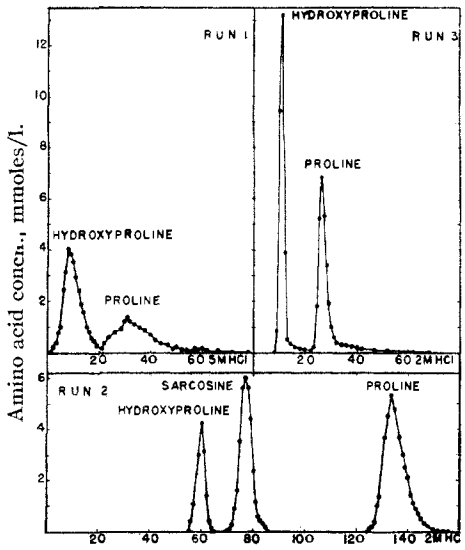

Fig. 1.—Effect of flow rate, resin particle size, and amount of resin on the separation of amino acids, using columns of Amberlite IR 100 (run 1), Dowex 50 (run 2), and Amberlite IR 120 (run 3).

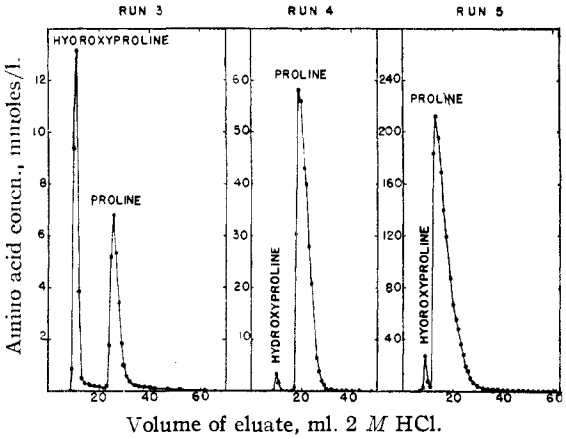

Fig. 2.—Effect of load on retardation volumes of amino acids, eluted from columns of Amberlite IR 120, using a mixture of 0.03 millimole of proline and 0.03 millimole of hydroxyproline (run 3), 0.3 millimole of proline (run 4), and 1.5 millimoles of proline (run 5).

combined influences of high flow rate and relatively large particles. Some channeling, due to uneven packing of the irregularly shaped particles may, also, have contributed to the poor results in the case of Amberlite IR 100.

The degree of agreement between the observed and the calculated retardation volumes for the Amberlite IR 120 and the Dowex 50 columns may be regarded as satisfactory in view of the approximations involved and the lack of precision in the values of K_H. In the case of Dowex 50, this agreement confirms the opinion that the exchange characteristics of this resin are similar to those of Amberlite IR 120.

In precision chromatography it is desirable to work with a large value of MT_{Rx}/s. It is evident, however, from the fifth run that good separations can be achieved at remarkably low values of this ratio provided the exchange constants of the components are well separated. In this run 0.173 g. of proline was separated from the hydroxyproline on a column of 5 g. of resin. That is to say a column with a capacity of 19 millimoles was adequate to resolve 1.5 millimoles of the amino acid. Elution chromatography on ion exchange columns promises to be valuable in preparative work as well as in the analysis of amino acid mixtures.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

A Procedure for the Ion Exchange Chromatography of the Amino Acids of Urine[1]

BY MARY E. CARSTEN

RECEIVED JULY 21, 1952

A method is presented for the chromatographic separation of the amino acids in urine. Ion exchange procedures are used for the removal of salts in a first step and the removal of most of the nitrogenous substances in a second step. Thereby, a crude ampholyte fraction is obtained without significant loss of amino acids. Separation of the single amino acids in this fraction is effected in a third step by ion exchange chromatography. The single amino acids can be identified by paper chromatography and by specific tests.

The overwhelming amounts of inorganic ions and of nitrogenous bases in biological fluids such as urine have been found to interfere with the chromatography of the amino acids on paper and on starch columns. Anticipating similar problems in the use of ion exchange columns we explored a method of preliminary desalting by ion exchange followed by precise chromatography on a cation exchange column. Recently, a similar procedure for removing salts has been applied by Piez, Tooper and Fosdick prior to paper chromatography.[2] On the other hand, Moore and Stein[3] avoided the desalting by using a sulfonated cation exchanger in its sodium form to chromatograph urinary amino acids. In their procedure, elution of the amino acids is effected with a series of buffers of increasing pH and resolution is improved by periodic modification of the temperature of the column. Our preliminary desalting has made it possible to eliminate the exacting control of pH and temperature neces-

(1) A preliminary report was presented at the Meeting of the American Society of Biological Chemists: M. E. Carsten, *Federation Proc.*, **10**, 170 (1951).

(2) K. A. Piez, E. B. Tooper and L. S. Fosdick, *J. Biol. Chem.*, **194**, 669 (1952).

(3) S. Moore and W. H. Stein, *ibid.*, **192**, 663 (1951).

sary in the procedure of Moore and Stein and may therefore be simpler although it uses three columns of ion exchange resins in place of two. Furthermore, Moore and Stein used a 40-g. column of Dowex 50 and a few milliliters of urine. The amounts of the individual amino acids thus recovered are sufficient only for colorimetric estimation. Their identification, therefore, depends upon their appearance in orderly sequence in the eluate. The casual intrusion of unidentified ninhydrin-reacting components, other than the recognized amino acids, may be expected to lead to some confusion in the interpretation of analyses.

Procedure

The procedure which will be described below was designed to deal with much larger amounts of urine so that the components of the chromatograms could, where necessary, be recovered and characterized. The principal involved is the preliminary use of an ion exchange cycle to remove interfering substances and to separate a crude fraction containing ampholytes of urine in a form suitable for precision chromatography. The urine is first perfused through a large column (column I) of the acid form of a sulfonated resin. All cations, including the ampholytes, are retained while the anions and the neutral constituents are discarded in the effluent (effluent I). The only important exception is taurine.[4] The sulfonic acid group of this ampholyte is so strong that the compound does not react as a cation toward the acid form of a sulfonic acid resin and most of it therefore is lost in the effluent. The ampholytes, together with the weak bases, are recovered from the column by elution (eluate I) with 2 *M* ammonia. The cations of the stronger bases are retained. After concentration, *in vacuo*, an aliquot of eluate I is passed through column II which is composed of a quaternary base resin in its basic form. This column retains the ampholytes and rejects the weak bases. The former are recovered by elution (eluate II) with 1 *M* hydrochloric acid. After concentration, *in vacuo*, eluate II is ready for chromatography by any chosen method. In the work which will be described below, elution chromatography on the hydrogen form of a sulfonated resin (column III) was employed, using 2 and 4 *M* hydrochloric acid as the eluent. Since columns I and II are only intended to effect separations on the basis of differences in ionic charge, they may be composed of resins of large particle size and may be operated at relatively rapid rates of flow. The third column should be operated with all the usual precautions of precision chromatography. The capacity of column I should be great enough to retain all cations in the sample of urine to be analyzed. A much smaller amount of resin will serve in column II since most of the anions of the urine have been discarded in the first step.

Fractionation of Urine

Experimental.—In order that adequate amounts of the components should be available for characterization, the preliminary procedure has been applied to 24-hour samples of urine. Four experiments will be reported. In the first, an artificial mixture, simulating the composition of urine, was carried through columns I and II. This was done to demonstrate that the amino acids could be recovered quantitatively and to trace the fate of other nitrogenous constituents. A sample of normal urine was then subjected to the complete procedure and a second sample of the same urine, to which 10 millimoles of amino acids had been added, was carried through column I only. Finally, a urine from a patient with rheumatoid arthritis was submitted to the complete procedure. The details of operation of columns I and II are summarized in Table I. The flow rates through columns I and II were about 18 and 9 ml./hour, respectively. Effluents and eluates were collected and analyzed for amino acids by a modified ninhydrin colorimetric method developed in this Laboratory.[5] Collection of each eluate was continued until the concentration of amino acids fell below 0.001 *M*. Analyses of other nitrogenous constituents were made by standard procedures.[6-8]

TABLE I

COLUMN CHARACTERISTICS

Experiment	I, simulated urine	II, normal urine	III, normal urine + 10 mmoles amino acids	IV, pathological urine
Column I				
Resin	Amberlite IR 100	Duolite C 3	Duolite C 3	Duolite C 3
Particle size, mesh	60–100	60–100	60–100	60–100
Wt. of resin, g.	280	250	250	250
Capacity, meq.	420	583	583	583
Amino acid input, mmoles	10	13.3	23.3	.....
Capacity/input	42	44	25	.....
Vol. of eluate, ml.	1930	1900	2400	930
Column II (Amberlite IRA-400, 60–100 mesh)				
Wt. of resin, g.	40	30	.....	15
Capacity, meq.	95	71	.....	36
Amino acid input, mmoles	5.6	7.6	.....	3.9
Capacity/input	17	9.3	.....	9.2
Vol. of eluate, ml.	500	500	.....	500

Results of Fractionation.—A summary of the results is given in Tables II and III.

The recovery of amino acids in eluates I and II from the simulated urine was close to 100%. In the case of urine some ninhydrin-reactive material escaped into the effluents. This may well be attributed to the presence in effluent I of taurine[4] and possibly of ninhydrin-reactive components of high molecular weight and, in effluent II, of weak primary amines. Furthermore, arginine may be incompletely adsorbed on column II because of its strongly basic properties. Even so these losses do not fully account for the low recoveries in step I. In part these may have been due to precipitation of cystine and of tyrosine and to some destruction of tryptophan. Moreover, any volatile amines which appeared in eluate I would have escaped detection because this fluid was concentrated *in vacuo* prior to analysis to remove ammonia. The analyses of effluent II are difficult to interpret because the ninhydrin-reactive material seems to diminish with time and therefore must include some unstable compound.

The satisfactory recoveries of amino acids from the simulated urine make it improbable that the

(4) R. Crokaert, S. Moore and E. J. Bigwood, *Bull. soc. chim. biol.*, **33**, 1209 (1951).

(5) W. Troll and R. K. Cannan, *Federation Proc.*, **10**, 260 (1951).

(6) O. Folin, *J. Biol. Chem.*, **17**, 469 (1914); **106**, 311 (1934).

(7) O. Folin and G. E. Youngburg, *ibid.*, **38**, 111 (1919).

(8) F. C. Koch and T. L. McMeekin, THIS JOURNAL, **46**, 2066 (1924).

TABLE II

DISTRIBUTION OF URINARY CONSTITUENTS BETWEEN EFFLUENT AND ELUATE IN STEP I

Run	I, simulated urine[a] mg. N		II, normal urine mg. N		III, normal urine + added amino acids[b] mg. N		IV, pathological urine mg. N	
Nitrogenous Constituent	Added	Recov.	Added	Recov.	Added	Recov.	Added	Recov.
			Effluent I					
Ninhydrin-reacting	140	4.76	186	10.8	326	11.1	...	14.9
A, Creatinine	372	4.31	350	4.72	350	4.72	342	3.13
B, Creatine	0	0	62	3.31	62	5.5	18	....
A + B	372	4.31	412	8.03	412	10.2	360	....
Uric acid	233	....	169	18.9	169	17.1	...	35.6
Urea	14,000	....	6325	1676	6325	2356	...	....
			Eluate I					
Ninhydrin-reacting	140	137	186	142	326	287	...	164
A, Creatinine	372	148	350	272	350	251	342	11.1
B, Creatine	0	55	62	14	62	28	18	29.8
A + B	372	203	412	286	412	279	360	40.9
Uric acid	233	....	169	0.97	169	1.62	...	....
Urea	14,000	....	6325	4464	6325	3106	...	....

[a] The simulated urine contained 0.5 mmole of each of the following: aspartic acid, β-alanine, histidine, lysine; 1 mmole of each of the following: alanine, phenylalanine, proline, serine, valine, glutamic acid; and 2 mmoles of glycine. [b] The amino acids were 2 mmoles of each of the following: alanine, phenylalanine, lysine, threonine and glutamic acid.

PER CENT. RECOVERY IN EFFLUENT + ELUATE OF STEP I

Run	I	II	III	IV
Ninhydrin-reacting	101	82	92	..
A, Creatinine	41	79	73	4
B, Creatine	...	28	54	..
A + B	56	71	70	..
Uric acid	...	12	11	..
Urea	...	97	86	..

TABLE III

DISTRIBUTION OF URINARY CONSTITUENTS BETWEEN EFFLUENT AND ELUATE IN STEP II

Run	I mg. N		II mg. N		IV mg. N	
Nitrogenous Constituent	Added	Recov.	Added	Recov.	Added	Recov.
			Effluent II			
Ninhydrin-reacting	78.2	1.82	106	14.6	54.9	12.7
A, Creatinine	84.5	0	204	53.3	3.72	0
B, Creatine	31	2.68	10	54	10.0	0.63
A + B	116	2.68	214	107	13.7	0.63
Uric acid	...	...	0.73	0	...	...
Urea	...	...	3348	2996	...	...
			Eluate II			
Ninhydrin-reacting	78.2	80.5	106	83.1	54.9	44.2
A, Creatinine	84.5	60.6	204	75.1	3.72	7.51
B, Creatine	31	36.0	10	0	10.0	3.8
A + B	116	96.6	214	75.1	13.7	11.3
Uric acid	...	2.33	0.73	0.19	...	4.14
Urea	...	30.0	3348	63.7	...	26.3

PER CENT. RECOVERY IN EFFLUENT + ELUATE OF STEP II

Run	I	II	IV
Ninhydrin-reacting	105	92	104
A, Creatinine	72	63	202
B, Creatine	125	540	44
A + B	86	85	87
Uric acid	...	3	...
Urea	...	91	...

low recoveries from the normal urine represented simple amino acids. This conclusion is fortified by a comparison of the recoveries from column I in experiments 2 and 3. It may be deduced that the 10 millimoles of amino acids added to the normal urine in experiment 3 were quantitatively recovered, although the recovery of the ninhydrin-reactive material native to the urine was only 82%. When column I, after elution with ammonia, was further extracted with 4 *M* hydrochloric acid small additional amounts of ninhydrin-reactive material were obtained. These probably consisted of bases stronger than ammonia.

The greater part of the urea was retained in the first column, as might be expected from the weakly basic properties of this substance. Only a trace of the urea in eluate I was retained by the second column. Most of the uric acid in the urine disappeared in the first step. It was evidently precipitated in the column. Indeed, a precipitate which was found in the first eluate was identified as uric acid.

The behavior of creatinine was peculiar. As was to be expected, most of it was retained in column I and reappeared in the first eluate. A part, however, appeared as creatine[9] and some of it stubbornly resisted elution with ammonia. When the course of elution was followed, it was found that creatinine began to appear only after elution of the amino acids was almost complete. Thereafter it continued to trickle out in low concentration indefinitely. After elution with 6 liters of ammonia about 80% had been recovered and a low concentration was still found in the eluate. It is interesting to note that very little creatinine was found in eluate I of experiment 4 because collection of the eluate was stopped at an earlier stage than in the other experiments.

Some interconversion of creatinine and creatine[9] occurred in column II. Since creatine is an ampholyte, it found its way into the second eluate, appearing in part as creatine and in part as creatinine.

In summary, it can be said that the ampholyte fraction contained no salts, 4% of the urea of urine, 2% of the uric acid, less than 45% of the creatinine and creatine, while the amino acids were essentially recovered.

(9) R. K. Cannan and A. Shore, *Biochem. J.*, **22**, 920 (1928).

Chromatography of the Ampholyte Fraction

Experimental.—Chromatography was performed on the ampholyte fractions from the normal urine and the pathological urine. In the former case a small aliquot of eluate II containing 0.285 millimole of amino acid was used. The aliquot from the pathological urine contained 0.379 millimole. The column was composed of 25 g. of Dowex 50. This had been cleaned first with alkali, then with acid, as described for other ion exchange resins,[10] and screened through a 325-mesh sieve. The exchange capacity of the column was 91.7 meq. The column was eluted with 2 *M* hydrochloric acid at a flow rate of 2.1 ml. per hour until 180 ml. was collected. Thereafter 4 *M* acid was used and elution continued until the total volume of the eluate was 400 ml. The eluate was collected in 1-ml. fractions with the aid of a Technicon Fraction Collector. Each fraction was analyzed colorimetrically for amino acids, using ninhydrin (normal urine)[5] or 1,2-naphthoquinone-4-sulfonic acid (pathological urine).[11] The results are displayed graphically in Figs. 1 and 2 where the concentration of amino acid (millimolar) is plotted against the volume (ml.) of eluate, after subtraction of the pore volume.[10]

TABLE IV

AMINO ACID COMPOSITION OF TWO 24-HOUR URINE SPECIMENS

Ampholyte	Normal urine, millimole	Normal urine, mg.	Pathological urine (rheumatoid arthritis), millimole	Pathological urine (rheumatoid arthritis), mg.
(1) Aspartic acid	0.192	25.6	0.567	75.5
(2) Serine	.230	24.2	.270	28.4
(A) Tetrapeptide (glycine + aspartic acid)	.296	....	.290	....
(3) Taurine[a]			.140	17.5
(4) Threonine[b]	1.50	179	.750	89.3
(5) Glutamic acid	0.155	22.8	.450	66.1
(6) Hydroxyproline	0.0120	1.57	.170	22.3
(7) Glycine	2.10	158	2.03	152
(8) Alanine	0.431	38.4	2.12[c]	189[c]
(9) α-Aminobutyric acid	.0855	8.81	0.0666	6.86
(10) Valine[b]	.794	93.0	.500	58.6
(B) Peptide	....	....	.0807	....
(12') Methionine sulfoxide	.133	21.9	....	....
(C) Peptide (alanine + glutamic acid)	....	....	.130	....
(11) Proline	.0153	1.76	.100	11.5
(12) Methionine	....	....	.0783	11.7
(D) Peptide	....	....	.0823	....
(E) Tetrapeptide (2 glycine: 1 alanine: 1 glutamic acid)	.0963	....	....	....
(F) Peptide	....	....	.193	....
(G) Peptide	.0765	....	.0760	....
(H) Peptide	....	....	.0864	....
(13) Isoleucine	.131	17.2	.160	21.0
(14) Leucine			.0864	11.3
(15) Ornithine	.0774	10.2	....	....
(16) Tyrosine	....	....	.270	48.9
(17) Lysine	.825[b]	121[b]	.0485	7.08
(18) Histidine	.918	142	.400	62.1
(19) Tryptophan	.121	24.7	....	....
(i)[d]	.457	....	.180	....
(20) Arginine	.0540	9.40	....	....
(21) Phenylalanine	.230	38.0	.330	54.5
(J)	....	....	.0815	....
(K)	....	....	.0657	....
Total	8.93		9.80	
Peptides and unknowns	0.926		1.27	
Amino acids	8.00	938	8.53	934

[a] Most of the taurine was removed in effluent I (see text). [b] Attention is drawn to the fact that these values are much higher than those obtained by microbiological assay. These anomalies are discussed in the text. [c] This is β-alanine (see text). [d] This component may be methylhistidine (see text).

(10) M. E. Carsten and R. K. Cannan, THIS JOURNAL, **74**, 5950 (1952).

(11) W. Troll and R. K. Cannan, unpublished.

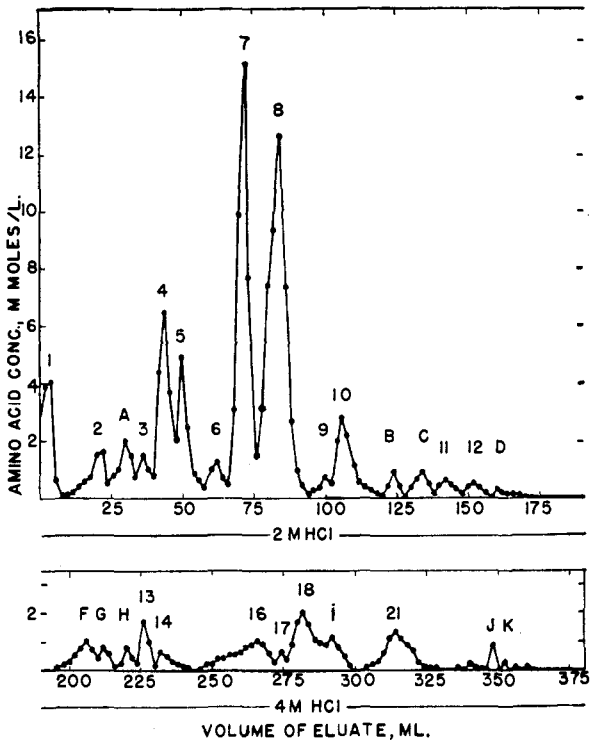

Fig. 1.—Chromatogram of amino acids and peptides found in a normal urine.

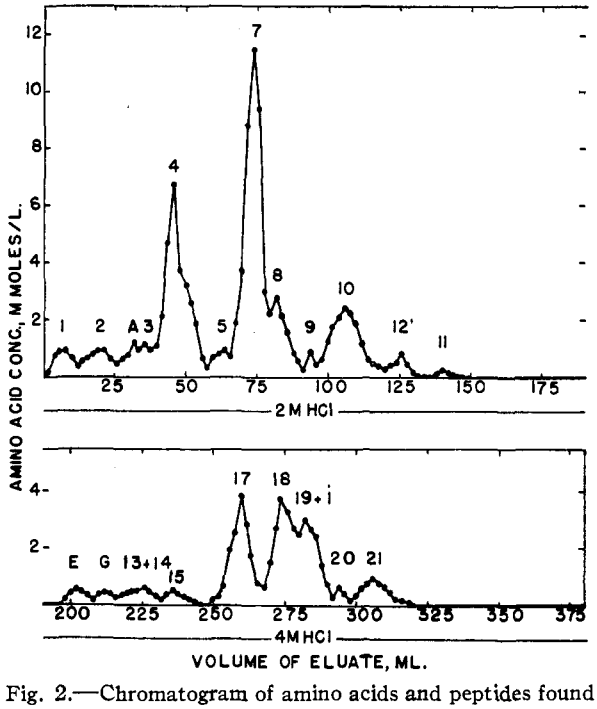

Fig. 2.—Chromatogram of amino acids and peptides found in urine of a patient with rheumatoid arthritis.

Results of Chromatography.—Twenty-one distinct components appear in Fig. 1 and 28 in Fig. 2. Those which have been identified as amino acids are numbered consecutively. Those which appear to be peptides or are of unknown composition are given the letters A to K. Quantitative estimation of each band was made by integrating the area in the band. Evidence that the bands are each com-

posed of a single component is based on paper chromatographic methods described below. In some cases, as mentioned below, the methods may fail to demonstrate the presence of more than one ampholyte. To this extent the quantitative data in Table IV are tentative.

Most of the components were identified by submitting aliquots to paper chromatography, using phenol–water or benzyl alcohol–water solvent pairs, and spraying with ninhydrin.[12] Comparison was made with authentic amino acids which were run simultaneously. When identity of R_F was established a second chromatogram with a mixture of the known and the unknown was run. If no separation developed, identity was regarded as established.

In addition, several components were also identified by specific reactions. Thus histidine was identified by Pauly's reaction,[13] tryptophan by its characteristic absorption spectrum.[14] Proline was quantitatively determined by its reaction with 1,2-naphthoquinone-4-sulfonic acid.[11] Hydroxyproline was determined in the non-chromatographed ampholyte fraction of the normal urine by the method of Troll and Cannan,[5] as the amount present was too small to show as a distinct peak in the chromatogram. Methionine and methionine sulfoxide were identified by submitting samples to paper chromatography before and after oxidation with hydrogen peroxide according to Dent.[15] The presence of creatinine in fractions 184 to 194 of the eluates was established by the Jaffe reaction.[6]

Components which were not identified as amino acids were hydrolyzed with 6 *M* hydrochloric acid at 110° for 24 hours. When the apparent amino acid content had increased by 100% or more the presence of a peptide was assumed and a paper chromatogram of the hydrolysate was made. In this way evidence was obtained of the presence in urine of the peptides listed in Table IV.

Discussion

The data in Table IV and Figs. 1 and 2 are not intended as definitive contributions to the question of the normal or abnormal distribution of amino acids in human urine. Such studies would involve the systematic investigation of many subjects under varied nutritional and physiological states. The purpose of the two chromatograms is to demonstrate that the ampholyte fraction which we have described is amenable to chromatography on an ion exchange column.

The total amounts of amino acids recovered agree well with the gasometric analyses of Van Slyke.[16] The presence of amines and peptides in urine also agrees with Van Slyke's conclusions which he based on the larger amounts of α-amino nitrogen in urine found by formol titration and by nitrous acid method than by gasometric ninhydrin analysis.

The ratio of the exchange capacity to the load of column III was 320 and 240, respectively, in the two experiments. The corresponding ratio in the work reported by Moore and Stein[3] must have been of the order of 3600, judging from the fact that they used for their experiment about one-tenth of the amount of amino acids used by us. Had the larger ratio been used in our experiments the resolution of the components would undoubtedly have been more nearly complete than that shown in Figs. 1 and 2.

Recoveries from column III were about 108%. It is probable that this high value is due to partial hydrolysis of peptides in the strongly acid column. When pure amino acids were run in the same column recoveries were all within 100 ± 3%.

The sequence of components is essentially the same in Figs. 1 and 2, except for some minor anomalies. Two peaks were identified as leucine and isoleucine in the chromatogram of the pathological urine, but only one was found in that of the normal urine. Tyrosine was identified only in the pathological urine while tryptophan, ornithine and arginine were found only in the normal urine. In view of the incomplete resolution of several of the peaks, it is probable that these missing components were buried in peaks attributed to other components. Tryptophan, for instance, as determined spectrophotometrically,[14] accounted for only 21% of the amino acid concentration in peak 19 + I. The unidentified component I may well be methylhistidine. This was not looked for since its presence as a major component of urine was not known[17] at the time this work was done. The tyrosine of the normal urine is probably hidden in the lysine peak; this may explain the seemingly high value for lysine in our analysis. The high values for valine and threonine as compared to microbiological assay[18] cannot be explained by the presence of any known amino acids. Ornithine and methionine sulfoxide in the normal urine may well be artifacts, the former caused by hydrolysis of arginine,[19] the latter by oxidation of methionine.[15]

The pathological urine contained much more proline and hydroxyproline than did the normal urine. Should this characteristic of the urine in rheumatoid arthritis be confirmed it may reflect a metabolic anomaly of this "collagenous disease."

The presence of a very large amount of β-alanine in the pathological urine will be noted. When this was found enquiry was made and it was discovered that the diet of the patient had been supplemented with large amounts of calcium pantothenate. The amount of β-alanine recovered from the urine corresponded to 5% of the daily intake of pantothenate. This free β-alanine may have arisen by partial hydrolysis of pantothenic acid on column I. No report has been found in the literature of the excretion of β-alanine following administration of pantothenic acid.

Evidence was obtained that some of the non-amino acid peaks contained peptides. Peak A in both urines behaved as a tetrapeptide consisting only of equal amounts of glycine and aspartic acid. A second tetrapeptide, E, was found in the

(12) A. J. P. Martin, *Ann. N. Y. Acad. Sci.*, **49**, 249 (1948).
(13) K. Urbach, *Proc. Soc. Expt. Biol. Med.*, **70**, 146 (1949).
(14) E. H. Holiday, *Biochem. J.*, **30**, 1795 (1936).
(15) C. E. Dent, *ibid.*, **43**, 169 (1948).
(16) D. D. Van Slyke, D. A. MacFadyen and P. B. Hamilton, *J. Biol. Chem.*, **150**, 251 (1943).
(17) S. P. Datta and H. Harris, *Nature*, **168**, 296 (1951).
(18) M. S. Dunn, M. N. Camien, S. Shankman and H. Block, *Arch. Biochem.*, **13**, 207 (1947).
(19) R. C. Warner, *J. Biol. Chem.*, **142**, 705 (1942).

normal urine, consisting of glycine, alanine and glutamic acid (2:1:1). The remaining peptides were all dipeptides. It is interesting to observe that all peptides identified contained one of the dicarboxylic amino acids. This may be one of the sources of the considerable increase in the amounts of dicarboxylic amino acids after hydrolysis of urine, found by Dunn and co-workers.[18]

Calculations have been made of the theoretical retardation volumes of several of those amino acids which emerged with the 2 *M* HCl eluate. The equation used for these calculations was given.[10] The theoretical retardation volumes for the amino acids behaving as single solutes are compared with the observed retardation volumes on column III in Table V.

In view of the complexity of the ampholyte fraction, agreement is surprisingly good.

TABLE V

RETARDATION VOLUMES OF AMINO ACIDS ELUTED WITH 2 *M* HCl FROM 25 G. COLUMN OF DOWEX 50

Run	II, 0.285 mmole of ampholyte fraction of normal urine		IV, 0.379 mmole of ampholyte fraction of pathological urine	
Amino acid	Retardation volume Obsd.	Calcd.	Retardation volume Obsd.	Calcd.
Threonine	46	46	44	47
Hydroxy-proline	64	54	62	53
Glycine	74	73	72	73
Alanine	82	83	84	73
Proline	140	118	142	114

Acknowledgment.—The author is indebted to Dr. R. Keith Cannan for his advice and criticism during the course of this work.

NEW YORK, N. Y.

[CONTRIBUTION FROM THE BIOCHEMICAL INSTITUTE AND THE DEPARTMENT OF CHEMISTRY, THE UNIVERSITY OF TEXAS, AND THE CLAYTON FOUNDATION FOR RESEARCH]

Synthesis of N-Phosphoryl Amino Acid Esters

BY SI-OH LI[1]

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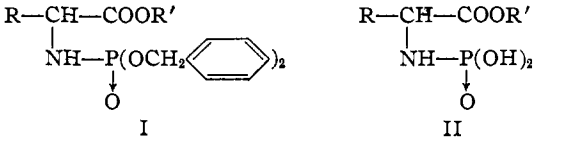
The synthesis of N-phosphoryl amino acid methyl esters of DL-phenylalanine and DL-tryptophan has been carried out by hydrogenolysis of the corresponding dibenzylphosphoryl compounds. The isolation of four dibenzylphosphoryl amino acid esters is reported.

Because of the increasing realization of the importance of phosphate compounds in biological systems, we have undertaken the preparation of some N-phosphoryl amino acid derivatives to be used as substrates for the study of the properties and physiological functions of phosphoamidase, an enzyme which specifically splits the P–N bond of the phosphoamide.[2] The direct N-phosphorylation of amino acids has been done by Neuberg and Oertel,[3] and later by Winnick and Scott,[4] using phosphorus oxychloride as the phosphorylating agent; however, the yields were low and the compounds thus isolated were readily hydrolyzed in acid solution.[4] Amino acid esters have been phosphorylated with diphenylphosphoryl chloride by Sciarini and Fruton[5] and with diisopropylphosphoryl chloride by Wagner-Jauregg, *et al.*[6] In both methods, the reaction took place without difficulty; however, the preparation of pure N-phosphoryl amino acid esters through hydrogenolysis of diphenylphosphoryl amino acid esters was not entirely successful.[5]

The method described in the present investigation is based on the reaction of dibenzylphosphoryl chloride (DBPCl) with an amine in organic solvents and the subsequent removal of the benzyl groups by hydrogenolysis with palladium oxide[7,8] to give compounds with structures (I) and (II), respectively.



I II

Thus an amino acid ester was first liberated from its hydrochloride salt according to the procedure of Hilmann[9] with slight modification. The free ester was then condensed with DBPCl in the presence of triethylamine. By this method the phosphorylation of about ten amino acid esters have so far been tried, but only four dibenzylphosphoryl amino acid esters have been isolated in good yield (Table I). These compounds are soluble in organic solvents but insoluble in water. Evidence for the attachment of the phosphoryl group onto the nitrogen atom in the phosphorylated compounds is given by the fact that these substances do not show on paper chromatograms treated with ninhydrin, whereas the unphosphorylated amino acid esters give characteristic spots.[10]

(1) Rosalie B. Hite Predoctorate Fellow, The University of Texas.

(2) H. Holter and S. O. Li, *Compt. rend. trav. lab. Carlsberg. Sér. chim.*, **27**, 393 (1951).

(3) C. Neuberg and W. Oertel, *Biochem. Z.*, **60**, 491 (1914).

(4) T. Winnick and E. M. Scott, *Arch. Biochem.*, **12**, 201 (1947).

(5) L. T. Sciarini and J. S. Fruton, THIS JOURNAL, **71**, 2940 (1949).

(6) T. Wagner-Jauregg, J. J. O'Neill and W. H. Summerson, *ibid.*, **73**, 5202 (1951).

(7) F. A. Atherton, H. T. Openshaw and A. R. Todd, *J. Chem. Soc.*, 382 (1945).

(8) S. O. Li, *Acta Chem. Scand.*, **4**, 610 (1950).

(9) G. Hilmann, *Z. Naturforsch*, **1**, 682 (1946).

(10) Contrary to general belief and to statements appearing in several standard reference books, many amino acid derivatives and other amines that do not possess a free α-carboxyl group will react with ninhydrin to give a distinct color reaction, *cf.* A. H. Cook and A. L. Levy, *J. Chem. Soc.*, 646 (1950). It is only when evolution of CO_2 is measured that the ninyhdrin reaction is specific for free α-amino acids.