved at 4° with toluene, were analysed, since random samples would not necessarily give comparable figures between different individuals. About 4 ml. samples of urine from normal adults were required, unless the specimens were unduly concentrated, and 1–2 ml. from pathological cases.

Blood samples were collected at midday, i.e. 4–5 hr. after food, to obtain virtually fasting plasma at a convenient time. At least 30 ml. of blood were collected from an arm vein into a centrifuge tube containing heparin, mixed and spun down at once. Immediately 5 ml. of plasma were added dropwise to each of two 25 ml. portions of 1% (w/v) picric acid solution (Hamilton & Van Slyke, 1943), well shaken and the protein precipitates spun down in the centrifuge. The measured supernatants, filtered through a dry cotton-wool plug to remove floating particles, were passed through a column of 0·5–1·0 g. fine Dowex 2 resin previously washed with 2 N-HCl and distilled water.

The column was then washed with distilled water until the effluent was no longer acid to indicator paper. The liberated HCl was partly neutralized by the addition of 0·4 ml. of N-NaOH and the combined effluent and washings were evaporated to 1–2 ml. in the rotary evaporator (Craig, Gregory & Hausmann, 1950). Samples B 8 and B 9 were freeze-dried at this stage to lessen the risk of hydrolysis. The residual liquid was washed on to a 100 cm. Dowex 50 resin column with pH 3·42 citrate buffer for analysis.

Paper chromatography. All specimens were examined by two-way paper chromatography on large sheets (18 in. × 22·5 in.) of Whatman no. 4 paper, using saturated aqueous phenol solution (in the presence of NH_3 vapour) and collidine–lutidine–water (+diethylamine vapour) as solvents (Dent, 1948). Desalted samples of urine equivalent

to 250 μg. total N (micro-Kjeldahl) were analysed and also samples containing ten times as much N (from normal subjects) and five times as much N (from cases with amino aciduria). The overloaded chromatogram made it possible to detect amino acids present only in trace amounts. Methionine and cystine were detected by oxidation with H_2O_2–ammonium molybdate mixture and subsequent paper chromatography (Dent, 1947). Chromatograms of 125 and 625 μl. of plasma ultrafiltrate were also run after electrolytic desalting. Since the plasma samples were obtained on a different day from that of the urine collection, a random urine specimen, obtained at the same time as the venepuncture, was subjected to paper chromatography to confirm that no gross change had occurred in the urinary pattern.

Urinary cystine determinations. The cystine content of the fresh urine was determined with a Tinsley polarograph, using the method of Reed (1942) removing proteins, if present, by ultrafiltration through a collodion membrane. The supporting buffer solutions were as used by Fowler, Harris & Warren (1952).

Ion-exchange chromatography. The method of Moore & Stein (1951) was used to determine the amino acids of blood plasma after satisfactory yields had been obtained on 12% cross-linked Dowex 50 resin with an acid hydrolysate of purified bovine plasma albumin. For urine analyses the 12% cross-linked resin was also used together with Stein's (1953) modified method. A fraction collector was used to obtain 1 ml. fractions. The positions of eluted peaks were checked by analysing mixtures of synthetic amino acids and testing alternate fractions by paper chromatography. To prepare specimens for the chromatograms, salts were removed by passage through a small column of Zeo-Karb 215 (about 0·7 g., 60–80 mesh/in.) in the acid form and the amino acids eluted by 0·1 N-NH_3. Separate determinations of urinary β-amino*iso*butyric acid were made on 100 cm. columns of 4% cross-linked Dowex 50 resin, eluting the amino acids in 1–5 ml. of urine (previously brought to pH 4·3–4·5 by addition of conc. HCl) by pH 4·75 sodium citrate buffer. After the first 70 ml. of effluent had been discarded, 1 ml. fractions were collected, the β-amino*iso*butyric acid being present at about 80–90 ml. The fractions were analysed by the colour reaction with ninhydrin–$SnCl_2$ reagent, as in the main method, but the concn. of amino acid was calculated by comparison with a calibration curve obtained with synthetic β-amino*iso*butyric acid at pH 4·75. When clear separations were not obtained at pH 4·75 satisfactory analyses were possible at pH 4·5. β-Alanine was eluted in the same range, but it rarely occurs in human urine and was neglected on the evidence of the two-way paper chromatograms. γ-Amino-*n*-butyric acid was probably included in the peak; however, it was equivalent to only 2–4 mg./day at the most.

Urinary amino acids. Some of the taurine results were unsatisfactory, owing to a coincident sudden rise in the pH of the eluate which made it impossible to neutralize exactly the appropriate fractions, with a corresponding inaccuracy in the ninhydrin colour yield. This was probably due to the liberation of sodium ions from the resin by excess of acid in the specimen (Dr J. P. Dustin, personal communication). No separation of aspartic acid from threonine would be expected, possibly owing to the highly acidic fore-run in the modified method of Stein (1953) and the combined peak was calculated as threonine. Paper chromatography suggested an excretion of less than 15 mg./day of aspartic acid by the healthy subjects, as no aspartic acid spot was detected by paper chromatography on analysis of the amount of urine containing 10 × 250 μg. of total N. Not even the slight separation of serine and asparagine reported by Stein (1953) was obtained under the present experimental conditions, and the combined peak was calculated as serine. This peak is probably also contaminated with glutamine as shown for rat-plasma analyses, (Rosen & Levenson, 1953) and for urine from Wilson's disease (Stein, Bearn & Moore, 1954). Minimal values were obtained for the basic amino acids and taurine (Stein, 1953).*

Plasma amino acids. Serine, asparagine and glutamine were again inseparable, being calculated as serine, and similarly tryptophan and ornithine overlapped to form a single peak. Satisfactory arginine peaks were not obtained, or expected.

Approximate plasma clearances. These were only approximate values, as the urine and blood samples were collected at different times. The figures were calculated from the formula: Approximate plasma clearance $= UV/P$ ml. of plasma cleared/min., where U represents the urinary amino acid (mg./100 ml.), P the plasma amino acid (mg./100 ml.), and V the urine volume (ml./min.). Values for U of <5 or <10 mg./day were included in the sum for calculating total clearances.

RESULTS

Normal urinary and plasma amino acids

*β-Amino*iso*butyric acid.* Urine from fifteen healthy males and nine females whose ages ranged from 20 to 55 years was analysed. They can be conveniently divided into three groups according to the amounts of β-amino*iso*butyric acid excreted. Group 1 consisted of nineteen subjects who excreted 4–28 mg./day. Group 2 consisted of three subjects who excreted 41, 43 and 55 mg./day respectively (the figure of 55 mg. was the average for two different days on which the figures obtained were 38 and 72 mg./day respectively). Group 3 consisted of two subjects who excreted 177 and 116 mg./day respectively (the latter was the average for two consecutive days on which the figures obtained were 99 and 134 mg./day respectively).

Other urinary amino acids. The daily histidine excretions for healthy British subjects were consistently much lower than those of Stein (1953) with the same technique. This suggests that the difference is due to a higher protein intake by individuals in the U.S.A., a suggestion which is confirmed by the much higher urinary nitrogen output of the latter. Feeding diets containing in-

* *Note added in proof.* The unidentified ampholytes present in normal urine, in amounts of less than 5 mg./day (Westall, 1955), were not investigated, although minor peaks similar to those of Stein (1953) were constantly observed in the elution diagrams.

creasing amounts of proteins in ordinary foods has resulted in increased histidine excretions (Eckhardt & Davidson, 1949). In addition to the histidine ingested in proteins, carnosine (β-alanylhistidine) present in the diet as a constituent of mammalian muscle (Zapp & Wilson, 1938) may act as a source of urinary histidine, the β-alanine being rapidly deaminated (Graf & Hoberman, 1950). Microbiological assays agree well with the lowest levels for histidine excretion reported in the literature (Frankl & Dunn, 1947), but analyses by chemical methods (such as the bromine colour reaction) tend to be higher, possibly owing to the inclusion of methylhistidines (Armstrong & Walker, 1932; Chatterway, 1947).

The influence of diet on methylhistidine excretion is also very marked, a British subject visiting the U.S.A. showing a change in the urinary pattern involving only histidine and methylhistidine but no other amino acids. Paper chromatography had shown initially only a faint histidine spot with an otherwise normal pattern, but on the more generous American diet a strong histidine spot with a faint methylhistidine spot was superimposed (Miss D. I. Fowler, unpublished work). Taurine excretions from satisfactory experiments were lower than those of Stein (1953), again suggesting a reflexion of the high level of meat consumption in the U.S.A. compared with that in this country.

Most of the urinary assays agree well with published values by other methods, with the following exceptions: for cystine, the polarograph gave values that were 21–48 mg./day higher than those obtained by the ion-exchange method for healthy subjects (Tables 1 and 2). Reed (1942) obtained a normal range of 40–80 mg./day by polarography, which compares with about 10 mg./day by the ion-exchange method (Stein, 1953). The former agrees well with the reported range of 35–63 mg./day by microbiological assay (Camien & Dunn, 1950), which tends to give high estimates. The reason for the different value may be that the polarograph determines some compound, in addition to free cystine, which is present in normal urine in almost constant but larger amounts. The polarographic method determines cysteine with the cystine, but the former is not likely to be present in view of the oxidizing conditions in urine; further, the nitroprusside reaction for –SH groups applied to urine (Brand, Harris & Biloon, 1930) is always negative unless cystine is first reduced to cysteine by the addition of cyanide. Some decomposition of cystine may occur on the resin column during the 2–3 days required to elute this amino acid, although Stein (1953) obtained quantitative recoveries of added cystine. Re-investigation of this problem by ion-exchange chromatography after oxidation of cystine to the more stable cysteic acid (Schram, Moore & Bigwood, 1954) should be a promising approach.

For urinary glutamic acid the higher values obtained by microbiological methods (Dunn, Camien, Shankman & Block, 1947; Steele, Sauberlich, Reynolds & Baumann, 1947) may well be due to the decomposition or microbiological availability of combined glutamic acid (Stein, 1953). Phenylacetylglutamine recently isolated from urine (Stein, Paladini, Hirs & Moore, 1954) might function in such a way. In the present investigation, in order to reduce losses of glutamine etc. specimens were preserved with toluene at 4° and analysed as soon as possible after the end of the collection period.

Results for alanine were lower than by the colorimetric method of Gutman & Alexander (1947), even without subtraction of the aminoadipic acid probably present (Stein, 1953). Methionine levels agreed well with those obtained by microbiological assay (Steele *et al.* 1947), but not with the very high and unlikely results of an unspecific oxidation method (Albanese, Frankston & Irby, 1944). Two urine samples each gave a peak in the position assigned to ethanolamine, the amount calculated being at the lower end of the range reported recently (Luck & Wilcox, 1953).

Normal plasma amino acids. The literature shows a lack of plasma levels for taurine, aspartic acid, proline, ornithine, ethanolamine, α-amino-*n*-butyric acid and β-amino*iso*butyric acid. Comparison of the values obtained in the present work with available plasma levels for the remaining amino acids (Table 3) showed the following differences: Use of a decarboxylase gave higher values for glutamic acid together with reduced glutamine levels (Krebs, Eggleston & Hems, 1949). Possibly this is due to hydrolysis of a labile precursor of glutamic acid, if not some of the glutamine itself, during the acid pretreatment of the plasma. Polarography applied to the determination of plasma cystine (Fowler *et al.* 1952; Dent, Senior & Walshe, 1954) gave results which agreed well with analyses on ion-exchange resins (Stein, Bearn & Moore, 1954), but the few values reported in the present paper were lower. The plasma citrulline content would appear to be low, as a definite peak was seen with only one sample and was equivalent to only about one-half of the value found by Archibald (1944). Resolution of the peak in the tryptophan–ornithine position was not attempted here. Microbiological values for methionine (Harper, Kinsell & Barton, 1947), threonine, isoleucine, leucine and histidine (Hier & Bergeim, 1946) seem all comparatively high (Table 3). Peptides or other derivatives present may be microbiologically available and may therefore be included in these assays.

Amino aciduria

Phenylketonuria. The values for urinary amino acids for the child with phenylketonuria were compared with those for two normal 1-year-old children, on a diet of cow's milk equivalent to 12–15 g. of protein/day (Jonxis & Huisman, 1953). Most of the values agreed with the normal controls within a factor of 2, with the outstanding exception of the phenylalanine, the value of 90 mg./day representing some thirty times that in the controls (Table 2). The minor increase for glycine may be dietary or inherent in the patient's genotype, while the slightly raised histidine almost certainly originated in the diet. The cystine value was very low but the polarographic figure was nearer to the normal. This abnormally high phenylalanine excretion, equivalent to nearly

Table 1. *Free urinary amino acids (mg./24 hr. collection) of healthy adults*

	Normal range: earlier values obtained by		Present values obtained by ion-exchange chromatography for				
			Males				
Amino acid	Ion exchange[a]	Other methods¶	U1	U2	U3	Female U15	'β' excretor U8, ♂
Taurine	86–294	—	81	61	35	80	61
Hydroxyproline	—	—	—	—	—	—	—
Aspartic acid*	<10	0–1[c, d]	17^{-}	30^{-}	37^{-}	11^{-}	18^{-}
Threonine	15–53	19–25[d]					
Serine	27–73	21–27[c]	44	134	100	30	53
Asparagine	34–92	—					
Sarcosine	—	—	5	9	9	<5	—
Glutamic acid	8–40	0–61[c, e]	7	12	19	5	<5
Proline	<10	7–15[c]	—	—	—	<5	<5
Citrulline	—	—	—	—	—	<5	<5
Glycine	68–199	120–737[e, f] (C)	68	112	148	24	84
Alanine†	21–71	—	17	29	37	12	22
Cystine‡	10–21	40–108[g, h] (P)	5 (26)	15 (63)	6 (27)	<5 (40)	<5 (27)
Valine	4–6	4–12[d, i]	<5	<5	8	<5	<5
Methionine	<5–10	2–8[c, d]	6	9	<5	<5	<5
Isoleucine	14–28	3–14[e, i]	<5	22	13	<5	14
Leucine	9–26	0–13[c]	10	9	<5	<5	<5
Tyrosine	15–49	11–44[d, j] (E)	18	24	26	11	25
Phenylalanine	9–31	3–34[c, d]	11	18	13	9	13
β-Amino*iso*butyric acid	—	—	18	13 (15)‖	9	5	185
γ-Amino-*n*-butyric acid	—	—	<5	<5 (7)	<5	<5	<5
3-Methylhistidine	50[b]	—	33	47 (47)	40	21	33
Histidine	113–320	60–188[c, d]	59	101 (131)	130	16	76
1-Methylhistidine	47–384	—	9	28	29	10	43
Hydroxylysine	—	—	—	—	—	—	—
Tryptophan	—	8–25[c, d]	?	?	—	?	—
Ornithine		—					
Lysine	7–48	1–47[c, i]	7	17	—	8	<5
Ethanolamine	—	5–57[k] (C)	11	—	—	<5	<5
Arginine	—	4–21[c, d]	?	?	?	24	?
Total§	789–1194	—	426	690	663	266	627
Urine volume (ml.)	900–2250	—	1630	1157	1470	840	1790
Total nitrogen (g.)	9·7–22·1	—	11·4	10·9	15·1	5·9	11·0
Body weight (kg.)	61·2–88·5	—	53·7	70·0	72·5	61·1	65·0

— Indicates that no peak was obtained in a position considered appropriate for a particular amino acid.
? Denotes that a peak was obtained but that its identity was uncertain owing to the anomalous position or asymmetry of the peak.
* ⁻ implies <15 mg./day of aspartic acid by paper chromatography.
† Alanine probably includes some aminoadipic acid (Stein, 1953).
‡ Polarographic values for cystine in parentheses.
§ Values given as <5 or <10 are not included in the totals.
‖ Values in parentheses for the basic amino acids under the heading U2 were obtained on the short (15 cm.) Dowex 50 column.
¶ Microbiological method unless otherwise stated.
C Colorimetric method. P Polarographic method. E Enzymic method.
[a] Stein, 1953. [b] Tallan *et al.* 1954. [c] Steele *et al.* 1947.
[d] Woodson, Hier, Soloman & Bergeim, 1948. [e] Dunn *et al.* 1947.
[f] Gutman & Alexander, 1947. [g] Reed, 1942. [h] Frankl & Dunn, 1947.
[i] Eckhardt & Davidson, 1949. [j] Lawrie, 1947. [k] Luck & Wilcox, 1953.

Table 2. *Free urinary amino acids (mg./24 hr. collection) in amino aciduria determined by ion-exchange chromatography*

Amino acid	Normal[a] adult (average)	Adult Fanconi syndrome			Hartnup syndrome		Osteomalacia		Cystinuria	Child Fanconi syndrome	Child phenylketonuria	Two normal children[b]	
		U5, ♀	U10, ♂	U11, ♂	U9, ♀	U12, ♂	U7, ♀	U13, ♂	U16, ♂	U14, ♀	U6, ♀	1	2
Taurine	156	29	113	124	23	23	127	56	98	30	7	—	—
Hydroxyproline	—	—	—	31	—	—	4	—	—	—	—	—	—
Aspartic acid*	<10 }	Trace }	605- (Aspartic acid + Threonine)	920- (Aspartic acid + Threonine)	617- (Aspartic acid + Threonine)	540- (Aspartic acid + Threonine)	30- (Aspartic acid + Threonine)	17- (Aspartic acid + Threonine)	5- (Aspartic acid + Threonine)	58- (Aspartic acid + Threonine)	5- (Aspartic acid + Threonine)	7·2	1·9
Threonine	28 }	142 }										4·8 }	
Serine	43 }	130 (Serine + Asparagine)	1200 (Serine + Asparagine)	1250 (Serine + Asparagine)	2290 (Serine + Asparagine)	1720 (Serine + Asparagine)	20 (Serine + Asparagine)	43 (Serine + Asparagine)	18 (Serine + Asparagine)	153 (Serine + Asparagine)	18 (Serine + Asparagine)		16·4
Asparagine	54 }											14·4 }	
Sarcosine	—	11	75	19	—	—	—	5	—	5	—	—	—
Glutamic acid	10	181	522	165	332	207	54	<5	<5	38	4	6·8	3·7
Proline	<10	218	1150	670	5	5	42	16	<5	18	—	3·4	0
Citrulline	—	82	—	380	30	77	7	—	—	—	—	—	—
Glycine	132	413	1010	980	440	278	438	226	140	116	41 }	14·0 (Glycine + Alanine)	26·3 (Glycine + Alanine)
Alanine†	46	237	1400	764	825	420	22	11 }	1050 (Alanine + Cystine)	106	9 }		
Cystine‡	10	58	84	100	39	48	<5	<5 }		11	0·1	4·7	3·8
		(92)	(190)	(195)	(108)	(80)	(27)	(29)	(870)	(48)	(9)		
Valine	<10	71	488	467	131	390	<5	11	?	77	4	1·6	2·5
Methionine	<10	15	91	77	46	75	<5	<5	6	7	1	3·4	2·4
Isoleucine	18	24	173	135	151	240	12	14	60	14	3	2·4	3·0
Leucine	14	35	310	244	120	280	<5	<5	20	54	1	4·7	6·1
Tyrosine	35	90	397	426	630	610	11	15	28	59	4	6·4	14·0
Phenylalanine	18	77	324	206	138	284	6	10	8	39	94	2·6	3·7
β-Amino*iso*butyric acid	—	9	49	158	18	13	21	26	6	36	2	2·3	0
γ-Amino-*n*-butyric acid	—	—	—	<5	5	<5	<5	<5	<5	<5	0·4	—	—
3-Methylhistidine	—	7	—	30	25	9	25	30	34	—	2	—	—
Histidine	216	36	500	620	770	470	43	51	91	19	34	27·0	14·3
1-Methylhistidine	180	7	129	270	Trace	10	178	214	75	—	0·5	—	—
Hydroxylysine	—	—	—	—	—	—	—	—	<5	—	—	—	—
Tryptophan } Ornithine }	—	—	93	350	—	—	—	—	340	—	—	—	—
Lysine	19	47	730	650	63	88	<5	<5	1400	27	4	2·2	3·8
Ethanolamine	—	47	—	—	—	—	—	—	<5	—	—	—	—
Arginine	<10	?	370	49	?	?	?	?	1300	?	—	4·1	3·0
Total§	979	1966	9813	9085	6653	5772	1040	745	4674	862	233	112	105
Urine volume (ml.)	1470	2050	3380	1580	2230	785	1645	2060	2930	1610	—	—	—
Total nitrogen (g.)	15·1	6·8	13·8	13·3	7·3	6·4	9·1	11·7	10·6	4·9	2·1–2·8	—	—
Body weight (kg.)	72·5	35·7	60·0	59·0	44·8	32·2	35·6	52·8	62·7	13·5	10·7	—	—

— Indicates that no peak was obtained in a position considered appropriate for a particular amino acid.
? Denotes that a peak was obtained but its identity was uncertain owing to the anomalous position or asymmetry of the peak.
* - Implies <30 mg./day of aspartic acid by paper chromatography.
‡ Polarographic values for cystine in parentheses.
[a] Stein (1953), by ion-exchange method.
† Alanine probably includes some aminoadipic acid (Stein, 1953).
§ Values given as <5 or <10 are not included in the totals.
[b] Jonxis & Huisman (1953), by ion-exchange method.

Table 3. *Free amino acids of blood plasma (mg./100 ml.)*

Amino acid	Earlier normal values by: Ion exchange[a]	Earlier normal values by: Other methods	Present values by ion-exchange chromatography for: Normal males B1*	Normal males B2	Normal female B15	β 'excretor' ♂, B8§	Adult Fanconi syndrome ♂, B10§	Hartnup syndrome ♀, B9
Taurine	0·4–0·8	—	1·24	0·74	0·34	0·74	0·62	0·82
Aspartic acid	Trace	—	0·2	0·06	—	—	0·03	—
Threonine	1·2–1·7	2·02[b]	1·47	1·34	1·51	1·34	1·15	0·61
Serine†	1·0–1·3	1·14[c] }	4·83 (Serine + Asparagine)	5·82 (Serine + Asparagine)	4·78 (Serine + Asparagine)	5·98 (Serine + Asparagine)	4·68 (Serine + Asparagine)	5·52 (Serine + Asparagine)
Asparagine	—	1·25[d] }						
Glutamic acid	0·4–1·2	3·41[e]	1·24	0·82	0·77	0·29	1·73	0·72
Citrulline	—	—	—	—	—	0·29	—	—
Proline	1·8–3·3	—	2·67	1·65	1·53	1·20	2·50	1·29
Glycine‡	1·5–2·0	1·77[f]	1·92	1·35	1·15	1·58	1·74	1·07
Alanine	3·0–3·7	3·97[f] }	3·36 (Alanine + Cystine)	3·38	2·40	2·66	3·86	2·10
Cystine	1·0–1·3	1·06[g] }		0·88	0·81	0·68	0·39	0·60
α-Amino-*n*-butyric acid	0·2–0·35	—	0·14	0·17	0·12	0·28	0·12	0·10
Valine	2·3–3·7	2·83[b]	2·16	2·52	1·93	1·51	1·70	1·14
Methionine	0·3–0·4	0·85[h]	0·59	0·40	0·22	0·34	0·54	0·28
Isoleucine	0·7–1·3	1·60[b]	1·11	0·87	0·72	0·81	0·97	0·48
Leucine	1·4–2·3	1·91[b]	1·45	1·48	1·10	1·23	1·38	0·81
Tyrosine	0·8–1·5	1·48[b]	0·94	1·11	0·87	0·65	0·92	0·66
Phenylalanine	0·7–1·0	1·38[b]	0·67	0·80	0·82	0·81	1·05	0·71
β-Amino*iso*butyric acid	—	—	0·28	Trace	—	0·15	Trace	—
Histidine	0·8–1·5	1·42[b]	0·74	0·93	1·02	1·10	1·05	0·43
Tryptophan	Trace	1·08[b] }	0·75 (Tryptophan + Ornithine)	0·78 (Tryptophan + Ornithine)	Trace }	0·60 (Tryptophan + Ornithine)	0·67 (Tryptophan + Ornithine)	0·46 (Tryptophan + Ornithine)
Ornithine	0·6–0·8	— }			0·63 }			
Lysine	2·5–3·0	2·95[b]	2·42	2·15	1·33	1·71	1·82	1·41
Ethanolamine	Trace	—	0·03	—	0·04	Trace	Trace	Trace
Arginine	1·2–2·0	2·34[b]	1·55	?	?	?	?	?
Total	25–28	—	29·8	27·3	22·1	24·0	26·9	19·2

* The same numbering of subjects has been used as in Tables 1 and 2.

† The serine/asparagine peak, probably also containing glutamine, was calculated as serine.

‡ The normal range given by Stein, Bearn & Moore (1954) for glycine also includes citrulline, not separated in their experiments.

§ Deproteinized plasma from B8 and B10 was freeze-dried; all others were evaporated *in vacuo* at low temperature.

[a] Stein, Bearn & Moore, 1954. [b] Hier & Bergeim, 1946. [c] Borden *et al.* 1950.
[d] Barry, 1953. [e] Krebs *et al.* 1949. [f] Christensen & Lynch, 1946.
[g] Dent *et al.* 1954. [h] Harper *et al.* 1947.

Table 4. *Approximate plasma clearances of amino acids*

ml. cleared per minute.

Amino acid	Normal males C1	Normal males C2	Normal female C15	β 'excretor' ♂, C8	Adult Fanconi syndrome ♂, C10	Hartnup syndrome ♀, C9
Taurine	4·6	6·1	1·6	5·7	12	1·9
Aspartic acid/threonine	0·6	1·5	0·5	0·9	34	70
Serine/asparagine	0·7	1·6	0·4	0·6	17	29
Glutamic acid	0·4	1·0	0·5	0·3	21	32
Citrulline	—	—	—	1·3	—	—
Proline	0·1	—	0·2	0·3	32	0·3
Glycine	2·5	5·7	1·5	3·7	41	29
Alanine }	0·5 (Alanine + Cystine)	0·6	0·3	0·6	25	27
Cystine }		1·2	0·6	0·6	15	4
Valine	0·2	0·1	0·2	0·3	20	8
Methionine	0·7	1·6	1·6	1·2	12	12
Isoleucine	0·3	1·7	0·5	1·2	12	22
Leucine	0·5	0·4	0·3	0·3	16	10
Tyrosine	1·3	1·5	0·9	2·7	30	66
Phenylalanine	1·1	1·5	0·8	1·1	20	14
β-Amino*iso*butyric acid	4·4	—	—	85	—	—
Histidine	5·5	7·5	1·1	4·8	25	122
Lysine	0·2	0·5	0·4	0·2	29	3
Clearance of total amino acids	1·1	1·9	1·0	1·6	25	25

one-half of the total amino acids in the urine, agrees well with other studies in phenylketonuria (Dann, Marples & Levene, 1943; Jervis, 1950).

Microbiological assay by Dr W. F. J. Cuthbertson of the plasma phenylalanine of the patient (while on a constant diet) 3 weeks and 1 week before the urine sample was collected gave results of 26 and 28 mg./100 ml. respectively. These values agree well with published values in phenylketonuria obtained by a similar method (Borek, Brecher, Jervis & Waelsch, 1950). The phenylalanine level was about fifteen times that for normal children. Its approximate plasma clearance was 0·2 ml./min., uncorrected for the reduced surface area in the case of a child. The four healthy adults showed approximate clearances of 0·8–1·5 for phenylalanine (Table 4), suggesting that its clearance in the phenylketonuric child was probably normal.

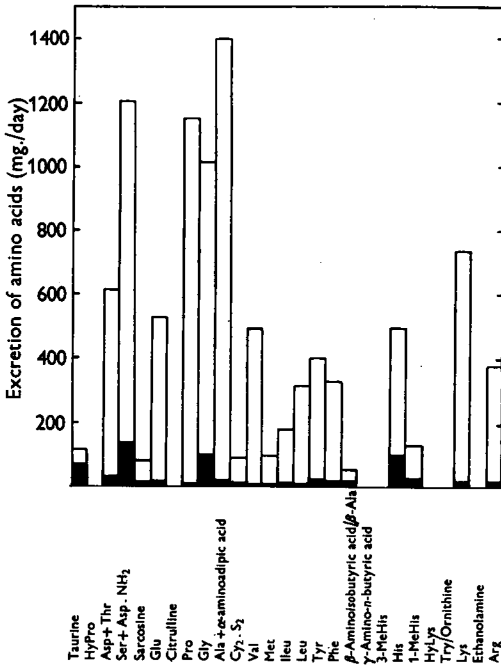

Fig. 1. Fanconi syndrome in the adult (♂, U10). Normal ♂, ■; pathological, □.

The Fanconi syndrome (child and adult forms). All the subjects studied showed excessive and qualitatively similar amino acid excretions, the totals varying from two to five times the average normal content (Fig. 1) and the amino aciduria being unaffected by treatment. Blood plasma analysed for one of the adult cases gave values similar to those obtained for the two healthy males and also to the figures already published for the normal (Stein, Bearn & Moore, 1954). The low cystine value may reflect its enhanced elimination in the urine and consequent loss from the blood, as occurs in cystinuria (Dent *et al.* 1954). Approximate plasma clearances for most of the amino acids were very high, agreeing well with the high clearances calculated from α-amino nitrogen analyses by the ninhydrin–CO_2 method (Dent, 1954).

The Hartnup syndrome. The characteristic urinary amino acid pattern revealed by paper chromatography (Dent, 1952) was confirmed, especially the high values for serine, asparagine and citrulline, the normal values for taurine, proline and β-amino*iso*butyric acid, and the low value for 1-methylhistidine (Fig. 2). The last was particularly important, as the histidine excretions were high in both cases, suggesting that the excretion of 1-methylhistidine here is not determined solely by

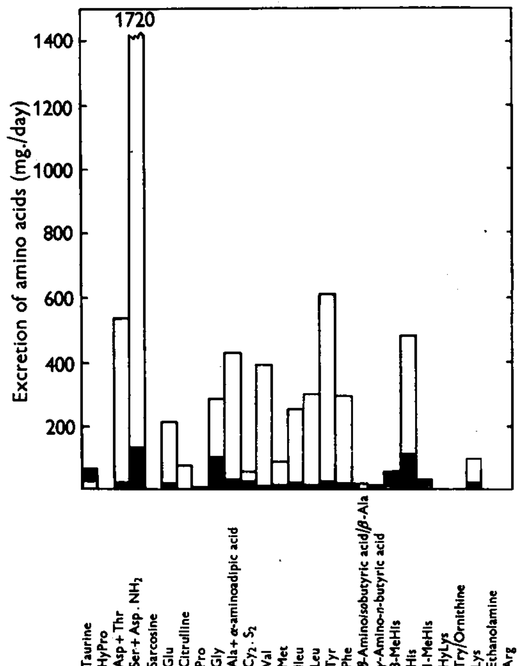

Fig. 2. Hartnup syndrome (♂, U12). Normal ♂, ■; pathological, □.

dietary habit. The high clearance of 122 for histidine in the female case, approximately equivalent to the average normal glomerular filtration rate for the adult, suggests a complete lack of reabsorption.

Cystinuria. The very high excretions for cystine, ornithine, lysine and arginine confirmed the excretion pattern for this disorder (Fig. 3). The presence of more than traces of tryptophan, which coincides with ornithine as a single peak, was

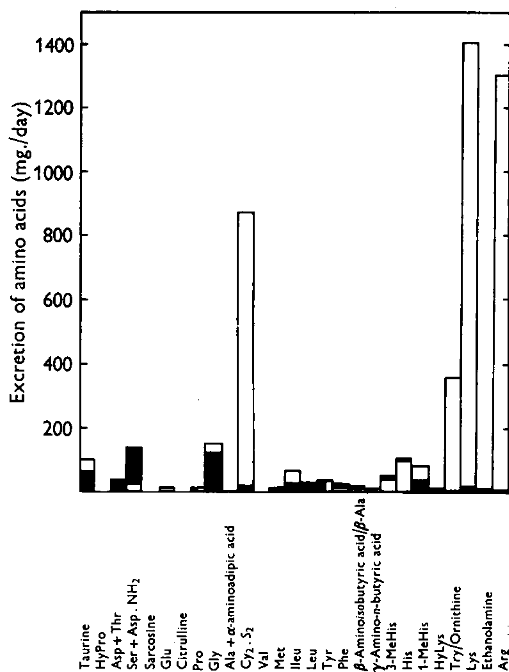

Fig. 3. Cystinuria (♂, U16). Normal ♂, ■; pathological, □.

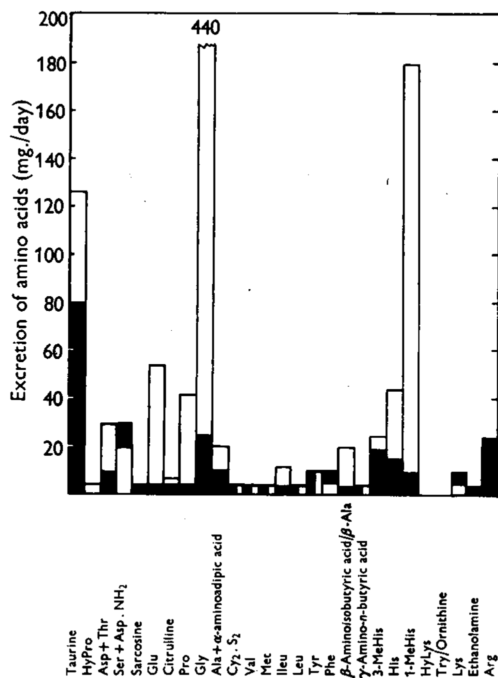

Fig. 4. Osteomalacia with excessive glycine excretion (♀, U7). Normal ♀, ■; pathological, □.

excluded by paper chromatography. An apparent twofold increase in isoleucine was noted, but may be an artifact (Stein, 1953). A small amount of hydroxylysine, not normally found in urine, was also detected, but a low taurine level (Stein, 1951) was not confirmed. Certain amino acids in the cystinuric urine were also assayed microbiologically by Miss E. B. Robson, using *Escherichia coli*. She found a value of 1·27 g./day for arginine, compared with 1·30 by ion exchange, and a value of 1·72 g./day for lysine, compared with 1·40 by ion exchange and 1·74 by paper electrophoresis (by Dr H. Harris).

Osteomalacia with excessive glycine excretion. The two patients studied were selected as the result of paper-chromatographic studies in this laboratory of a large number of people with a similar clinical condition (idiopathic or vitamin D-resistant osteomalacia). In both of these patients the output of glycine, proline and 1-methylhistidine was raised, according to the results of ion-exchange chromatography. In addition, the female patient showed an increased taurine excretion, probably of dietary origin, and small amounts of hydroxyproline and citrulline in the urine (Fig. 4). The glycine excretions seemed approximately constant for each individual, when estimated by paper chromatography, and were not influenced when phosphate excretion was greatly altered by variations in intake.

DISCUSSION

Numerically the approximate plasma clearances of amino acids for healthy humans form two badly defined groups, high values being obtained for histidine, taurine and glycine and low values for most of the other amino acids. With the exception of glycine, excretion of the first group appears to be grossly influenced by dietary intake, and the second group is little influenced by such exogenous factors.

High values for the plasma 'clearance' of histidine have also been calculated on the results of microbiological assay (Sheffner, Kirsner & Palmer, 1948). The actual figures are not at all comparable, since these workers calculated from the total plasma and urinary amino acids assayed after hydrolysis. Further, their use of the formula (plasma clearance $= U\sqrt{V/P}$) was not justified, as recent evidence suggests that the rate of amino nitrogen excretion does not vary with urine volume as does that of urea (Dent, 1954). The high plasma clearance of histidine may account, in part at least, for the increased histidine excretion by pregnant women (Armstrong & Walker, 1932; Soupart, 1952). An already low renal threshold, if associated with a slightly raised plasma level after the increased turnover in amino acid metabolism, would lead to an increase in the amount of histidine

eliminated. Simultaneous analyses of plasma and urine throughout pregnancy are necessary to test such an hypothesis.

Calculation of plasma clearances for the methylhistidines was not possible, as the plasma levels are too low to permit accurate determination. The high level of the methylhistidines in human urine, compared with their very low plasma level, suggests that the tubular reabsorption of these amino acids is very inefficient or that they are formed by the kidney. Urinary 1-methylhistidine is thought to arise from ingested anserine (β-alanyl-1-methylhistidine), which is well known to occur in most vertebrate muscle (Datta & Harris, 1951). The isomeric 3-methylhistidine recently isolated from urine (Tallan, Stein & Moore, 1954) may be derived from a similar β-alanyl peptide, but such a derivative has not yet been shown to occur in any tissue or body fluid.

The taurine excretion may be correlated with the dietary intake of protein, but certain inbred mice show differences in taurine excretion (Harris & Searle, 1952), apparently due to genetically determined differences in the renal threshold for taurine. Taurine almost certainly arises mainly from the metabolism of cystine or cysteine (Awapara & Wingo, 1953), these being readily interchangeable by reduction–oxidation. Many detoxication mechanisms result in the synthesis of more acidic compounds which are said to be more readily eliminated by the kidney than their precursors (Quick, 1932). Hence the high clearance of taurine may be associated with its high acidity. However, since other, only slightly less acidic, amino acids (e.g. aspartic acid) are excreted in low concentrations, this explanation can only be tentative in the absence of other evidence.

The significance of the high plasma clearance for glycine is unknown, although the first member of any homologous series of organic compounds usually shows anomalies in chemical and physiological reactions. Glycine can certainly exhibit unorthodox metabolic behaviour (Bach, 1952), and its competition with phosphate for renal reabsorption may well be a further manifestation. However, alanine infusions are also known to depress phosphate reabsorption (Ayer, Schiess & Pitts, 1947).

The second group, with low plasma clearances, includes most of the common amino acids. These amino acids are excreted at levels characteristic for the individual in a pattern little influenced by exogenous factors. It has been demonstrated by paper chromatography that in normal subjects three patterns of amino acid excretion commonly prevail, as far as the principal constituents are concerned, involving, namely: (1) glycine alone; (2) glycine and taurine; (3) glycine and β-amino-*iso*butyric acid (Dent & Harris, 1951). Each particular type seems constant, and is attributed to genetic as opposed to environmental factors, although, as shown above, taurine may play a double role.

The approximate plasma clearance of β-amino-*iso*butyric acid for an hereditary 'excretor' of this substance is particularly interesting, the approximate value of 85 ml. of plasma cleared/min., compared with the average value of 127 for glomerular filtration rate (Smith, Goldring & Chasis, 1938), suggests that it undergoes little if any reabsorption from the renal tubule, although the other amino acids were excreted in normal amounts by this subject. These clearance data recall the situation in human cystinuria, where the clearance of cystine is approximately equal to that of inulin, also suggesting an absence of renal tubular reabsorption (Dent *et al.* 1954). Cystinuria, too, is well known to be under genetic control (Dent & Harris, 1951).

Each type of hereditary amino aciduria may be considered in relation to the 'overflow' and 'renal' types of amino aciduria (Dent, 1954). Only phenylketonuria, of the types considered here, may be attributed to the 'overflow' mechanism. The urinary output of phenylalanine increases solely as the result of the raised plasma concentration, in full agreement with the report that the enzyme responsible for the oxidation of phenylalanine to tyrosine is absent from the liver in this disorder (Jervis, 1953). The low value for urinary tyrosine was interesting, as this amino acid is not considered to be essential for normal individuals but may be essential in phenylketonuria if, as is generally believed, phenylketonurics are unable to form any tyrosine at all from phenylalanine or its metabolic products.

The raised clearance values clearly place the amino aciduria of the adult form of the Fanconi syndrome in the class arising from an inborn defect of renal reabsorption as originally suggested for the child form by Fanconi (1936). Reports that the plasma α-amino nitrogen and the distribution of amino acids in an adult case were both normal agree with this theory (Dent, 1947).

In the Hartnup syndrome the low plasma levels for many of the individual amino acids and the low sum total for these levels combined with the raised clearances again point to defective renal reabsorption. This conceivably shifts the equilibrium of competing factors influencing the plasma level sufficiently to result in a low plasma concentration of most of the amino acids. Why this is not observed also in the Fanconi syndrome is not obvious.

The characteristic excessive excretions of ornithine, lysine and arginine, as well as cystine,

were confirmed in cystinuria. Recent studies on the plasma cystine have revealed a statistically significant low plasma level in cystinuria (Dent *et al.* 1954) consistent with other evidence that this disorder constitutes a severe renal defect of tubular function (Dent & Rose, 1951).

Deranged phosphate metabolism associated with excessive glycine excretion, which occurred in the patients with idiopathic osteomalacia, may be of theoretical significance in view of the known competition of phosphate and glycine for reabsorption from the kidney tubule (Ayer *et al.* 1947). The increased urinary glycine and proline may be connected with the high content of these compounds in collagen (Block & Bolling, 1951), part of the organic matrix which forms bone after calcification. Collagen also contains a large amount of hydroxyproline (Neuman & Logan, 1950), but as only one patient excreted a detectable amount the finding may not be typical. The significance of the citrulline in this patient's urine and the high excretion of 1-methylhistidine in both cases cannot be explained. The condition may even be a variation of normality comparable with β-amino*iso*butyric aciduria, but the final opinion must await further investigations specially directed towards a study of the plasma clearance of glycine in such people and in healthy individuals.

The determination of plasma clearances for the amino acids by ion-exchange chromatography affords an elegant means of classifying a given amino aciduria, whether it be inherent or induced by infective or chemical agents. The improved gradient-elution method described since the present study was completed (Moore & Stein, 1954) could be used to study a number of problems. For example, the mechanism of the amino aciduria occurring in galactosaemia (Holzel, Komrower & Wilson, 1952) could be determined. Many toxic substances may inhibit renal reabsorption, but the evidence is often scanty or merely qualitative. Cases of amino aciduria following burns (Rosen & Levenson, 1953; Nardi, 1954) and poisoning by lead (Wilson, Thomson & Dent, 1953), uranium (Voegtlin & Hodge, 1949), 'Lysol' (Spencer & Franglen, 1952) or maleic acid (Harrison & Harrison, 1954) merit re-investigation.

SUMMARY

1. By ion-exchange chromatography daily excretions of amino acids and midday plasma levels were determined for a number of healthy adults and for patients with amino aciduria.

2. The normal subjects and each of the patients studied showed distinctive patterns of amino acid excretion, patients with the same syndrome showing characteristic similar patterns.

3. In phenylketonuria a high phenylalanine output and a raised plasma level were found with a normal clearance. This suggests an overflow mechanism due only to the renal threshold of phenylalanine being exceeded.

4. Conversely, low or normal plasma levels were found with the amino acidurias observed in the adult form of the Fanconi syndrome, the Hartnup syndrome and β-amino*iso*butyric aciduria. This suggests defective renal tubular reabsorption of specific amino acids consistent with previous views based on paper chromatography. The approximate plasma clearances of certain amino acids were of the order to be expected of the glomerular filtration rate.

5. The ion-exchange method also proved convenient for the quantitative determination of β-amino*iso*butyric acid. Assays on specimens from twenty-four healthy subjects gave results compatible with published genetical researches using paper chromatography.

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The Enzymic Activation of Cholic Acid by Guinea-pig-Liver Microsomes

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Cholic acid is found in bile conjugated with the two amino acids glycine and taurine. For a long time this process of conjugation has been thought to occur in the liver, although nothing was known about the mechanism by which the peptide-like bond of taurocholic acid is formed. During the study of this problem it was found (Elliott, 1955) that formation of an hydroxamic acid occurs in preparations of guinea-pig-liver microsomes. This is dependent on the presence of cholic acid and of coenzyme A (CoA) and adenosine triphosphate (ATP). The present paper is concerned with this activation of cholic acid.

EXPERIMENTAL

Materials

ATP. This was isolated from rabbit muscle by the method of Needham (1942). The potassium salt was prepared from dibarium ATP by dissolving the latter in the minimum volume of N-HCl, running this solution through