CONJUGATED AMINO ACIDS IN PLASMA OF PATIENTS WITH UREMIA *

By GEORGE W. FRIMPTER,† DAVID D. THOMPSON AND E. HUGH LUCKEY

(From the Department of Medicine, Cornell University Medical College and The New York Hospital, New York, N. Y.)

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In patients with renal insufficiency, the elevated plasma nonprotein nitrogen cannot be accounted for solely by the rise in the urea nitrogen (2). It is known that other nitrogenous materials such as creatinine and uric acid contribute to this elevation. Since free amino acids also contribute to the nonprotein, nonurea nitrogen of plasma, several investigators have determined the amino acid composition of plasma from both normal subjects and patients with uremia. They have shown that in uremia, concentrations of the individual plasma are normal or that such alterations in concentrations as exist are slight and probably insignificant (3–7). In addition, the total α-amino nitrogen in plasma of patients with uremia remains approximately normal (3–10). However, studies in which deproteinized plasma was hydrolyzed revealed that some amino acids are variably increased in concentration (3–6). These findings suggested the presence of one or more amino acid conjugates in the plasma of patients with uremia.

The investigations described in this paper were undertaken to characterize further the amino acid conjugates in the plasma of patients with uremia. The general plan of the study consisted of measurement of α-amino nitrogen in deproteinized plasma of normal subjects, and of patients with uremia before and after hydrolysis. By means of dialysis through cellophane, rough indications were obtained of the molecular size of conjugates containing nitrogen. By chromatographic separation and serial fraction collections, the sources of conjugate nitrogen were concentrated. Attempts were then made to identify the compounds accounting for the increase in α-amino nitrogen of plasma after hydrolysis.

METHODS

Patients. Patients used in this study had been hospitalized with symptomatic chronic renal insufficiency due to a variety of renal diseases including chronic glomerulonephritis, chronic pyelonephritis, nephrosclerosis, and congenital polycystic kidney disease. None had liver disease, diabetes mellitus, or other metabolic diseases. None had recent surgery, wounds, or infarction. None was receiving a blood transfusion at the time a specimen was obtained or within 24 hours prior to collection. Blood specimens were taken from an antecubital vein without stasis, through an 18-gage needle into a siliconized glass syringe, and transferred immediately into a siliconized Erlenmeyer flask containing 0.1 mg of dried sodium heparin per 10 ml of blood. The cells were separated promptly by centrifugation and the plasma was processed immediately.

Alpha-amino nitrogen before and after hydrolysis. Plasma proteins were removed by precipitation with 1 per cent picric acid (10). The picric acid was subsequently removed by passage of a measured portion of the filtrate through a Dowex-2 resin column as described by Stein and Moore (11). Ultrafiltration through Visking cellophane was performed at 4° C under 400 pounds per square inch of N_2 pressure (12). An aliquot of each filtrate was then hydrolyzed by sealing it with an equal volume of 12 N hydrochloric acid in a Pyrex test tube under nitrogen, and heating at 110° for 22 hours. The acid was subsequently removed on a rotary evaporator. The ninhydrin reaction was performed in triplicate at pH 2.5 by the gasometric ninhydrin method of Hamilton and Van Slyke (10).

Because some CO_2 evolves from urea in the ninhydrin reaction, it was found desirable to remove the urea. Triplicate aliquots of each specimen, before and after hydrolysis, were placed in the ninhydrin reaction vessels, buffered to pH 6.1, and incubated 12 hours at 40° C with urease (10). The urease had previously been purified by dialysis to remove canavanine and other ninhydrin-reacting substances, thus reducing the "blank" determination (13). After 12 hours of incubation the reaction mixture was found to be free of urea, as determined by the method of Van Slyke and Cullen (14). Under the conditions described, less than 12 hours' incubation with

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† Senior Research Fellow, New York Heart Association.

urease was inadequate. The 12 hours of incubation did not alter the α-amino nitrogen determination of sample plasmas. Recovery of α-amino nitrogen added as glycine and glutamic acid to uremic plasma was 100 ± 2 per cent. Plasma urea nitrogen was performed in duplicate (14); uremic plasma specimens were diluted with water (usually 1:10) so that all titrations were performed in the same range.

Dialysis of amino acid conjugates. After removal of picrate, plasma filtrates were concentrated 4× on a rotary evaporator and adjusted to pH 3.4. Using the Craig, King and Stracher dialysis cell (15), 2 ml of the specimen was placed in an acetylated 18/32 Visking cellophane sac which was then transferred through a series of test tubes containing 5 ml of 0.01 M acetic acid, pH 3.4 (15). Analyses performed on the dialysate, on an aliquot of the initial specimen, and on the material remaining in the sac at the end of the experiment included: colorimetric ninhydrin after alkaline hydrolysis in triplicate (16), urea (14), glucose (17).

Chromatographic separation of amino acid conjugates on ion-exchange resins. A picric acid plasma filtrate was added to the top of a 1.8 × 165 cm column of Amberlite 1R 120 resin (18) equilibrated with 0.2 N, pH 2.20 sodium citrate (19), or 0.2 N, pH 3.25 sodium citrate buffer (20). These were pumped through at a rate of 50 ml per hour, and approximately 300 3.0-ml fractions were collected. Unhydrolyzed aliquots of these fractions were analyzed for color developed with ninhydrin (21). Additional aliquots of these same fractions were subjected to an arbitrary acid hydrolysis (1 hour in boiling water bath with equal volume of 6 N HCl), were neutralized with NaOH and the color developed with ninhydrin was determined. Identity and location of amino acids in the eluate were established by use of known standards and paper chromatography. Urea was identified by the urease reaction (14). Fractions containing substances localized by the ninhydrin reactions before and after hydrolysis were pooled, and the volume was reduced on a rotary evaporator. Analysis for peptide was performed by the biuret (22) and the Lowry-Folin reactions (23). Chromatography on a basic resin, Dowex 1-× 4 (acetate), was performed according to the method of Stein, Paladini, Hirs and Moore (24). Glucose was determined by the Somogyi reagent (17). Phosphate was determined by the method of Lowry and Lopez (25) before and after acid hydrolysis of the specimens.

TABLE I

Free and conjugated α-amino nitrogen

	Plasma urea N	α-Amino N	Post-hydrolysis α-amino N	Conjugated α-amino N
	mg %	*mg %*	*mg %*	*mg %*
	I. Normal subjects			
1	11.5	4.9	5.7	0.8
2	12.0	4.4	5.1	0.7
3	11.8	3.9	4.9	1.0
4	13.3	3.4	3.6	0.2
5	11.4	4.2	4.3	0.1
Mean	12.0	4.1	4.7	0.6
	II. Uremic patients			
1	318	4.4	6.2	1.8
2, a*	177	3.4	6.1	2.7
b†	235	4.1	11.2	7.1
3	175	5.4	11.8	6.4
4	187	5.7	7.2	1.5
5	122	5.3	7.8	2.5
6	200	4.4	7.2	2.8
7	220	5.3	7.0	1.7
8	183	3.6	5.9	2.3
9	95	4.3	6.2	1.9
10	240	5.5	8.1	2.6
Mean	195	4.7	7.7	3.0

* On admission.
† Two weeks later.

TABLE II

Picric acid versus ultrafiltration

	Plasma urea N	α-Amino N	Post-hydrolysis α-amino N	Conjugated α-amino N
	mg %	*mg %*	*mg %*	*mg %*
Normal				
Picric acid	11.4	4.2	4.3	0.1
Ultrafiltration		4.5	4.6	0.1
Uremic				
Picric acid	240	5.5	8.1	2.6
Ultrafiltration		5.6	7.5	1.9

RESULTS

Alpha-amino nitrogen before and after hydrolysis. In ten patients with uremia, free α-amino nitrogen in plasma ranged from 3.4 to 5.7 mg per 100 ml, with a mean of 4.7. In five healthy control subjects, the α-amino nitrogen ranged from 3.4 to 4.9 mg per 100 ml, with a mean of 4.1. These values for free plasma α-amino nitrogen are in accord with normal values established by other workers (9, 10, 26, 27). After hydrolysis there was a mean increase of 0.6 mg per 100 ml (range, 0.1 to 1.0) in the normal plasmas. In deproteinized plasmas from patients with uremia, this increase ranged from 1.5 to 7.1 mg per 100 ml, with a mean increase of 3.0. The data are presented in Table I. Similar results were obtained when plasma was deproteinized by ultrafiltration (Table II). Only a small number of ultrafiltrations was performed, since the method involves definite possibilities for error in procedure, as well as in theory. While the limited data precluded statisti-

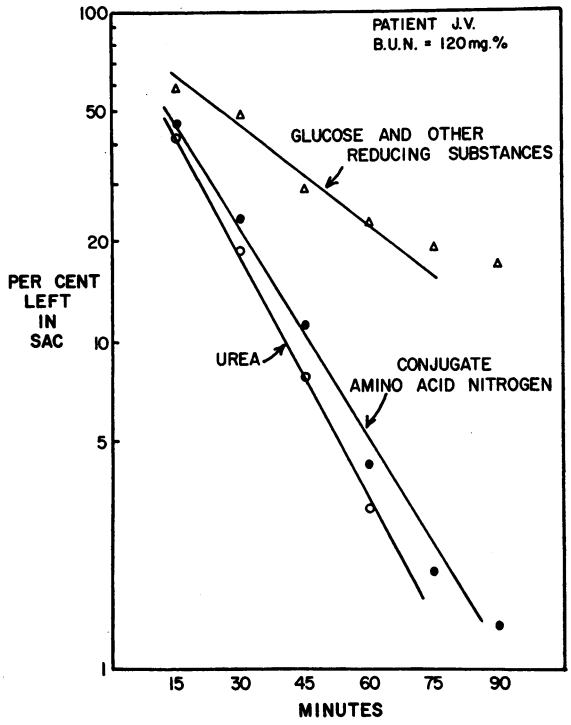

FIG. 1. CONJUGATED AMINO N DIALYZED THROUGH CELLOPHANE AT A RATE FASTER THAN UREA BUT SLOWER THAN GLUCOSE.

cal comparison, it was our impression that picric acid deproteinization, although less convenient than ultrafiltration, gives more consistent results. This agrees with the findings of other workers (11).

Dialysis of amino acid conjugates. The compounds which gave rise to the increased α-amino nitrogen upon hydrolysis dialyzed rapidly through cellophane. Efforts to increase the selectivity of the experiment by slowing the rate of dialysis were only partially successful, although acetylation of the membrane and dialyzing into 0.01 M acetic acid did slow the rate, as noted by Craig and co-workers (15). After one experiment, manometric CO_2-ninhydrin determinations on the dialysate were made, but dialysis of the unknown proceeded so rapidly that the concentration of α-amino nitrogen (after hydrolysis) in the dialysate fell off quickly. That is, only the earliest tubes of dialysate contained any reliably detectable α-amino nitrogen. The colorimetric ninhydrin method was applied and proved to be more sensitive than the manometric method. In these experiments alkaline hydrolysis was selected because any ammonia formed would be driven off. In specimens from normal subjects, there was essentially no conjugated amino nitrogen, as shown by a lack of change in ninhydrin color after hydrolysis. In specimens from patients with uremia, conjugate N dialyzed at a rate slower than that of urea, but faster than that of glucose (Figure 1). When the simple tripeptide glycylglycylglycine (mol wt 189) was added to deproteinized normal plasma, it dialyzed at the same rate as glucose (mol wt 185). Similar results were obtained on specimens deproteinized by ultrafiltration.

Chromatographic separation of amino acid conjugates on ion-exchange resins. Figure 2 illustrates the amino acid pattern obtained by ion-exchange chromatography on Amberlite 1R 120 of picric acid-deproteinized normal plasma. It can be seen that no additional peaks resulted upon hydrolysis; i.e., no conjugated amino N was detected in the range examined. At the end of the scheme shown in Figure 2, the pH was progressively raised, resulting in separation and elution of the acidic, neutral, and basic amino acids in series (20) (*vide infra*).

Figure 3 illustrates the typical result obtained in an identical experiment with a similarly prepared plasma from a patient with uremia. After hydrolysis of aliquots of the fractions, three distinct peaks, labeled A, B, and C were found where there was little or no color prior to hydrolysis. Chromatography of the contents of peak C on Dowex-1 resulted in a single peak detectable only by performing the ninhydrin color after hydrolysis. Hydrolysis of pooled fractions was performed in a sealed glass tube with 6 N HCl, after which the HCl was evaporated. When the desalted (28) specimen was examined by two-dimensional paper chromatography, a single spot of glutamic acid was identified. An aliquot of the hydrolysate, which was subjected to quantitative analysis by an ion-exchange chromatography system employing an automatic recorder (27), revealed only glutamic acid and ammonia. This substance, therefore, had the properties of phenylacetylglutamine (PAG) as determined by Stein, Paladini, Hirs and Moore (24).

Synthetic PAG was obtained from Dr. William Stein and Dr. Stanford Moore of The Rockefeller Institute. Additional synthetic PAG was pre-

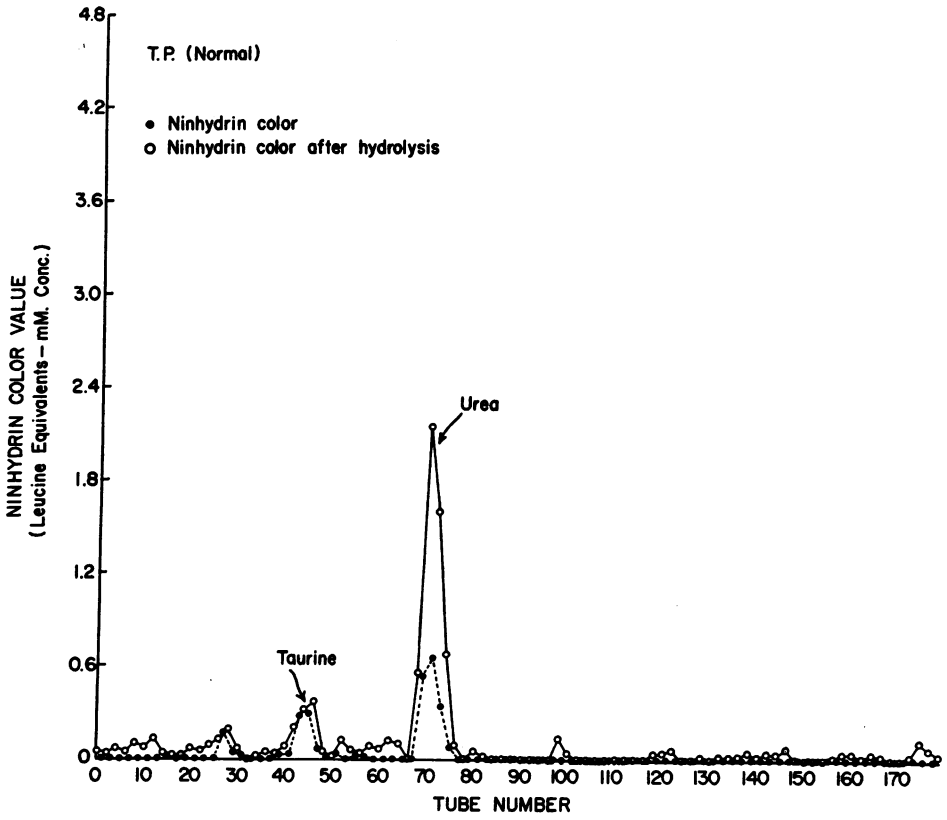

FIG. 2. ION-EXCHANGE CHROMATOGRAM ON DEPROTEINIZED PLASMA FROM NORMAL SUBJECT.

pared in our laboratory according to the method of Thierfelder and Sherwin (*29*). Chromatography of both samples of synthetic PAG specimens on the Amberlite 1R 120 preparative column followed by the arbitrary 1 hour hydrolysis of aliquots of effluent fractions yielded over-all recoveries of 0.93, 1.14, 1.19, 0.93, and 1.18, with a mean of 1.1. The yields greater than unity may be due to ammonia liberated from glutamine together with the α-amino N of glutamic acid. The mean factor (1.1), was used to estimate PAG concentration in the plasma of 10 patients with uremia, as shown in Table III.

Peaks A and B contained carbohydrate, identified as the plasma glucose. This glucose caused considerable humin formation on hydrolysis of concentrated fractions, and otherwise complicated the further handling of these unknown substances. It seemed probable that the contents of the peaks were acidic and would be retained on a basic resin, as was PAG. However, attempts at further separation by chromatography using various systems employing basic resins were not successful, the unknown usually eluting in a broad smear without peaks. The same negative results were obtained with paper chromatography and paper electrophoresis. When the contents of peak B were subjected to acid hydrolysis in a sealed tube and were chromatographed on the analytical ion-exchange resin system (30), several acidic

TABLE III

Plasma urea N and phenylacetylglutamine levels in uremic patients

	Urea N	PAG
	mg %	
A.S.	200	5.9
L.O.	194	3.5
E.R.	240	5.2
Y.B.	217	12.2
V.W.	200	20.0
J.W.	248	8.1
H.B.	144	5.1
R.B.	133	3.2
J.A.	222	8.2
G.T.	295	5.5

and neutral amino acids were identified, as shown in Figure 4. The prominent peak at 80 effluent ml displayed an optical density at 440 mμ which is higher than that seen with urea which is eluted in this area. An aliquot of the hydrolysate (prior to this chromatography) gave a negative urease reaction. The contents of this peak were not identified, but a dense brownish mass, probably representing a fraction of the soluble humin, was eluted from the column at a time corresponding to this peak. No basic amino acids were present. Chromatography of the unhydrolyzed specimen yielded a small ninhydrin-positive peak emerging before the position of taurine. Peak A yielded the same amino acids after acid hydrolysis as did peak B.

Other amino acid conjugates. The large increase in ninhydrin color after hydrolysis of the tubes containing urea suggested that this peak might contain other amino acid conjugates. However, when the tubes were pooled, the volume reduced by evaporation, and the contents hydrolyzed, no amino acids were found upon ion-exchange chromatography. The concentration of taurine was not specifically determined in the plasmas studied but appeared to be variable. No additional amino acids were found underlying the taurine peak. In two experiments, the automatic recording apparatus employing a 150 × 0.9 cm resin column for acidic and neutral ninhydrin-reacting components and a 50 × 0.9 cm column for basic components (30) was modified to allow splitting of the effluent stream at the bottom of the column (31). One-tenth of the effluent was directed through the reaction bath and photometers of the apparatus in the normal fashion. The remainder was directed to a fraction collector, and over 600 2.0-ml fractions were collected. The experiment was continued for several hours past the position of β-amino*iso*butyric acid on the 150 cm column and the emergence of arginine on the 50 cm column. In one experiment, both alkaline and acid hydrolysis were performed on aliquots of each fraction. In the second experiment, only

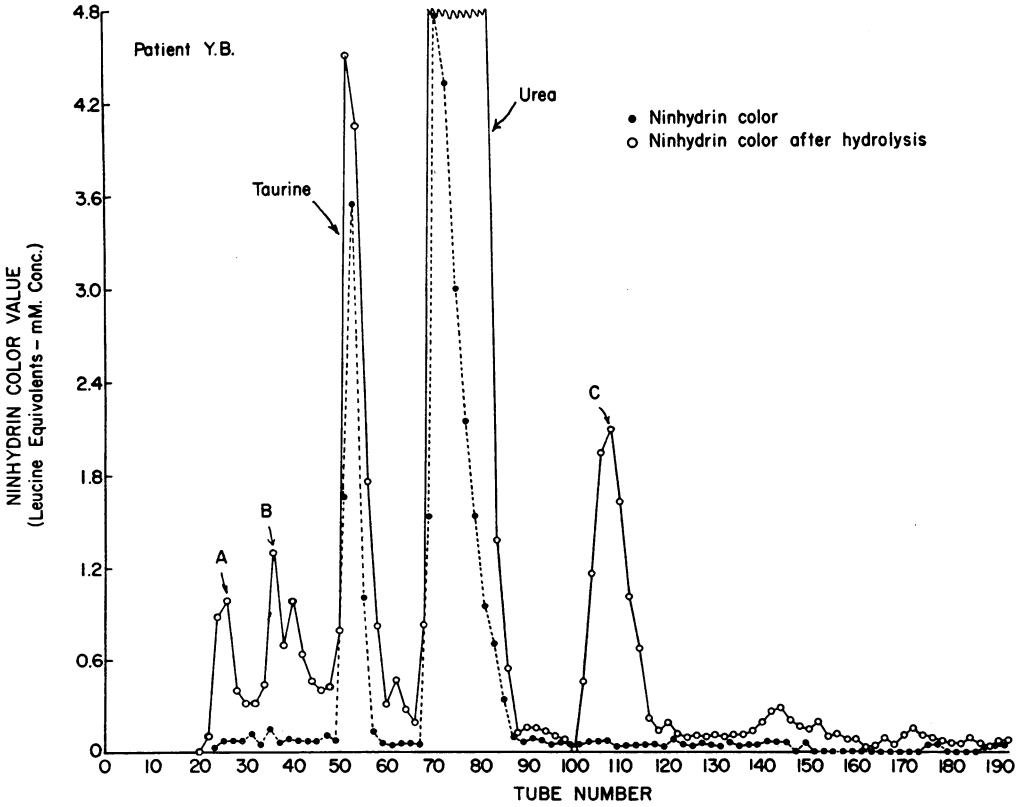

FIG. 3. ION-EXCHANGE CHROMATOGRAM ON DEPROTEINIZED PLASMA FROM A UREMIC SUBJECT. Plasma urea N 217 mg per 100 ml, phenylacetylglutamine 12.2 mg per 100 ml.

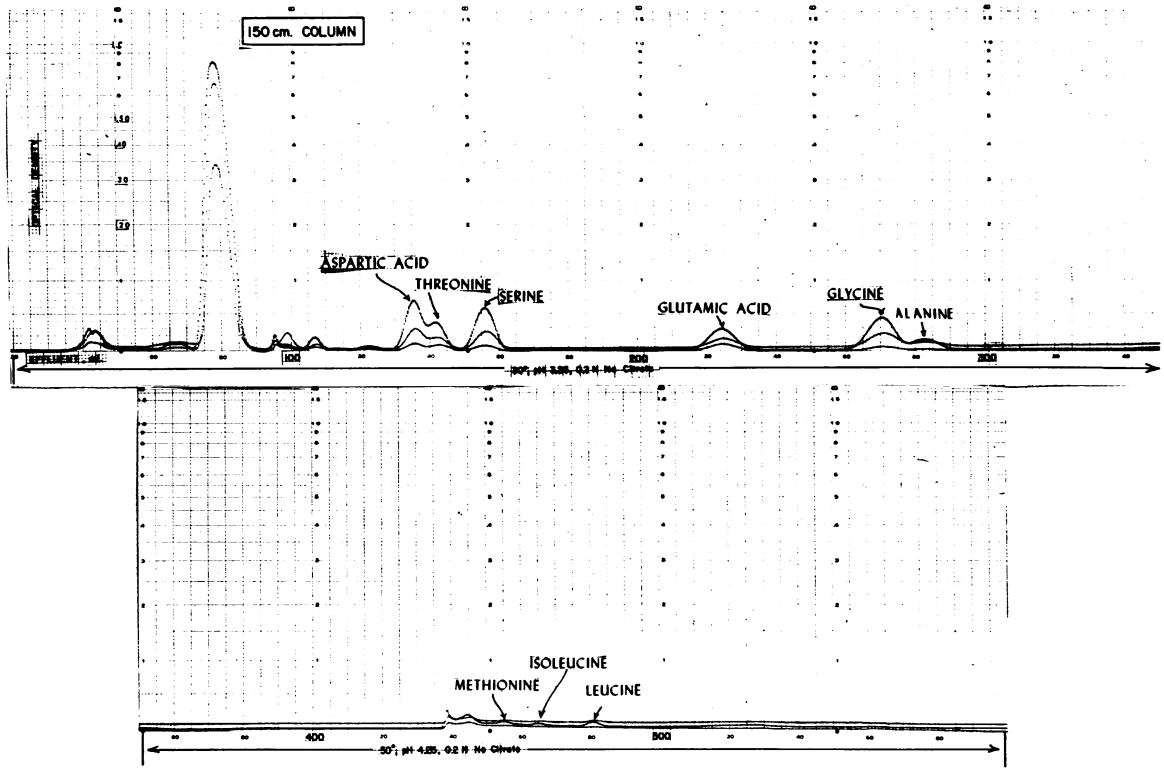

FIG. 4. RECORDING FROM AUTOMATIC AMINO ACID ION-EXCHANGE CHROMATOGRAM OF CONTENTS OF PEAK B, FIGURE 3, AFTER ACID HYDROLYSIS. The material in the early part of the record represents decomposition products from the plasma glucose and citrate buffer from the preparatory separation (see text).

the previously described 1 hour acid hydrolysis was used. In these experiments no prominent conjugate other than PAG and the peaks A and B were noted. A small post-hydrolysis peak was found between the peaks of glutamine-asparagine and proline. This peak contained only glycine after hydrolysis. The contents of tubes corresponding to the peaks of plasma amino acids were pooled, hydrolyzed, de-salted, and examined by paper chromatography. In no instance was there found any additional amino acid which would indicate a conjugate obscured on the chromatograph recording by the expected amino acid.

Because of our failure to locate hippuric acid, a conjugate known to be present in normal urine (24), radioactive hippuric acid was synthesized from C^{14}-glycine and benzoyl chloride (32). On the 1.8 × 165 cm column, C^{14}-hippuric acid was not eluted with the pH 2.2 buffer but was eluted with pH 3.25 buffer in a complex peak which also contained serine, glutamine, and asparagine. On the 0.9 cm column of the automatic apparatus, the C^{14}-hippuric acid was eluted in a position corresponding to that of the unknown glycine-containing peak in plasma from patients with uremia.

DISCUSSION

The finding of increased α-amino nitrogen after hydrolysis of deproteinized plasma from patients with uremia confirms the findings of previous investigators (2–7). The dialysis experiments indicated that the substances giving rise to increased ninhydrin color after alkaline hydrolysis were of low molecular weight.

Our observations indicate that deproteinized plasma from patients with uremia contains amino acid conjugates which were separated into three groups on an ion-exchange resin column. One of these contained phenylacetylglutamine as the sole conjugate. Although not previously demonstrated, PAG might be expected to be found free in the plasma of patients with uremia, in view of the quantity normally excreted. Stein and co-workers

reported a normal daily excretion of 250 to 500 mg (24). However, the *amount* of PAG apparently present in the plasmas from patients with uremia was surprisingly large. The fact that none was detected in the plasma specimens from normal subjects suggests that PAG is secreted by the renal tubules, as are similar acyl amino acids (33). Furthermore, there is evidence that excreted PAG is synthesized in part in the renal tubular cells (34).

It is interesting to compare the apparent retention of PAG with that of hippuric acid, a similar amino acid conjugate. Stein and colleagues reported a normal daily excretion of hippuric acid of 1.0 to 2.5 g (24). In contrast to PAG, only relatively small amounts of hippuric acid were apparently present in our plasmas from patients with uremia. This might have been partially attributable to the method employed, since PAG would liberate NH_3 on acid hydrolysis and thereby increase the ninhydrin color value, whereas hippuric acid would not. Also, hippuric acid was eluted at a more complicated position in the ion-exchange chromatogram. Alternative explanations for the relatively low plasma hippuric acid should be considered. First, there might be a decrease in the formation of hippurate. The origin of benzoic acid to form urinary hippuric acid is not known and may be, at least in part, dietary. These patients had poor dietary intake. Second, the production of PAG might be increased in some way, perhaps as the result of accelerated endogenous protein catabolism. The answer to the problem must be complicated and may include consideration of the differential effect of the uremic state upon many enzyme systems.

The presence of large amounts of PAG in the plasma of patients with uremia raised the question of whether the precursors might be increased in concentration. One of these, phenylacetic acid (PAA), has been shown to have untoward effects in man (35). However, plasma concentrations of glutamine in patients with uremia are essentially normal (3–6) and should be adequate to combine with PAA. We were unable to demonstrate the presence of PAA in plasma from patients with uremia. Whole plasma from such patients in amounts up to 250 ml (pooled) was acidified and extracted 5 × with dichloromethane. Evaporation yielded an oily, brownish material which was more plentiful and darker than that similarly obtained from plasma from normal subjects. This material was subjected to paper chromatography according to the method of Ladd and Nossal (36). No PAA was found in the extracts, although standard organic acids chromatographed well. This does not rule out the possibility that PAA may be present in the plasma of patients with uremia; current methods for detection of PAA lack sensitivity and specificity.

The question of the effects of PAG in such large amounts in the plasma naturally arises. We have not studied the toxicity of PAG, nor have others reported on this subject. A large number of nonprotein nitrogenous substances is known to be present in increased amounts in the plasma in uremia. These include urea, creatinine, phenols, and so forth. Although each of these substances may have untoward effects under certain circumstances, it is generally accepted that the uremic syndrome is due to a multiplicity of factors rather than a single agent (37, 38).

Moldave and Meister noted that the enzyme of human liver and kidney which is responsible for the conjugation of glutamine with phenylacetate to form PAG also catalyzes the reaction of phenylacetate with glycine to form a slight amount of phenylacetylglycine *in vitro* (34). C^{14}-phenylacetylglycine was synthesized by reacting C^{14}-glycine with phenylacetyl chloride (29). This substance was eluted at 612 effluent ml from the 1.8 × 165 cm column with the pH 2.2 buffer. Nothing was found in this area in our studies of patients with uremia. Therefore, phenylacetylglycine was not present in the plasma of our patients with uremia.

The material in the peaks labeled A and B has not been completely identified. Although some amino acids were identified after hydrolysis, the usual tests for proteins and peptides were negative. The manner of deproteinization and the dialysis experiments also tend to militate against the presence of peptides. This confirms the observations of Christensen and co-workers, who were unable to find an increase in nondialyzable α-amino N in deproteinized uremic plasma (39). Significant amounts of phosphate were not present in spite of the fact that phosphoethanolamine,

glycerophosphoethanolamine, and phosphoserine are eluted before taurine in the method of Spackman, Stein and Moore (30). Since one organic acid, phenylacetic acid, is a constituent of one conjugate (peak C) it is conceivable that other conjugates (peaks A and B) represent conglomerates of amino acids with other organic substances.

The two types of study, *1*) α-amino N before and after hydrolysis, and *2*) chromatographic separation of amino acid conjugates on ion-exchange resins, were not performed on the same specimens. It is apparent that even 20 mg per 100 ml of PAG would yield only about 1 mg per 100 ml of α-amino N. It is difficult to estimate the amount of α-amino N contributed by the conjugates which were incompletely identified. However, it may be anticipated that other analytical procedures will reveal additional sources for the α-amino N liberated by acid hydrolysis.

SUMMARY AND CONCLUSIONS

1. We have confirmed the observation that the α-amino nitrogen in deproteinized plasma from patients with uremia is approximately normal. Acid hydrolysis of deproteinized plasma of uremic patients resulted in an increase in α-amino nitrogen of 1.5 to 7.1 mg per 100 ml, with a mean increase of 3.0 (65 per cent), compared with a mean of 0.6 mg per 100 ml (11 per cent) in normal plasmas.

2. The substances apparently giving rise to the increased α-amino nitrogen dialyzed through cellophane at a rate slower than that of urea, but faster than that of glucose.

3. Amino acid conjugates were separated on ion-exchange resins. Phenylacetylglutamine was found in amounts up to 20 mg per 100 ml in plasma of patients with chronic uremia. Thus, phenylacetylglutamine accounts for a portion of the rise in α-amino nitrogen after hydrolysis of uremic plasmas. No correlation of the concentrations of phenylacetylglutamine with plasma urea nitrogen levels was apparent.

4. Evidence for the presence of other conjugates is offered. The amino acids present in these conjugates were identified. The structure of the organic compounds containing these amino acids has not been determined and requires further study.

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