UNBOUND AMINO ACID CONCENTRATIONS IN HUMAN BLOOD PLASMAS

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Studies of small molecules in plasma have indicated the importance of defining concentrations in terms of both "unbound" and "total." For example, McLean and Hastings (1) showed calcium was both bound and free in plasma, but that the free form was regulatory in the body. Further, Davis (2) found it was the unbound concentrations of the sulfonamides in the plasma which were active. In addition to these, cholesterol, fatty acids, bilirubin, estriol, chloride and many others have been observed to bind with various affinities to the plasma proteins [see review by Surgenor (3)].

The amino acids in blood may be considered to exist in one or more of the following forms:

a) combined into proteins or other substances by covalent linkages (peptide bonds and possibly other stable bonds).
b) combined with proteins or other substances by weak forces.
c) present within the various formed elements of the blood (erythrocytes, leucocytes, platelets).
d) present in plasma as individual unbound molecules.

The unbound plasma amino acid concentrations have been considered as the concentrations present in form d), and have been measured experimentally after equilibration at nearly physiological condition of pH of freshly prepared plasma across cellophane membranes known to be freely permeable to the amino acid molecules. Many of the earlier estimates have failed to remove completely the formed elements of the blood to exclude form c), or have involved long durations of manipulation and/or temperature changes so that proteolytic and other enzymes may have produced changes in the concentration of unbound amino acids.

The total free plasma amino acid concentrations may be considered to be the concentrations present in forms b) and d), and have been measured experimentally after precipitation of freshly prepared plasma by agents such as trichloroacetic acid, picric acid, and phosphotungstic acid. These substances cause large changes in pH, and denaturation of some or all of the proteins present.

In this report, a method is described for measuring the amino acid levels in the unbound form d). Employment of nonwettable surfaces for blood collection, immediate cooling of the specimens, early cold centrifugation, rapid dialysis, and avoidance of changes in pH or other destructive steps in the analysis of the dialysates are believed to be necessary for this purpose.

Concentrations of the amino acids are determined by paper chromatography. In order to secure satisfactory chromatograms, the developing solvents are saturated with buffered sodium chloride and the papers are pretreated with salt. Under these conditions, the physiological salts of the plasma do not interfere with chromatography. A desalting step with its possible destructive effect is avoided. Salt-saturated developing solutions have been reported previously (4), but the use of such solvents with salt-treated papers does not appear to have been published.

METHODS

Preparation of protein free concentrates

A syringe and needle treated to make their surfaces nonwettable (5) and containing one-third ml. of heparin solution (1,000 U.S.P. units per ml.) are used to collect 25 ml. of blood. The blood is placed immediately in a vinyl plastic bag which is submerged in ice water for

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prompt transportation to the laboratory. The plasma is separated by centrifugation in a refrigerated centrifuge at 1,750 times gravity for 40 minutes and removed from the bag by compression. Ten ml. is dialyzed at 2° C. for two hours against 65 ml. of distilled water, using the thin layer technique of Seegers (6). A sealed glass tube, which is weighted with lead pellets and placed inside the dialysis bag, is 16 mm. in outside diameter and 215 mm. in length. The dialysis membrane is Nojax Casing, size 23/32, obtained from the Visking Corporation, Chicago, Illinois. This arrangement produces a layer of plasma 1 to 2 mm. thick. Agitation is accomplished by suspending the membrane from a stirring motor assembly controlled to alternate between 30 second periods of rotation at 90 r.p.m. and equal periods of nonrotation. This method of agitation was suggested by Dr. N. A. Saroff, Naval Research Institute, Bethesda, Maryland. Following the dialysis, the dialysate is stirred for 10 minutes with 1 ml. of wet, mixed anion exchange resin, Rohm and Haas XE-114, mesh size 50-100, of which one part is the ethylenediaminetetraacetate salt and three parts are the chloride salt. This step reduces distortions of some of the amino acid zones on the chromatograms, presumably through removal of bivalent cations. It has been found not to remove any of the amino acids.

Sixty ml. of the dialysate is then concentrated to dryness from the frozen state. At this point the residue may be safely stored at −10° C. For applications to the papers the residue is dissolved in 0.4 ml. of water. The solution volume with this amount of water is found to be approximatey 0.43 ml. for dialysates derived from normal plasma.

Pretreatment of papers

Whatman No. 1 and Whatman No. 4 papers, size 18¼ by 22½ inches, are used. The pretreatment consists of dipping the papers in a borate buffer solution, pH 8, containing 3.5 Gm. H_3BO_3, 0.25 Gm. NaOH, and 30 Gm. NaCl per liter of water and suspending the papers in a nonwrinkled condition to dry. Manipulation of the wet papers is facilitated by attaching one end to a wooden clamp consisting of two slats (1 by 1 by 24 inches) held together by two bolts and winged nuts.

Application of dialysate concentrates and standard solutions to the papers

One dimensional chromatograms are run in the long direction of the paper. A separate paper is prepared for each staining reagent except Sakaguchi's. This reagent is applied to the chromatogram developed for and stained with Ehrlich's reagent after the results of the latter test have been recorded. The pretreated papers are placed in a support to facilitate applications of the amino acid solutions (7). These are applied to the papers with a 3 μl. pipette in an arrangement similar to that used for quantitative analysis by McFarren and Mills (8), except that aliquots of 3, 6 and 12 μl. of the unknown, and aliquots of 3, 6, 9, and 12 μl. of the standard solution are used. The composition of the standard solution is listed in Column A of Table I.

TABLE I

Composition and chromatographic characteristics of the amino acid mixture

	Standard solution mM/L. (A)	R_F values	
		Solvent A (B)	Solvent B (C)
Alanine	4.3	0.10	0.20
Allantoin		0.14	
α-amino n-butyric	0.5	0.22	0.50
Arginine	1.0	0.06	0.44
Asparatate		0.007	0.02
Citrulline		0.055	0.08
Cystine		0.006 (0.20)*	0.05
Glutamate	1.7	0.01	0.04
Glutamine	6.8	0.04	0.22
Glycine	3.4	0.053	0.07
Histidine	1.7	0.065	0.54
Isoleucine	1.0	0.46	0.75
Leucine	1.5	0.50	0.75
Lysine	2.2	0.032	0.20
Methionine	0.5	0.29	0.68
Ornithine	1.4	0.028	0.12
Phenylalanine	0.8	0.40	0.82
Proline	2.4	0.14	0.71
Serine	1.7	0.053	0.05
Taurine		0.06	0.04
Threonine	2.2	0.08	0.14
Tryptophan	0.7	0.35	0.77
Tyrosine	1.2	0.26	0.49
Urea	68.0	0.38	
Valine	3.0	0.32	0.60

* This amino acid frequently splits into two zones in this solvent.

Development

After the applications have been made, the papers are hydrated by placing them in a cold room until they have adsorbed enough moisture to make them feel damp. Following hydration, the papers are immediately placed in chromatographic chambers prepared for each solvent, as indicated below, and descending development started. The preferred solvent for determining each amino acid is listed under the heading Staining Techniques. The solvents used are prepared as follows:

Solvent A. Isopropanol, reagent grade, is mixed with borate buffer solution of the same composition as used for pretreatment of the papers in the ratio of volumes 80:20. Solid sodium chloride is then added with agitation until a small amount of salt remains undissolved in the bottom of the flask. The organic layer is decanted, part is poured over adsorbent papers attached to the sides of the chamber to hasten saturation of the vapor phase, and the remainder is used for development of chromatograms. It has been found advantageous to start development immediately after the solvent has been poured over the papers on the sides of the chambers.

Solvent B. Liquified phenol (Mallinckrodt, Gilt Label) is mixed with borate buffer solution and concentrated ammonium hydroxide in the volume ratios of 90.5:9:0.5.

The solvent is saturated with sodium chloride and the organic layer is decanted for use in development. With this solvent, improved chromatograms are obtained by saturating the vapor phase in the chamber with water rather than with the developing solvent. This is accomplished by covering the bottom of the chamber with water.

The migration rates (R_F values) of amino acids and several other substances in these solvents are listed in Columns B and C of Table I. Solvent A is the more stable of the two and gives the more reproducible results. It is used in preference to solvent B when choice permits.

Staining techniques

After development, the papers are dried in a mechanically vented oven at 60° C. prior to staining.

Ninhydrin-collidine reagent. This stain is prepared by dissolving 0.5 Gm. of ninhydrin in 100 ml. of a mixture of acetone, glacial acetic acid, lactic acid (85 per cent), methylcellosolve, water and collidine (2,4,6-trimethylpyridine) in the volume proportions 66 : 9.5 : 0.5 : 10 : 10 : 4, respectively. The collidine is added immediately before use. The papers are dipped in this reagent and heated at 60° C. for 15 minutes for color development. The lactic acid enhances the ninhydrin stain, and the collidine causes color contrast in the stains of the amino acids (9). The following amino acids or combination of amino acids are determined by this reagent:

Alanine, arginine, glutamic acid, glutamine, lysine, ornithine, threonine and a combined value for *serine* and *glycine* are determined on a Whatman No. 4 paper developed for 64 hours in solvent A.

Glycine is determined on a Whatman No. 1 paper developed for eight hours in solvent B.

α-amino n-butyric acid, isoleucine, leucine and a combined value for *methionine, tryptophan* and *valine* are determined on a Whatman No. 1 paper developed for 16 hours in solvent A. A corrected value for valine is obtained by subtracting the tryptophan and methionine values found with the special reagents described below.

Special reagents. The low plasma concentration of some amino acids and the low staining intensities of others with the ninhydrin-collidine reagent makes it desirable to use more sensitive staining reagents for their determination. These tests are run on Whatman No. 1 papers developed for 16 hours in solvent A.

Histidine and *tyrosine* are determined by Pauly's reagent as modified by Bolling, Sober, and Block (10).

Tryptophan and *urea* are determined by Ehrlich's reagent as modified by Smith (11). Allantoin is also stained by this reagent and its color appears occasionally in some analyses.

Arginine may be determined by Sakaguchi's reagent as modified by Acher and Crocker (12). This is sometimes helpful because arginine is in low concentration in plasma and cannot always be determined by the ninhydrin-collidine reagent. When this occurs, Sakaguchi's reagent is applied over the stain of Ehrlich's reagent, making it unnecessary to prepare an additional paper.

Proline, phenylalanine and *tyrosine* are determined by isatin, using a modification of the method of Saifer and Oreskes (13). Five-tenths Gm. isatin is dissolved in a mixture of acetone, acetic acid and water in the volume proportions of 88 : 4 : 8, respectively. The paper is dipped in this reagent and heated for one hour at 60° C. for color development. Colors from other amino acids do not interfere under these conditions. The tyrosine values determined by this reagent are usually averaged with those obtained by Pauly's reagent.

Methionine is determined by the platinum reagent of Toennies and Kolb (14). Cystine is also observed when this reagent is used, but elongation of its zone with occasional splitting into two zones makes its determination inexact.

Quantitation

The amounts present on the chromatograms are determined visually by comparison of the size and intensities of the zones of the unknowns with those of the standards. The values observed are multiplied by 1.04 to compensate for losses occurring during analysis. The principal loss is the amount absorbed on the dialyzing membrane.

An alternate method of quantitation is to measure the maximum intensities of the zones on the paper photometrically, as described by Block, Durrum, and Zweig (15). This method in general is not recommended for the present types of chromatograms. Whereas its accuracy is comparable to that obtained visually for amino acid zones of high intensity, it is less accurate for weakly pigmented or irregular zones. Further, the variation in color of zones brought out by the ninhydrin-collidine and special reagents complicates photometry by requiring measurements at a diversity of wavelengths. Such color differences assist visual evaluation by clearer delineation of the zones. Photometry is also slower. For example, chromatograms from 14 dialysates require approximately 40 hours for photometric analysis, whereas visual analysis can be accomplished in 6 to 8 hours.

RESULTS AND DISCUSSION

Amino acid binding to non-dialyzable molecules in plasma

Amino acid binding was measured by recovery studies. A fresh sample of plasma was divided into 14 parts. Three parts were analyzed to establish the amino acid concentrations in the original plasma and 11 parts were analyzed after various mixtures of amino acids had been added. Recoveries were calculated and the data shown in Table II. With the exception of tryptophan and glutamic acid, no significant difference is indicated between the calculated values and the amounts found ($p > 0.06$). It should be noted

TABLE II

Recovery of amino acids added to plasma

	Average amounts added	Total amounts calculated present	Total amounts found	Standard deviation	Coefficient of variation	Per cent recovery
	μM/L.	μM/L.	μM/L.			
Alanine	133	352	350	18.0	5.2	99
α-amino n-butyric acid	16	30	29	2.4	8.5	96
Arginine	32	105	106	10.0	9.4	105
Glutamic acid	72	72	89	5.6	6.3	131
Glutamine	215	870	880	54.3	6.2	105
Glycine	111	293	303	22.4	7.4	109
Histidine	53	142	153	9.1	5.9	120
Isoleucine	32	87	85	7.8	9.2	92
Leucine	48	144	134	7.7	5.8	81
Lysine	70	228	240	19.3	8.1	117
Methionine	16	34	32	5.8	18.0	87
Ornithine	65	140	135	14.8	11.0	92
Phenylalanine	27	68	70	8.4	12.0	106
Proline	75	167	162	14.0	8.7	92
Threonine	70	170	166	17.9	10.8	94
Tryptophan	28	28	7		18.0*	<28
Tyrosine	37	87	81	8.0	10.0	83
Valine	96	248	243	9.7	4.0	95
Serine and glycine	161	507	510	35.7	7.0	101
Urea	2,136	6,320	6,440	354.0	5.5	105

* Calculated from other data.

that the amount of amino acids added averaged only 40 per cent of the total present. Recoveries calculated for these added amounts reflect experimental variation two and one half times larger than if recoveries had been calculated on the total present.

Tryptophan showed distinct binding. Approximately 70 per cent of the added tryptophan was absent from the dialysate, but could be recovered from the solution inside the bag if the proteins were precipitated with trichloroacetic acid. This observation explains the low tryptophan values

TABLE III

Comparison of amino acid concentrations in plasma obtained by two techniques

	Values by rapid dialysis and salt-saturated chromatography solvents			Values from Stein and Moore (16)*	
	No.	Mean	Range	Mean	Range
		μM/L.	μM/L.	μM/L.	μM/L.
Alanine	15	320	200–450	383	338–418
α-amino n-butyric acid	15	20	10– 30	29	21– 34
Arginine	15	68	50– 96	87	70–111
Glutamic acid	15	<20	0– 25	48	29– 78
Glutamine	15	450	320–780		
Glycine	13	235	140–290	205	178–231
Histidine	15	88	60–110	74	51– 95
Isoleucine	14	57	35– 90	68	52–100
Leucine	15	79	50–105	129	108–175
Lysine	10	143	105–190	186	171–207
Methionine	13	28	10– 45	25	22– 29
Ornithine	5	58	46– 75	55	47– 61
Phenylalanine	14	56	35– 75	51	42– 58
Proline	15	142	100–180	205	160–292
Serine and glycine	15	320	270–390	312	274–340
Taurine	2	10	0– 20	44	33– 66
Threonine	15	135	100–180	117	102–144
Tryptophan	11	21	14– 26		
Tyrosine	15	70	35– 90	57	48– 80
Valine	15	174	110–220	246	202–317

* Converted from mg. per 100 ml.

obtained in the plasma analyses of the next section.

Glutamic acid was found only in those specimens to which it had been added. However, as shown by the table, the amount found was in excess of the amount added. This excess corresponds to a concentration of 17 μM in the original plasma, a value which is reasonable because concentrations less than 20 μM generally cannot be determined by the procedure used.

These recovery studies indicate that except for tryptophan the amino acids are not reversibly bound to the large molecules of plasma. It is possible that binding does occur between amino acids and small dialyzable molecules. However, chromatography of dialysates at neutral pH in a much concentrated form showed no characteristic variation of the R_F value of any amino acid and suggests that binding with smaller molecules probably does not occur to any appreciable extent.

Equilibrium binding studies will not indicate degradations of labile complexes when these occur irreversibly. Since such degradations are more likely to occur under acid conditions and least likely to occur at neutrality, analyses by both methods are compared in the next section.

Unbound amino acid levels in normal fasting plasmas

Fifteen normal, fasting plasmas were analyzed by methods described above and the results reported in Table III. For comparison, Stein and Moore's results of plasma from five fasting normal subjects, analyzed by picric acid deproteinization and a resin column effluent technique, are included (16). In addition to the amino acids listed in the table, Stein and Moore reported determinations of aspartic acid, asparagine, the methyl histidines, serine and cystine plus cysteine but did not report values for tryptophan and glutamine which were destroyed in their analysis. Neither cystine nor cysteine is determined separately. The other three substances, as well as citrulline, are not observed on the chromatograms, presumably because their concentrations are too low.

Values obtained by these two methods for many of the amino acids agree rather closely. Levels of alanine, glycine, histidine, isoleucine, methionine, phenylalanine, threonine, tyrosine and a combined value for serine and glycine are essentially the same. Somewhat less good agreement is shown between the values for α-amino n-butyric acid, arginine, leucine, lysine, proline and valine. The values of Stein and Moore are 25 to 60 per cent higher for these amino acids. In view of the variation in normal individuals and the small number of analyses reported, differences in these values are not of established significance.

The average glutamic acid concentration as determined by rapid dialysis and paper chromatography is, on the other hand, significantly less than the value reported by Stein and Moore, or, for that matter, significantly less than any other known report of glutamic acid concentrations in plasma. In the study of the 15 normal samples, a mean value less than 20 μM was indicated. Glutamic acid was found in only two of the analyses at levels of 20 and 25 μM. The average value reported by Stein and Moore of five analyses was 48 μM. Krebs, Eggleston, and Hems (17) reported an average value of 238 μM (3.5 mg. per 100 ml.). Walshe (18), using Dent's procedure of electrolytic desalting with paper chromatography, found glutamic acid in such high concentration it was considered a major component of the "free" amino acids in plasma. To explain these large differences, it seems likely that the technique of the latter investigators degraded a labile plasma substance, presumably by the acid conditions employed. Stein and Moore suggest that some glutamic acid may come from hydrolysis of glutamine in their analysis. It is also possible that glutamic acid may leak from the erythrocytes during the handling of whole blood.

Determinations of glutamine in this study, in which an average concentration of 450 μM was obtained, agree reasonably well with the values reported by Archibald (19), and Krebs, Eggleston, and Hems (17). These observers made determinations by use of glutaminase. They reported average values of 8.3 and 5.8 mg. per 100 ml. of plasma or 570 and 400 μM per liter, respectively.

Large differences occur between tryptophan values found in this study and values reported by Hier and Bergeim (20), and Johnson and Bergeim (21). They used microbiological assays on acid-deproteinized supernatant solutions. The mean unbound value for the 11 normal samples reported in Table III is 21 μM, whereas they obtained values in normal plasma of 55 μM (1.11 mg. per 100

TABLE IV

Effect of surgery on the plasma levels of unbound amino acids *

	Patients in good nutritional status			Patients emaciated			Mean percentage change in levels immediately post-operative observed in 16 patients by Everson and Fritschel (23)
	No. subjects	Mean levels before operation μM/L. plasma	Mean percentage change in levels after operation	No. subjects	Mean levels before operation μM/L. plasma	Mean percentage change in levels after operation	
Alanine	5	230	+ 2 ± 8†	3	120	+53 ± 4†	
α-amino n-butyric acid	3	30	−27 ± 13	2	25	−20 ± 16	
Arginine	5	80	−43 ± 8	3	47	+13 ± 9	−28
Glutamine	5	368	−32 ± 10	3	233	− 4 ± 6	
Histidine	5	94	−28 ± 11	3	100	−17 ± 21	−12
Leucine‡	5	170	−65 ± 6	3	113	−14 ± 11	− 8
Isoleucine‡	5			3			−23
Lysine	5	114	−33 ± 7	3	67	− 6 ± 10	−15
Methionine	3	40	−32 ± 5	3	20	− 5 ± 0	−26
Phenylalanine	2	65	−35 ± 16	1	50	0	−26
Proline	3	217	−42 ± 9	2	85	0 ± 12	
Serine and glycine	3	237	−27 ± 5	2	150	−10 ± 10	
Threonine	5	100	−22 ± 14	2	57	+17 ± 13	−30
Tryptophan	5	44	−37 ± 6	3	23	+ 9 ± 33	−40
Tyrosine	5	66	−41 ± 10	3	50	−14 ± 23	
Valine	5	254	−48 ± 3	3	127	+ 5 ± 3	−12
Average % change			−34			0	−20

* At the time this study was undertaken analyses for some of the amino acid had not been developed.
† Standard deviations of the mean percentage changes.
‡ Leucine and isoleucine values were combined in this study.

ml.) and 64 μM (1.3 mg. per 100 ml.), respectively. These differences in concentration arising from different procedures of analyses coincide with the observation made in the binding study of this amino acid in the previous section. It should be further noted that the levels of unbound tryptophan reported in Table III are probably high because the dilution occurring during dialysis tends to remove more tryptophan from the binding site. This binding is being further studied.

Another observation made in studying the unbound amino acid levels in normal plasma is the low levels of taurine. This substance has been reported present in plasma by other investigators (16, 22). In an effort to determine trace amounts of taurine, zones were eluted from several chromatograms, concentrated, and rechromatogramed in another solvent. By this technique, plasma taurine concentration was estimated at approximately 10 μM. In plasma from whole blood exposed to wettable surfaces, or plasma which had not been centrifuged adequately to remove most of the leucocytes and platelets, higher levels were obtained. These observations indicate that taurine may be derived from the leucocytes and platelets. In unpublished experiments of the authors, taurine has been found in very high levels in these cells.

Additional studies

The effect of surgery on the plasma levels of amino acids was studied in eight patients undergoing operations of one to three hours duration. Either cyclopropane, ether, or spinal anesthesia was administered. The patients' diagnoses were subacute cholecystitis, chronic cholelithiasis (two), varicose veins, umbilical hernia, carcinoma of the duodenum, burns complicated by chronic alcoholism, and severe burns, respectively. The first five patients were in reasonably good nutritional status. The latter three were emaciated, with the two burn patients undoubtedly in negative nitrogen balance. The patient with carcinoma of the duodenum had lost 60 pounds in the period six months preceeding the operation. Blood specimens were drawn immediately before and immediately after surgery.

The data from this study are summarized in Table IV. The patients are grouped according to their nutritional condition. The preoperative amino acid levels of those in good nutritional status are in the general range of the normals reported in Table III. In this group an average decrease in amino acid level of 34 per cent occurred after surgery. This decrease is in agree-

ment with the observations of Everson and Fritschel (23) in their study of the postoperative changes of the essential amino acids in 16 patients, although the magnitude of the change we observed is larger than theirs. With the emaciated group, a decrease was not observed. In this group the preoperative amino acid levels were low, and the additional stress of surgery did not further depress them.

Alanine showed anomalous behavior. Its postoperative concentration in relation to the other amino acids increased about 50 per cent in both groups of patients studied. This increase does not appear to have been reported previously.

SUMMARY

1. A technique is reported for assaying unbound amino acids in human plasma. Containers with nonwettable surfaces are used during collection and separation of the cells to avoid release of cell substance. The amino acids are separated from the plasma proteins by rapid dialysis and analyzed by paper chromatography in salt-saturated solvents on salt-saturated papers. This method avoids a desalting step. Physiological pH is maintained throughout and low temperatures maintained up to the start of the chromatograms. In addition to amino acids, urea may also be determined by this method.

2. Unbound concentrations of eighteen individual amino acids and a combined value for serine and glycine have been determined in plasma samples from fifteen normal subjects. With the exception of tryptophan and glutamic acid, agreement with other assays has been obtained.

3. The unbound concentration of glutamic acid and tryptophan are found to be very low in plasma. At physiological pH, tryptophan is bound to a nondialyzable plasma substance, probably protein, but is released upon precipitation of the proteins with trichloroacetic acid.

4. Recovery studies of amino acids added to plasma show that none were bound except tryptophan.

5. Plasma levels of amino acids of patients in good nutritional status dropped an average of 34 per cent immediately after surgery. In three emaciated patients whose preoperative levels were approximately 65 per cent of normal levels, no amino acid drop occurred after surgery.

6. Alanine showed anomalous behavior. In the normal patients, the alanine level remained the same while the average of all the amino acids decreased 34 per cent. In the emaciated patients, the alanine level increased 50 per cent while the average of all amino acids remained the same. Thus, alanine in relation to the other amino acids increased postoperatively in both groups of patients studied.

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