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Research Article

STUDIES OF UNBOUND AMINO ACID DISTRIBUTIONS IN PLASMA, ERYTHROCYTES, LEUKOCYTES AND URINE OF NORMAL HUMAN SUBJECTS

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Understanding of the role of the blood in amino acid transport and metabolism requires information not only about the plasma concentrations and turnover of these substances, but also about their distribution between the plasma and the formed elements of the blood.

Many workers (1–4) have measured the concentrations of unbound amino acids in plasma and several have studied their contents in erythrocytes (5) and leukocytes (6, 7). Simultaneous measurements of unbound amino acids in two or more of the blood compartments are few and limited (8, 9).

The present communication describes methods for the measurement of concurrent concentrations of unbound amino acids in plasma, erythrocytes, leukocytes and urine, and reports the results obtained from the study of normal fasting subjects.

METHODS

Collection of samples. Subjects were instructed to fast from 7 p.m. the evening before until the blood had been drawn. Between 8 and 10 a.m., 50 to 60 ml of blood was collected by the method of Lund, McMenamy and Neville (10) using one of the following resins for decalcification: Rohm and Haas anion exchange resin XE-114, mesh size 20 to 50, one part of which was cycled with ethylenediamine-tetraacetate (EDTA), two parts with chloride and one part uncharged; or, Rohm and Haas anion exchange resin XE-168, mesh size 20 to 50, two parts of which were cycled with EDTA, two parts with chloride and one part uncharged.[1] Decalcified blood as opposed to heparin-treated or citrated blood gave a much better yield of leukocytes. Containers of the blood were immediately placed in ice water for trans-

portation to the laboratory except where otherwise specified. Blood analyses were commenced within one-half hour after the blood collection. Urines were collected during the latter part of the fasting period over a measured time interval of 6 to 8 hours with thymol as preservative.

Preparation of the plasma, erythrocytes and leukocyte samples for analysis. Surfaces that came in contact with blood or blood cells were treated to make them non-wettable (11). Glass was first cleaned with a detergent solution, rinsed thoroughly with water, dried, exposed for several seconds to vapors of Dri-Film 9977 (General Electric Co., Chemical Division), flushed with air, and finally rinsed thoroughly with water. Metal surfaces were cleaned, placed in a boiling solution of 1 per cent Arquad (Armour and Co.) for 2 minutes, then thoroughly rinsed with water.

Solutions used in sample preparation were kept at 2° C unless otherwise stated. For plasma and erythrocyte analyses a 12 ml aliquot of blood was centrifuged in a 15 ml plastic test tube at 1,700 × G for 40 minutes. Four ml of the plasma was removed and dialyzed by the thin layer technique (1), except that here the plasma was placed in Visking tubing, size 22/32, and dialyzed for 2 hours against 25 ml of water. The dialysate was dried from the frozen state in a 25 ml round bottom flask.

The remainder of the contents of the plastic centrifuge tube was frozen, the tube was then cut in two below the residual plasma layer and approximately 4.4 g of frozen red cells was weighed accurately from the plug. The red cells were lysed by thawing, refreezing and rethawing, diluted to a volume of 12 ml with water, placed inside Visking tubing size 23/32, and dialyzed for 2 hours against 40 ml of water. The dialysate of the red cells was agitated for several minutes with 0.5 ml freshly washed EDTA-chloride resin prepared as described under *Collection of samples*. This procedure removes material from the dialysate which causes spreading of the glutamic acid and O-phosphoethanolamine zones. It is filtered and dried from the frozen state in a 50 ml round bottom flask.

To obtain white cells for analysis, 40 to 50 ml of blood from a subject with a normal white count (smaller amounts of blood may be used from patients with elevated counts) was diluted with two parts of a 3 per cent dextran solution at 2° C, gently mixed for 5 minutes

[1] After autoclaving, the latter resin sheds a small amount of a ninhydrin-positive substance with an Rf value between valine and α-amino-n-butyric acid; this substance did not interfere with the analyses.

Concentration of unbound amino acids in plasma

	Standard solution	No. of subjects	Mean
	mmoles/L		*µmoles/kg water*
Alanine	2.14	15	399 ± 25*
α-Amino-*n*-butyric acid	0.32	11	28 ± 3
Arginine	0.53	13	68 ± 6
Glutamic acid	1.94	15	<28
Glutamine	3.75	15	512 ± 24
Histidine	0.74	12	89 ± 9
Lysine	1.88	15	204 ± 13
Methionine	0.32	12	22 ± 2
Ornithine	0.85	11	85 ± 8
Phenylalanine	0.53	14	54 ± 4
Proline	1.07	15	185 ± 15
Threonine	1.39	15	165 ± 6
Tryptophan	0.43	15	(18)†
Tyrosine	0.74	15	70 ± 4
Valine	1.49	15	203 ± 11
Ergothioneine	2.07	15	<10
Ethanolamine	0.74	15	<10
Leucine plus isoleucine	1.60‡	13	202 ± 18
Serine plus glycine	4.27§	14	609 ± 26
Urea	34.4	13	5,382 ± 267

* Standard deviation of the mean.
† Estimated; see text.
‡ Leucine, 0.96 mmole; isoleucine, 0.64 mmole.
§ Serine, 1.5 mmoles; glycine, 2.77 mmoles.

and allowed to stand 20 minutes (12). The dextran solution contained 3 per cent dextran H (R. K. Laros and Co. Bethlehem, Pa.), 0.1 per cent dextrose, and 0.15 M NaCl; it had been filtered through Seitz filters to remove the insoluble particles and decalcified, since calcium leaks from the Seitz filters, with a column of resin (50 ml of the EDTA-chloride resin, described above, to 1,000 of dextran solution).[2] After standing 20 minutes, the supernatant solution was siphoned into a 100 ml pear shaped oil centrifuge tube, the tip of which had been modified by attachment of a Tygon plastic tube (5/16 in. OD, ⅟₁₆ in. wall thickness, 2.5 in. long) with the lower end thermally sealed. The solution was centrifuged at 100 × G for 1 hour. The white cell layer was cut from the tube and suspended in 0.5 ml of a 1 per cent acetylated human serum albumin solution in 0.15 M NaCl. The albumin was mildly acetylated using conditions described for Experiment 135 DI, Table I (13), to prevent its binding of tryptophan. The white cell counts and platelet counts were made in quadruplicate on aliquots of the plug suspension. A recovery of 70 to 90 per cent of the white cells was obtained. The plug suspension contained less than 5 per cent of the total blood platelets. Differential counts made on the plug suspension and whole blood did not vary significantly. An aliquot of the plug suspension was centrifuged in a microtube for 20

² A dextran (grade H, clinical, lot H 1257) was recently obtained from Laros and Co.; it is free of insoluble particles and can be used without filtering and decalcification. This type was not used in the experiments reported here.

minutes at 7,800 × G using the centrifuge described by Gold and Solomon (14). The relative volume of packed white cells was taken as the volume fraction of cells in the plug suspension.

The volume of the remainder of the plug suspension, usually 0.6 to 0.8 ml, was measured and made up to 2 ml with water. The cells were lysed by freezing and thawing and dialyzed in Visking tubing size 22/32 for 2 hours against 25 ml of water by the thin layer technique. The dialysate was treated with resin in the same manner as was described for the erythrocyte dialysates and dried from the frozen state in a 25 ml round bottom flask.

The residues of the dialysates from the plasma were dissolved in 0.345 ml of Tergitol-Safranin solution, the composition of which was as follows: 0.05 per cent Tergitol Nonionic NPX (Carbide and Carbon Chemical Co.), 0.005 per cent Safranin O dye (National Aniline Division, Allied Chemical and Dye Corp.) and 0.4 per cent EDTA trisodium salt. The wetting agent in this solution facilitates dissolving the residue in the flask and makes micropipetting more reproducible; the dye serves as a marker to aid in counting the multiple applications of the concentrates placed on the papers; EDTA is added as a general precaution to prevent the interference of bivalent cations in chromatography. The residues of the dialysates of the erythrocytes were dissolved in the Tergitol-Safranin solution on the basis of 0.054 ml per g packed cells dialyzed. The residues of the dialysates of the leukocytes were dissolved in the Tergitol-Safranin solution on the basis of 0.862 ml per ml of packed cells dialyzed. In the calculations, a 14.7 per cent increase in volume of the Tergitol-Safranin solution was allowed for the salts present.

Preparation of the urine samples for analysis. An aliquot of urine equivalent to a 12 minute excretion period was passed at 2° C through a resin column containing 4 ml of wet resin of the same type and prepared in the same manner as the resin used for the collection of blood. The flow rate was adjusted to about 1 ml per minute. The column was washed with a 10 mM solution of NaCl to give a total volume of effluent and wash of 40 ml. The resin step removes substances from the urine which might otherwise precipitate when the urine is concentrated and interfere with chromatography; amino acids were not retained by the resin. A sample of the mixed urine and wash solution was removed for total amino acid estimation (see below). The remainder was lyophilized in a 50 ml round bottom flask.

Concentrations of unbound amino acids in urine vary considerably. In order to estimate the approximate concentration for chromatography, a 50 µl aliquot of the mixed urine and resin wash was placed on Whatman no. 1 paper which had previously been dipped in 0.1 per cent NaOH solution and dried. Fifty µl aliquots of solutions of alanine at concentrations of 0.5, 1, 2 and 4 mM were placed in the vicinity of the samples. The paper was dipped in the ninhydrin reagent, less collidine (1), and placed in an oven for 15 minutes at 60° C for color development. Where the intensity of the zone of the

TABLE II

Urinary amino acid excretion rates of normal subjects (μmoles per hour)

	Standard solution	No. of subjects	Fasting urines (paper chromatography)		Nonfasting urines (resin column)*	
			Mean	Range	Mean	Range
	mmoles/L					
Alanine	3.0	15	7.8	3.0–16.0	21.6	9.4–33.0
α-Amino-*n*-butyric acid	0.5	5	<2.0			
Arginine	0.0	15	<1.0		<2.4	
Aspartic acid	0.0	8	<1.0		· <3.0	
Glutamic acid	1.0	9	<1.0		<3.0	
Glutamine	4.0	15	17.1	6.0–28.0		
Histidine	8.0	14	32.4†	6.0–80.0	102.0†	41.0–138.0
Lysine	2.0	14	8.4	3.3–22.0	5.4	2.9–14.2
Methionine	0.5	15	<1.0		<3.0	
Ornithine	1.0	15	<2.0			
Phenylalanine	1.0	15	<2.0	<1.5–3.0	4.5	2.5–7.5
Proline	1.0	15	<1.0		<3.5	
Threonine	3.0	14	7.6	4.2–22.0	9.8	5.2–17.5
Tryptophan	0.5	14	<1.7	<0.5–5.0		
Tyrosine	1.0	15	3.1	1.5–6.0	8.0	3.5–11.5
Valine	1.0	15	<2.0		<3.5	
Ethanolamine	2.0	14	14.1	7.8–24.0		
Guanidoacetic acid	2.0	5	9.6	4.0–20.0		
Leucine plus isoleucine	3.5‡	15	<7.0		10.2	6.7–16.3
Serine plus glycine	10.0§	15	61.3	26.0–93.0	90.0	46.0–135.0
Taurine	5.0	15	24.1	13.0–31.0	52.0	28.4–100.0
Urea	1,000.0	15	11,450.0	3,800.0–24,000.0		

* Stein's values, 8 subjects (19).
† Includes the methyl histidines.
‡ Leucine, 2.0 mmoles; isoleucine, 1.5 mmoles.
§ Serine, 3.0 mmoles; glycine, 7.0 mmoles.

sample was between that of the 0.5 and 1 mM alanine standard, the dried residue from the urine was dissolved in 0.5 ml of the above described Tergitol-Safranin dye solution; where the intensity was between the 1 and 2 mM standard, it was dissolved in 1 ml of the Tergitol-Safranin solution; and where it was between the 2 and 4 mM standards, it was dissolved in 2 ml of the solution.

Results showed that the salts and other substances that dissolved in the Tergitol-Safranin solution increased its volume approximately 20 per cent. This increase in volume was not measured in each case but was assumed, and in the calculations appropriate corrections were made.

Chromatography. The developing solutions and staining reagents were the same as used in our earlier method for plasma amino acid determinations except for the following: *a*) 2, 4 and 8 μl aliquots of samples and 2, 3, 4, 6 and 8 μl aliquots of standard solution were placed on the chromatograms; *b*) S and S paper no. 589 (green ribbon) was used for the amino acid analysis determined by the ninhydrin-collidine and the isatin reagents; *c*) the determination of glycine with Solvent B (1) was omitted, since quantitative estimates made from chromatograms developed in this solvent have been found to be poor; furthermore, when taurine is present in large amounts, it interferes with the glycine zone. *d*) *p*-Dimethylamino-cinnamaldehyde was used in lieu of *p*-dimethylamino-benzaldehyde in Ehrlich's reagent (15); and *e*) all periods of 16 hour development, except for the paper used to determine histidine and tyrosine, were reduced to 6

hours. It was found that substances on the chromatograms gave more intense stains after a 6 hour development period than after a 16 hour period; this modification, however, was a compromise since leucine and isoleucine were not resolved separately in the shorter time interval. The composition of the standard amino acid solution used for plasma, red cells and white cells is given in Table I; that for the urine amino acid determinations is given in Table II.[3] To minimize deterioration of the standard solution, 5 ml aliquots were freeze-dried and stored at − 5° C. Glutamine, which was not stable in the presence of other amino acids in the dried preparations, and urea, which caused difficulty in drying of the amino acid solutions, were added to the Tergitol-Safranin solution which was used to reconstitute dried aliquots of the standard at approximately monthly intervals.

Estimations of the concentrations of the compounds were as previously described (1). The taurine concentration in the white cells was estimated by using the serine and glycine zone of the standard. The concentration of arginine, which has the same Rf value as taurine,

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[3] All compounds were obtained from Mann Research Laboratories, New York, N. Y., except urea which was obtained from Merck and Co., Rahway, N. J., and ethanolamine which was obtained from Fisher Scientific Co., New York, N. Y. The compounds were dried *in vacuo* prior to weighing.

was found to be very low in the cells and its contribution to the intensity of the taurine zone was negligible; this was always confirmed by the Sakaguchi test. The concentration of O-phosphoethanolamine, which in Solvent A (1) has an Rf value approximately one-half that of glutamic acid, was estimated on the chromatograms developed 64 hours using the glutamic acid zone as a standard. Ergothioneine concentration was estimated on the papers treated with Pauly's reagent; its Rf value was slightly faster than histidine. Ethanolamine and β-amino isobutyric acid concentrations were estimated on the papers treated with the ninhydrin-collidine reagent after 6 hours' development. The Rf value of ethanolamine was slightly faster than valine; the Rf value of β-amino isobutyric acid was slightly faster than alanine. The concentration of guanidoacetic acid was determined by the Sakaguchi test; its Rf value was slightly faster than arginine. Glutathionine and diglutathionine, both major ninhydrin-positive components of erythrocytes, did not move from the origin of application on chromatograms developed in Solvent A and were not determined. Histidine and methyl histidines (the latter are present in moderate amounts in urine) are reported as a combined value, since the isopropanol solvent does not resolve these compounds.

To express the amino acid values on the basis of a kilogram of water, plasma was taken to be 92 per cent and leukocytes 74 per cent water by volume. The erythrocytes were taken to be 65 per cent and urine 100 per cent water by weight.

Statistics. The individual amino acid values found in the study of the plasma, erythrocytes and leukocytes were assumed to be normally distributed. This assumption was reasonably satisfactory in most instances. In some leukocyte analyses, a skew distribution was evident for substances in low concentration. No attempt was made to adjust for this, since its effect was never such as to invalidate any of the conclusions drawn. Skewness was of such magnitude in some of the urine studies that ranges of the excretion rates were given rather than an expression of variability by a standard deviation notation.

The test statistic $t = \dfrac{\bar{x} - \bar{y}}{\sqrt{s_x^2/n_x + s_y^2/n_y}}$ was used to determine whether the mean of one sample population was equal to the mean of another sample population (16). The degree of freedom for this test statistic (referred to hereafter as the t test) was obtained from the formula:

$$\frac{(s_x^2/n_x + s_y^2/n_y)^2}{\dfrac{s_x^4/n_x^2}{(n_x + 1)} + \dfrac{s_y^4/n_y^2}{(n_y + 1)}} - 2$$

where s_x and n_x were, respectively, the standard deviation and number of samples from population x; and s_y and n_y were the same parameters from population y. The level of significance, α, that is, the probability of rejecting the hypothesis that the two means were equal when in fact they were equal, was taken as 0.05 for a two-sided test. Since in some categories the number of

subjects was small, the β error, which is the probability that the significant difference would not be redetected, in another set of experiments in which the same number of samples was employed, was large. The β error has therefore been estimated for analyses where significant differences have been found.

The sample correlation coefficient for regression analysis, R, was calculated to detect associations between concentrations intracellularly and extracellularly. Association probabilities greater than 0.95 (a rejection of $p = O$ at an α level of 0.05) are noted in the tables by the symbol "+." The symbol O indicates a failure of this test to detect an association.

RESULTS AND DISCUSSION

Reliability of the analytical methods

The precision of analysis and recovery of added amino acids have been previously studied for plasma (1). Investigations as to the suitability of the salt-saturated chromatographic technique for the analysis of erythrocytes, leukocytes and urine are reported here.

Recovery of amino acids added to lysed erythrocyte solutions and variability of the erythrocyte analyses. Amino acids added to the lysed erythrocyte solutions were recovered quantitatively in a 2 hour period of dialysis (Table III). When the duration of dialysis was extended up to 16 hours, variable increases in the concentration of amino acids in the dialysate were observed. For 2 hours of dialysis this amounted to less than 10 per cent and was not corrected for. The increase appears to be partly enzymatically induced, since it could be diminished by addition of enzyme inhibitors such as dodecyl sulfate, EDTA and N-maleylimide to the lysed erythrocyte solution prior to dialysis. The accuracy of the erythrocyte analyses as computed from the average variability of the data in Table III, was similar to that of the plasma analyses (1).

The red cell samples were found to be slightly contaminated with white cells. By comparison of the taurine levels found in the red cells with those found in the white cells, and assuming that taurine was absent in red cells, it was shown that this contamination could not increase the red cell levels of the other substances determined by more than 2 per cent.

Reproducibility of leukocyte analyses. In view of the small amounts of solution available, paper chromatography proved to be a very useful tech-

TABLE III

Recovery of amino acids added to lysed erythrocyte solutions

	Average amts. added*	Total amts. calc. present*	Total amts. found*	Re-covery
		μmoles/kg water		*%*
Alanine	601	972	1,010	106
α-Amino-*n*-butyric acid	72	102	110	111
Glutamic acid	313	1,066	1,180	136
Glutamine	970	1,700	1,830	113
Histidine	243	· 360	387	. 111
Lysine	315	487	415	77
Methionine	72	102	92	86
Ornithine	193	336	350	107
Phenylalanine	120	191	162	76
Proline	340	468	423	87
Threonine	315	446	420	92
Tryptophan	77	95	82	83
Tyrosine	169	241	220	88
Valine	435	667	645	95
Leucine plus isoleucine†	362	565	557	98
Serine plus glycine‡	726	1,553	1,680	117
Urea	9,700	16,360	16,700	103

* The mean of duplicate analyses.
† Leucine 217; isoleucine 145.
‡ Serine 242; glycine 484.

nique for the analysis of unbound amino acid concentrations in the leukocytes. The amino acid values obtained from the lysed leukocyte solutions, as opposed to the values obtained from the lysed erythrocyte solutions, were found not to change significantly on extension of the dialysis period from 2 to 4 hours; furthermore, duplicate analyses of the leukocytes were only slightly less reproducible than those of plasma analyses (Table IV). This stability of the leukocyte suspensions was surprising, since these cells are generally considered to have more active proteolytic enzymes than the erythrocytes. Undoubtedly an important factor in this instance was the relative volume of lysed cells dialyzed; the volumes of the leukocytes were ⅟₂₀ to ⅟₄₀ the volume of the erythrocytes.

Effect of temperature on the concentrations in the cells and plasma. Since it was desirable for stability purposes to chill the blood to 2° C for its transportation to the laboratory and separation of the cells, it became necessary to obtain some estimate of the effect of cooling on the amino acid distributions. Whole blood was drawn and the plasma and cells separated as rapidly as possible at 37° C. This posed no difficulty for the plasma and erythrocytes, since separations were made within 30

TABLE IV

Reproducibility of unbound amino acid analyses of lysed leukocytes *

	Average of means	Standard deviation	Coefficient of variation	Coefficient of variation of plasma analyses†
	μmoles/kg water			
Alanine	2,180	143	7	5.2
Glutamic acid	3,720	560	15	9.4
Glutamine	635	131	8	6.2
Histidine	374	83	22	5.9
Lysine	1,300	78	6	8.1
Methionine‡	540	47	9	18.0
Ornithine	1,300	163	13	11.0
Phenylalanine‡	730	78	11	12.0
Proline	648	134	21	8.7
Threonine	1,190	185	16	10.8
Tryptophan‡	160	23	14	18.0
Tyrosine	392	73	18	10.0
Valine	873	115	13	4.0
Ethanolamine‡	148	57	38	
Leucine plus isoleucine	1,320	111	9	7.5
Serine plus glycine	6,084	840	14	7.0
Taurine	27,800	937	4	
O-phospho-ethanolamine	7,940	446	6	

* Lysed cells of two subjects were analyzed, each in triplicate. Four periods of dialysis were for 2 hours, two periods were for 4 hours.
† See reference (1).
‡ Three determinations only.

minutes after drawing the blood by increasing the centrifugal force to 2,500 × G. For the leukocytes, centrifugation of the dextran supernatant solution at 37° C gave a packed cell plug which was frequently irreversibly clumped even though the time of centrifugation was reduced; in 12 attempts only 3 samples redispersed. The results of these studies are shown in Table V.

With the exception of two compounds, ornithine in the plasma and phenylalanine in the erythrocytes, there were no significant differences as evaluated by the *t* test in the analyses conducted at 2° and 37° C. The values for ornithine in the plasma and phenylalanine in the erythrocytes differed by amounts which were at the border line of significance ($\alpha = 0.05$). Furthermore the β error was large because of the small

TABLE V

*Concentrations of amino acids in plasma, erythrocytes and leukocytes at 37° C ***

	A Plasma		B Erythrocytes		C Erythrocyte-plasma ratio		D Leukocytes	
	Mean	PM[†]	Mean	PM	Mean	PM	Mean	PM
Alanine	398 ± 27[‡]	ns	477 ± 30	ns	1.22 ± 0.30	ns	2,860 ± 940	ns
α-Amino-n-butyric acid	25 ± 5	ns	26 ± 1	ns	1.14 ± 0.40	ns		
Arginine	57 ± 3	ns						
Glutamic acid			465 ± 33	ns			2,520 ± 670	ns
Glutamine	421 ± 60	ns	477 ± 34	ns	1.17 ± 0.26	ns	1,660 ± 100	ns
Histidine	87 ± 12§	ns	121 ± 21	ns	1.35 ± 0.52§	ns	950‖	ns
Lysine	210 ± 18	ns	202 ± 10	ns	1.00 ± 0.26	ns	1,910 ± 540	ns
Methionine			51 ± 9	ns				
Ornithine	135 ± 20	SH(0.50)	202 ± 20	ns	1.50 ± 0.40	ns	2,170 ± 520	ns
Phenylalanine	67 ± 7	ns	82 ± 9	SH(0.50)	1.26 ± 0.21	ns		
Proline	200 ± 14	ns	226 ± 17	ns	1.15 ± 0.12	ns		
Threonine	179 ± 11	ns	183 ± 8	ns	1.04 ± 0.18	ns	1,740 ± 370	ns
Tryptophan			35 ± 4	ns				
Tyrosine	80 ± 3	ns	82 ± 6	ns	1.03 ± 0.10	ns		
Valine	205 ± 11	ns	221 ± 16	ns	1.08 ± 0.16	ns	1,010 ± 420	ns
Ergothioneine	<10		508 ± 160	ns			<300	
Ethanolamine	<10		<10				<300	
Leucine plus isoleucine	233 ± 8	ns	296 ± 19	ns	1.29 ± 0.17	ns	2,860‖	ns
Serine plus glycine	687 ± 66	ns	1,091 ± 77	ns	1.62 ± 0.28	ns	11,900 ± 2,450	ns
Taurine			<70				36,400 ± 8,240	ns
Urea	5,413 ± 190	ns	5,887 ± 600	ns	1.09 ± 0.18	ns		
O-phospho-ethanolamine							3,550 ± 1,540	ns
Cell volume¶							0.501 ± 0.121	ns

* The plasma and erythrocyte values are the mean from four subjects. The leukocyte values are the mean of analyses of three subjects. The low yields of leukocytes at this higher temperature did not give sufficient material to determine several of the amino acids. The values are expressed as μmoles per kilogram water.

† Comparison of the mean at 37° C with the appropriate mean at 2° C taken from Table I, VI, or VII; ns = not significantly different; S = significantly different; H or L = a high or low value, respectively, at 37° C. The number in the parentheses is the β error.

‡ Standard deviation of the mean.

§ Three determinations.

‖ One determination.

¶ Volume of one billion leukocytes in milliliters.

number of samples determined. To investigate further whether these concentration differences were real, additional ornithine and phenylalanine determinations were made at 2° and 37° C with blood from the same donor. In this instance the values at the two temperatures agreed in duplicate analyses within 15 per cent, which is well within the experimental variability of the method and makes it unlikely that the differences found be-fore were significant. Given as micromoles per kilogram water, ornithine was 118, at 2° and 113 at 37° C, phenylalanine was 50 at 2° and 58 at 37° C. To obtain more power in our statistical treatment, a sign-rank test was conducted on all the data. The analyses were normalized by di-viding the difference in the means at the two temperatures by the standard deviation. By this test the results of the plasma and leukocyte analy-

ses were found to be identical at the two temperatures whereas the erythrocyte determinations were significantly higher at 37° than at 2° C. Further evidence of higher values for the erythrocytes at 37° C was seen by comparison of the totals of the amino acid concentrations in which the values at 37° were 7 per cent higher than those at 2° C. In that the plasma amino acid values did not differ whether determinations were conducted at the higher or lower temperature, it can be inferred that the changes in the erythrocyte concentrations were not induced by a distributional shift but, rather, that they were due to the lower stability of the erythrocytes at 37° C.

From these results it was concluded that chilling the blood to 2° C for up to 2 hours would not cause major changes in the unbound amino acid distributions.

Reliability of the urine amino acid determinations. The excretion rates of most compounds in urine vary from large amounts to nil. This, combined with the unusually large number of compounds which may be excreted (17–19) leads to some uncertainty as to whether the amino acid zones on the chromatograms were pure zones or zones in which the amino acid was mixed with other substances. Several steps in the analytical procedure tended to reduce this uncertainty: *a*) the passage of chilled urine through a short resin column (described above) removed all colored and cold-insoluble solid material, and presumably other substances, but not the amino acids or urea; *b*) the characteristic colors shown by many of the amino acids with the ninhydrin-collidine reagent (1) helped greatly in preventing false identification of substances; and *c*) the variety of staining reagents used in each analysis assisted in identifying zones that contain mixtures of substances. Furthermore, in some instances, zones were rechromatographed in different solvents to determine whether they contained mixtures of compounds. An important factor with the salt-saturated systems was the tendency for a large number of unknown substances to move with slow Rf values and not to be encountered in the regions of the amino acid zones. Exceptions to this were the slow moving amino acids, aspartic and glutamic. In about one-third of the urines, substances interfered with the analysis of these two amino acids. In the remaining urines no color zones were found

at glutamic acid and aspartic acid locations. Whereas paper chromatography has certain drawbacks for urine analyses, it does not lead to the destruction of glutamine, tryptophan or possibly other labile substances which are destroyed by conditions used in resin column analyses (19).

A recovery study of the substances present in the urine standard added to urine specimens indicated no loss during the procedure. Recoveries of 83 to 132 per cent were obtained. The coefficient of variation averaged about 7, which is similar to the accuracy obtained in the other analyses using salt-saturated paper chromatography.

Normal fasting values in the body fluids

Unbound amino acid concentrations in plasma. The concentrations of unbound amino acids found in plasma are reported in Table I. These values agree reasonably well with those previously reported in which larger aliquots were used for analysis, except that the combined values of serine and glycine in the plasma are higher than those reported previously (1). Plasma values for tryptophan are not given. Tryptophan was largely bound by the plasma albumin and the conditions under which the present experiments were conducted would not give a correct unbound value for tryptophan.[4] One anomalously high glutamic acid value was found (70 μmoles per kg water); all other subjects had values lower than 30 μmoles per kg water.

Unbound amino acid concentrations in erythrocytes. The concentrations of the amino acids in the erythrocytes and the erythrocyte-plasma concentration ratios are given in Table VI. The means of the plasma and the erythrocyte concentrations were statistically the same for alanine, α-amino-*n*-butyric acid, glutamine, lysine, methionine, phenylalanine, proline, threonine, tyrosine, valine, leucine plus isoleucine, and urea. In testing the distribution of molecules which carry net charges, a Donnan ratio of 1.4 was used between the plasma and erythrocytes. The average of the erythrocyte-plasma ratios for these substances, excluding that of methionine whose ratio was high and irregular, was 1.10. The increase over the expected ratio of 1.0 is believed to be an artifact induced by enzymatic activity in the erythrocytes

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[4] McMenamy, R. H., and Lund, C. C. To be published.

TABLE VI

Unbound amino acid concentrations in erythrocytes

	Erythrocytes		Erythrocyte-plasma ratio			
	No. of subjects		No. of subjects	Mean	PM*	PC†
		μmoles/kg water				
Alanine	15	427 ± 25‡	15	1.08 ± 0.04	ns	+
α-Amino-n-butyric acid	9	25 ± 2	9	0.96 ± 0.08	ns	+
Arginine	11	<12	10	low	SL(<0.01)	ID§
Glutamic acid	15	374 ± 33	15	high	SH(<0.01)	ID§
Glutamine	15	601 ± 46	15	1.27 ± 0.10	ns	0
Histidine	14	131 ± 8	12	1.58 ± 0.12	SH(0.10)	0
Lysine	15	233 ± 24	15	1.15 ± 0.09	ns	0
Methionine	12	30 ± 4	12	1.63 ± 0.34	ns	0
Ornithine	14	185 ± 13	11	2.50 ± 0.11	SH(<0.01)	0
Phenylalanine	14	59 ± 4	14	1.18 ± 0.28	ns	0
Proline	15	191 ± 11	15	1.13 ± 0.06	ns	+
Threonine	15	157 ± 9	15	0.97 ± 0.06	ns	0
Tryptophan	15	21 ± 4		(1.2)‖		
Tyrosine	15	72 ± 5	15	1.03 ± 0.08	ns	+
Valine	15	223 ± 12	15	1.12 ± 0.05	ns	+
Ergothioneine	13	458 ± 51	14	high	SH(<0.01)	ID§
Ethanolamine	15	<10				
Leucine plus isoleucine	14	211 ± 21	13	1.16 ± 0.09	ns	+
Serine plus glycine	14	948 ± 48	14	1.59 ± 0.10	SH(0.01)	0
Taurine	8	<70				
Urea	13	6,000 ± 370	13	1.14 ± 0.05	ns	+

* A comparison of the mean value in the erythrocytes to the mean value in the plasma; see footnote † Table V for terms.

† Probability that there is a correlation between the concentration in the erythrocytes and the concentration in the plasma; + = correlation indicated, 0 = correlation not supported.

‡ Standard deviation of the mean.

§ Indeterminate, concentration of substance in one medium is missing.

‖ Estimated; see text.

(see previous discussion); and, if such an increase is allowed, the probability of equivalence of means of the above list of compounds is further improved. On the other hand, the mean concentrations of arginine, glutamic acid, histidine, ornithine, ergothioneine, and serine plus glycine were significantly different, implying that a concentration gradient across the red cell membrane exists for many substances. Aspartic acid was also undoubtely concentrated by the erythrocytes; it was frequently seen on the chromatograms of the red cell dialysates but never on those of the plasma. With the possible exception of histidine, concentration gradients of similar magnitude have been ob-

served by others (8, 9, 20, 21). Tryptophan, in contrast to the plasma studies, was not bound to a nondialyzable component of lysed red cells (see Table III) and, therefore, concentrations obtained for it by dialysis were reliable. The erythrocyte-plasma ratio for the unbound tryptophan concentration was estimated by using an average unbound plasma tryptophan value of 17 μmoles per kg water.[4]

The low concentrations of arginine and the elevated levels of ornithine in red cells are of interest in view of the presence of arginase in these cells (22). The observed concentration gradients may be due to the slow transport of these compounds

across the red cell membrane in the presence of relatively high arginase activity. Such a phenomenon as this had been attributed to Ehrlich ascites carcinoma cells by Johnstone (23).

The hypothesis: "Is the concentration of the substance in the erythrocytes influenced by the concentration in the plasma?" was examined by computation of the correlation coefficient R (see column p_C, Table VI). A correlation with a probability ≥ 0.95 was found for alanine, α-amino-n-butyric acid, proline, tyrosine, valine, leucine plus isoleucine, and urea implying that a concentration dependence exists between the two media. An association in concentration intracellularly and extracellularly was not found for several of the compounds; however, by the very nature of the mathematical test this cannot be taken as an index of no dependence. Furthermore, in this investigation normal fasting subjects were studied and no large fluctuation in concentrations was imposed which would have made the correlation tests more sensitive. A detailed study of the relationship between the erythrocyte-plasma concentrations is in progress.

Unbound amino acid concentration in leukocytes. The amino acid concentrations in the leukocytes of normal subjects were considerably higher than those in plasma or erythrocytes (Table VII). When compared with plasma glutamic acid, ornithine, serine plus glycine, taurine, and O-phosphoethanolamine were concentrated 20 times or more by the leukocytes, whereas the other amino acids (excluding arginine) were concentrated on the average 9.9 times (range 4.8 to 14.5). It was of interest that glutamic acid, ornithine, and serine plus glycine also showed an enhanced concentration relative to the other amino acids in the erythrocytes, and it is implied that the concentrative potential for both the erythrocytes and leukocytes is large for these substances. Arginine was generally low in the leukocytes, although an occasionally high result was found, and ornithine, as noted above, was elevated. This again may be due to the arginase which has been found in the leukocytes (24). α-Amino-n-butyric acid was probably present in the leukocytes; its quantity was not estimated due to the interference on the chromatograms of an unknown ninhydrin-positive substance. The leukocyte-plasma ratio for trypto-

phan was estimated by using an average plasma concentration for tryptophan of 17 μmoles per kg water.[4] It also should be noted that whereas there was a direct correlation between the concentration of many amino acids in plasma and in erythrocytes, this was not observed in the leukocyte-plasma distribution (see column p_C, Table VII). This may well be due to the small difference in concentration found in bloods of subjects in the fasting state.

The concentration of amino acids in the leukocytes is of the approximate order of magnitude found in other mammalian tissues (25, 26). This is not unexpected, since the leukocytes appear to perform most of the general metabolic functions of such cells.

Unbound amino acid excretion rates in urine. Williams and co-workers (18) found that the 24-hour excretion patterns of amino acids were reasonably constant from day to day in any one subject. We studied the excretion rate of two fasting individuals by means of urines collected for shorter periods on different days and also found for each of these that the rates were reasonably constant from day to day (Table VIII). With the exception of taurine, the mean variation was less than 40 per cent. This variation is small in view of the large differences in excretion rates of urines from different individuals (see later results). The excretion rates were not related to the volume of urine voided during the period.

The effect of food ingestion on excretion is shown by comparison of columns A and C of Table IX. When subjected to a sign-rank test the differences between these columns were highly significant. All substances were consistently excreted at a higher rate in the nonfasting period, averaging 60 per cent higher than in the fasting period. This was in qualitative agreement with α-amino nitrogen values of 6.6 mg per hour for nonfasting individuals, and 4.4 mg per hour for fasting individuals reported by Eckhardt, Cooper, Faloon and Davidson (27). The increased excretion in the nonfasting period was approximately the same for each amino acid. In unpublished experiments it has been found that the unbound amino acids increased an average of 20 per cent in the plasma of five normal adults 2 hours after a typical 25 g protein meal. This suggests that

TABLE VII

Unbound amino acid concentrations in leukocytes

| | Leukocytes | | Leukocyte-plasma ratio | | | |
	No. of subjects		No of subjects	Mean	PM*	Pc†
		μmoles/kg water				
Alanine	15	2,881 + 256‡	15	7.5 ± 0.9‡	SH	0
Arginine	13	<290 ± [46]	13	<4.2 ± [1.2]	ID§	
Glutamic acid	15	2,745 ± 251		very high	SH	
Glutamine	15	2,650 ± 251	15	5.2 ± 0.6	SH	0
Histidine	10	762 ± 70	9	9.8 ± 1.5	SH	0
Lysine	15	2,111 ± 216	15	10.2 ± 1.1	SH	0
Methionine	12	391 ± 54	11	14.5 ± 2.1	SH	0
Ornithine	12	1,767 ± 113	9	20.6 ± 2.0	SH	0
Phenylalanine	9	647 ± 105	8	13.6 ± 2.3	SH	0
Proline	15	862 ± 79	15	4.8 ± 0.4	SH	0
Threonine	14	2,345 ± 174	13	14.4 ± 1.3	SH	0
Tryptophan	10	222 ± 31		(11.7)‖	SH	
Tyrosine	15	480 ± 97	15	12.0 ± 1.2	SH	0
Valine	15	1,335 ± 132	15	6.6 ± 0.6	SH	0
Ergothioneine	15	<300				
Ethanolamine	15	<250				
Leucine plus isoleucine	13	1,999 ± 195	12	10.4 ± 1.2	SH	0
Serine plus glycine	15	13,021 ± 1,480	14	22.1 ± 2.8	SH	0
Taurine	15	28,683 ± 2,726		very high	SH	
O-phospho-ethanolamine	8	2,651 ± 389		very high	SH	
Cell volume¶	13	0.604 ± 0.028				

* A comparison of the mean in the leukocytes and plasma; see footnote † Table V for terms.   The β error is less than 0.01 in all instances.
   † Probability that there is a correlation between the concentration in the leukocytes and the concentration in the plasma; + = correlation indicated, 0 = correlation not indicated.
   ‡ Standard deviation of the mean.   Values in brackets are approximate.
   § Indeterminate, concentration of substance in one medium was missing.
   ‖ Estimated; see text.
   ¶ Volume of one billion leukocytes.

the increased excretion is caused by the post-prandial rise in the plasma amino acids. The fasting urine specimens collected as described in the experimental procedure should be largely free of this diet effect. Excretion rates thus measured should be more directly related to the fasting blood amino acid levels than are specimens collected through a 24 hour period which encompasses fasting and nonfasting intervals.

In column B of Table IX the excretion rates of subjects undergoing moderate exercise (an hour's walk of 3 to 4 miles between the hours of 8 and 10 a.m.) are given. This was an attempt to see whether such activity influenced the amino acid excretion. The differences between the overnight specimens and these latter samples were not significant. The similarity of the excretion rates of the two periods also implied that a 5 hour interval after the meal was sufficient to obtain constant fasting values. Diurnal variations were not studied in this work.

The urine excretion rates of the 15 normal fasting subjects on whom the blood analyses were conducted are presented in Table II. For comparison, Stein's values obtained by resin column chromatography on 24-hour collections of nonfasting

TABLE VIII

Urinary excretion of amino acids of two normal subjects on different days

	Subject A			Subject B		
	Day 1	Day 10	Day 135	Day 1	Day 10	Day 135
	μmoles/hr			*μmoles/hr*		
Alanine	11.0	12.0	9.8	11.3	8.4	7.7
Glutamine	28.0	38.0	26.3	27.8	22.0	26.9
Histidine plus methyl histidines	64.0	81.6	62.5	56.4	42.0	42.3
Lysine	9.0	18.0	9.9	7.0	4.2	8.7
Threonine		13.2	8.2		5.2	
Tryptophan	<2.0	4.2	<2.0	<2.0	1.1	
Tyrosine	3.5	3.6	<2.0	3.5	5.2	
Serine plus glycine	79.0	114.0	66.0	155.0	105.0	96.0
Taurine	17.0	26.0	13.1	11.3	14.7	4.2
Volume of urine excreted, ml/hr	139	120	164	70.5	52.5	23

subjects are included in the table (19). The generally higher values reported by Stein were probably due to the analyses of nonfasting specimens. In both studies there was considerable variation in the excretion rates of different subjects. This variability was noted previously by Williams and colleagues (18) and was used as a basis for demonstrating individual differences. The amino acids frequently found excreted in high amounts in normal urines were taurine, threonine, glycine plus serine, histidine plus methyl histidines, alanine, glutamine and lysine. Excretion rates of certain other amino acids (proline, leucine, isoleucine, arginine, glutamic acid, aspartic acid, valine, α-amino-*n*-butyric acid, and phenylalanine) were usually low in the fasting specimens of normal individuals. These results agree qualitatively with those of other workers (17–19, 28).

TABLE IX

The effect of food intake and exercise on the urinary excretion of amino acids *

	Fasting A Midnight–7 a.m.	Exercise (fasting) B 8–10 a.m.	Nonfasting C 10 a.m.–midnight
Alanine	10.7 ± 0.8†	11.4 ± 0.9	22.0 ± 2.7
Glutamine	29.1 ± 3.5	34.2 ± 1.1	47.8 ± 7.8
Histidine plus methyl histidines	61.0 ± 8.3	59.9 ± 3.6	85.0 ± 13.0
Lysine	9.8 ± 3.0	8.0 ± 1.3	13.0 ± 2.9
Threonine	<8.5	<9.8	13.7 ± 3.5
Tryptophan	<1.1	<1.9	3.4 ± 1.2
Tyrosine	4.2 ± 0.4	6.0 ± 0.9	8.7 ± 1.0
Serine plus glycine	114.0 ± 16.0	138.0 ± 20.0	180.0 ± 12.0
Taurine	17.4 ± 3.3	26.0 ± 2.6	30.6 ± 7.2
Volume of urine excreted, ml/hr	96.0	106.0	86.0

* Averages of two experiments on each of two subjects. The meals eaten were their usual ones and contained on each day at least 75 g of protein. The values are expressed as micromoles excreted per hour.
† The standard deviation of the mean. Alanine, tyrosine, and serine plus glycine were on the basis of the *t* test significantly higher in column C than A. When compared by the sign-rank test columns A and B were not significantly different, whereas columns A and C were different at a high level of significance (α = 0.008).

SUMMARY

1. The concentrations of unbound amino acids in the plasma, erythrocytes and leukocytes, and rates of excretion of amino acids in the urine of normal human subjects have been determined using salt-saturated paper chromatography for analysis. The variability of the determinations and the effect of temperature on the distribution of the cell components have also been investigated.

2. The mean concentrations of values in the plasma and erythrocytes for alanine, α-amino-n-butyric acid, glutamine, methionine, phenylalanine, proline, threonine, tyrosine, valine, leucine plus isoleucine, and urea did not differ significantly. On the other hand the mean concentration of arginine, glutamic acid, histidine, ornithine, serine plus glycine, and undoubtedly aspartic acid were significantly different in the plasma and the erythrocytes. When examined by a linear regression analysis a positive correlation for an association in concentration between the erythrocytes and plasma was found for alanine, α-amino-n-butyric acid, proline, tyrosine, valine, leucine plus isoleucine, and urea. This supports a diffusion-controlled distribution for these latter substances.

3. The concentrations of amino acids in the leukocytes, with the exception of arginine which was generally too low to be measured, were much higher than those found in the plasma. The leukocyte-plasma ratios ranged from 4.8 to 14.5 for most of the amino acids. Glutamic acid, ornithine, and combined serine plus glycine were concentrated in leukocytes at leukocyte-plasma ratios of 20 or higher. Taurine and O-phosphoethanolamine, which were in very high concentration in the leukocytes, were not found in the plasma analysis, and only small concentrations of taurine were observed in the red cells. A linear regression analysis showed no relationship between the concentrations of any of the amino acids in the plasma and leukocytes.

4. In the urine studies a reasonably constant excretion rate for the same individual was observed from day to day, whereas a large variation was found in the excretion rates among different individuals. The excretion rates of amino acids during a nonfasting period were on the average 60 per cent higher than during the fasting period.

Exercise of a moderate extent had little effect on the excretion rates.

REFERENCES

1. McMenamy, R. H., Lund, C. C., and Oncley, J. L. Unbound amino acid concentrations in human blood plasmas. J. clin. Invest. 1957, 36, 1672.
2. Stein, W. H., and Moore, S. The free amino acids of human blood plasma. J. biol. Chem. 1954, 211, 915.
3. Levenson, S. M., Howard, J. M., and Rosen, H. Studies of the plasma amino acids and amino conjugates in patients with severe battle wounds. Surg. Gynec. Obstet. 1955, 101, 35.
4. Stein, W. H., Bearn, A. G., and Moore, S. The amino acid content of the blood and urine in Wilson's disease. J. clin. Invest. 1954, 33, 410.
5. Iyer, G. Y. N. Paper-chromatographic study of free amino acids in normal and anaemic erythrocytes. Indian J. med. Res. 1957, 45, 213.
6. Nour-Eldin, F., and Wilkinson, J. F. Amino-acid content of white blood cells in human leukemias. Brit. J. Haemat. 1955, 1, 358.
7. Rouser, G. Free or easily extractable amino acids in blood cells and body fluids in The Leukemias: Etiology, Pathophysiology and Treatment. J. W. Rebuck, F. H. Bethell and R. W. Monto, Eds. New York, Academic Press, 1957, p. 361.
8. Christensen, H. N., Cooper, P. F., Jr., Johnson, R. D., and Lynch, E. L. Glycine and alanine concentrations of body fluids; experimental modification. J. biol. Chem. 1947, 168, 191.
9. Johnson, C. A., and Bergeim, O. The distribution of free amino acids between erythrocytes and plasma in man. J. biol. Chem. 1951, 188, 833.
10. Lund, C. C., McMenamy, R. H., and Neville, G. J. A syringe attachment for decalcification of small quantities of blood. Amer. J. clin. Path. 1957, 28, 328.
11. Tullis, J. L., and Rochow, E. G. Non-wettable surfaces. Blood 1952, 7, 850.
12. Skoog, W. A., and Beck, W. S. Studies on the fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes. Blood 1956, 11, 436.
13. McMenamy, R. H., and Oncley, J. L. The specific binding of L-tryptophan to serum albumin. J. biol. Chem. 1958, 233, 1436.
14. Gold, G. L., and Solomon, A. K. The transport of sodium into human erythrocytes in vivo. J. gen. Physiol. 1955, 38, 389.
15. Mason, J. H., and Archer, A. A. P. G. Use of

p-dimethylaminocinnamaldehyde as spray reagent for indole derivatives on paper. Biochem. J. 1958, **69**, 60P.

16. Bowker, A. H., and Lieberman, G. J. Handbook of Industrial Statistics. Englewood Cliffs, N. J., Prentice-Hall, 1955, p. 872.

17. Westall, R. G. The amino acids and other ampholytes of urine; unidentified substances excreted in normal human urine. Biochem. J. 1955, **60**, 247.

18. Williams, R. J., Berry, H. K., and Cain, L. Biochemical Institute Studies IV. Univ. of Texas Publication, no. 5109, May 1, 1951, pp. 7, 70, 150.

19. Stein, W. H. A chromatographic investigation of the amino acid constitutents of normal urine. J. biol. Chem. 1953, **201**, 45.

20. Melville, D. B., and Lubschez, R. A method for the determination of ergothioneine in blood. J. biol. Chem. 1953, **200**, 275.

21. Jocelyn, P. C. The distribution of ergothioneine in blood as determined by a new method of estimation. Biochem. J. 1958, **70**, 656.

22. Covolo, G. C., and West, R. The activity of arginase in red blood cells. J. clin. Endocr. 1947, **7**, 325.

23. Johnstone, R. M. Arginine uptake and arginase activity in Ehrlich ascites carcinoma cells. Canad. J. Biochem. 1959, **37**, 589.

24. Borghetti, A., and Scarpioni, L. Arginase activity of human leukocytes. Enzymologia 1956, **17**, 338.

25. Tallan, H. H., Moore, S., and Stein, W. H. Studies on the free amino acids and related compounds in the tissues of the cat. J. biol. Chem. 1954, **211**, 927.

26. Piez, K. A., and Eagle, H. The free amino acid pool of cultured human cells. J. biol. Chem. 1958, **231**, 533.

27. Eckhardt, R. D., Cooper, A. M., Faloon, W. W., and Davidson, C. S. The urinary excretion of amino acids in man. Trans. N. Y. Acad. Sci. 1948, **10**, 284.

28. Doolan, P. D., Harper, H. A., Hutchin, M. E., and Shreeve, W. W. Renal clearance of eighteen individual amino acids in human subjects. J. clin. Invest. 1955, **34**, 1247.