THE FRACTIONATION AND ESTIMATION OF FREE AMINO ACIDS IN SERUM

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(Received April 18th, 1959)

Recent reports[1] have shown that there is an altered amino acid metabolism in rheumatoid arthritis, and it was considered of importance to study this abnormality in greater detail. However, no suitable chemical method for the quantitative estimation of amino acids in blood was available, other than the elegant but complex technique of MOORE AND STEIN[2], and consequently a simpler method has been developed in this department.

Acting on a suggestion by D. BIDMEAD, the analytical problem was divided into three parts, (a) removal of protein and salts from the serum, (b) separation into individual amino acids and (c) quantitative estimation of each amino acid.

BOULANGER, BISERTE AND COURTOT[3] have shown that the usual reagents used for precipitating proteins can cause losses of numerous amino acids, and also excess reagent must be removed[4]. Preliminary experiments with some other deproteinising agents, including picric acid followed by Dowex-2[5], alcohol[6], acetic acid at controlled pH[7], and the extraction of dried plasma with acid acetone[8] or butanol–phenol mixtures, gave extracts which were not suitable for our purposes. These methods were also criticised by AWAPARA AND SATO[9]. JIRGL[10] has employed a mixture of alcohol and acetone followed by ethanol to prepare blood extracts for chromatography, but only 12 amino acids were shown to be present in such extracts.

STEIN AND MOORE[11], and FLOCK *et al.*[12] have shown that the process of electrolytic desalting, used by many authors, may cause losses of certain amino acids. An alternative chemical technique has been employed by MCCOLLUM AND RIDER[13] who used acetone containing *dl*-camphorsulphonic acid to extract the amino acids from salt, followed by saturation with ammonia gas to liberate the dissolved acids.

However, ion exchange resins can be used for the simultaneous removal of both salts and protein (see *e.g.* REDFIELD[14], CARSTEN[15], PIEZ *et al.*[16]). Recently COCHRANE *et al.*[17] have passed serum through a column of Zeocarb 225(H) which adsorbed the amino acids but allowed the proteins, sugars, acids and other compounds to be washed through. Elution with 5 *M* ammonia extracted the amino acids free of salts. Unfortunately no detailed practical account was given. We have investigated this system and have found that the extracts obtained are suitable for subsequent fractionation by electrophoresis.

Two-way paper chromatography offers a convenient way of separating complex

mixtures of amino acids, but the spots are often diffuse and may consist of overlapping compounds (see *e.g.* GORDON AND NARDI[8], FLOCK *et al.*[12]). It was considered a better approach technically to separate the mixed amino acids into groups by electrophoresis followed by the analysis of each group by descending paper chromatography.

Initial experiments showed that high voltage electrophoresis in barbitone buffer (pH 8.2) would separate serum extracts into 4 groups of amino acids, but the technique was discarded partly through practical difficulties and partly due to the necessity for removing the barbitone buffer prior to paper chromatography. Eventually a method was adopted which employed electrophoresis in *N* acetic acid buffer (after DURRUM[18]). By this means it was found possible to split the free amino acids of serum into 6 groups which could then be located on the paper by a non-destructive fluorescence method (COOK AND LUSCOMBE[19]). Each group was then separated into its component amino acids by descending paper chromatography employing a solution of butanol, ethanol, ethyl acetate and dilute acetic acid. The individual acids were measured as the stable pink complexes formed on addition of cadmium sulphate to the blue colour produced by ninhydrin and amino acids[20]. Salts of zinc, nickel, copper and cobalt were substituted for cadmium sulphate but were not satisfactory.

This paper presents a detailed account of the method together with some preliminary results from its application to normal sera.

MATERIALS AND METHOD

Materials

Zeocarb 225H was prepared by washing approximately 400 g (60–80 mesh) with 1 l of 6 *N* ammonia followed by 5 l of distilled water. The material was then treated with 100 ml of 40 % HCl, 300 ml of 20 % HCl, and washed with water until the washings were free from chloride. The resin was stored under water until required, but some decomposition occurs after 3–4 weeks, as shown by a yellow colour in the supernatant water.

Power-pack for high voltage electrophoresis (1350 V) was designed and built by A. J. S. McMillan, 5 Oakfield Road, Bristol 8, Great Britain.

Method

Extraction of amino acids from serum. Practical experience showed that consistent results were not obtained by shaking the serum with added resin, and consequently the following column technique was adopted. A column of Zeocarb 225(H), 5 cm long, 0.4 cm diameter, is prepared by pouring an aqueous suspension on to a glass wool plug in the bottom of a Pyrex glass tube with a 1 mm constriction at one end, and a 20 ml bulb reservoir at the other.

1.5 ml of fresh serum is passed slowly through the column which is then washed with 50 ml of water to remove protein, sugars etc. The amino acids are eluted with 20 ml of 10 % NH_4OH which is collected in a 50 ml conical flask and evaporated off under reduced pressure in a vacuum desiccator containing NaOH pellets and fused

$CaCl_2$. The residue is dissolved in 5 ml of warm water and 4 ml pipetted into a small test tube, 4 in. × ½ in., which had been previously treated with Silicone Repelcote (Hopkins and Williams). The water is again evaporated off under reduced pressure over NaOH pellets.

In this way, an extract, equivalent to 1.2 ml of serum, can be concentrated over a small area in a test tube, from which it can be conveniently removed quantitatively with a small volume of liquid.

Electrophoresis. The blood extract is partitioned into groups of amino acids by electrophoresis in *N* acetic acid on Whatman 3 MM paper in the conventional type of horizontal apparatus (see *e.g.* McDONALD[21], WIELAND AND FISCHER[22]).

The serum extract is transferred to a strip of Whatman 3 MM paper, 5 cm wide and 20 in. long, by a technique designed to obtain even distribution along the load line, and to prevent amino acids running along the edges of the paper. The dry residue is dissolved in 0.05 ml of warm water and evenly distributed over an area of paper 4 cm × 3 cm adjacent to the load line which is 2½ in. from the anode end of the strip. This is allowed to partially dry, and the tube rinsed out with a further 0.05 ml of water which is transferred to the paper in a similar fashion and evaporated by a cold air blast. The strip is clamped in the electrophoretic apparatus, and wetted evenly with *N* acetic acid from both ends, so that the advancing liquid fronts meet at the loading line. In this way the compounds within the loading rectangle are dissolved up, and distributed along the starting line. Electrophoresis is carried out for 2½–3 h at 1,350 V, with a current passing of 2½ mA/strip of paper 5 cm wide. At the end of this time the paper is dried *in situ* by a stream of cold air.

Early experiments demonstrated that to obtain reasonable separations it was essential to minimise electro-osmotic flow of buffer solution, and the customary wet anode has therefore been substituted by a 26 SWG platinum wire sandwiched between two perspex strips which are pressed together by elastic bands. When in operation the end of the paper is folded and nipped between the perspex strips, thus forcing the wire into the wet paper surface and ensuring a good electrical contact. Fig. 1 describes the paper and electrode layout.

After electrophoresis, the dried paper strips are immersed in acetone containing 0.1 % *o*-coumaric acid and dried at room temperature for 4–5 min, followed by heating in an oven at 100° for 1½–2 min. Exposure to ultra violet light from a Mazda MBW/U bulb produces yellow fluorescent bands of amino acid–coumaric acid complex, which can be easily outlined with pencil. The imino acids, proline and hydroxyproline, can also be visualised. Overheating and over exposure to ultra violet radiation should be avoided[19].

The most rapidly moving band containing ornithine, lysine, histidine and arginine, is sometimes rather diffuse and difficult to see, but it can be concentrated by ascending chromatography with water followed by drying and relocating by the ultra violet technique.

The outlined bands are excised, dropped into test tubes and extracted with 5 ml of water for at least 2 h with occasional shaking. Suitable aliquots of the solutions,

usually 3–4 ml, are measured out into test tubes, 4 in. × ½ in., previously treated with Silicone Repelcote, and evaporated under suction in a desiccator over NaOH pellets.

The dry residue, concentrated over a very small area in the bottom of the tube, is dissolved in 0.02 ml of water, and applied as a thin streak[23], 2 cm long, to a sheet of Whatman No. 1 filter paper 22½ in. long by 8 in. wide, drying being hastened by a cold air blast. The test tube is washed out twice more with 0.02 ml of water.

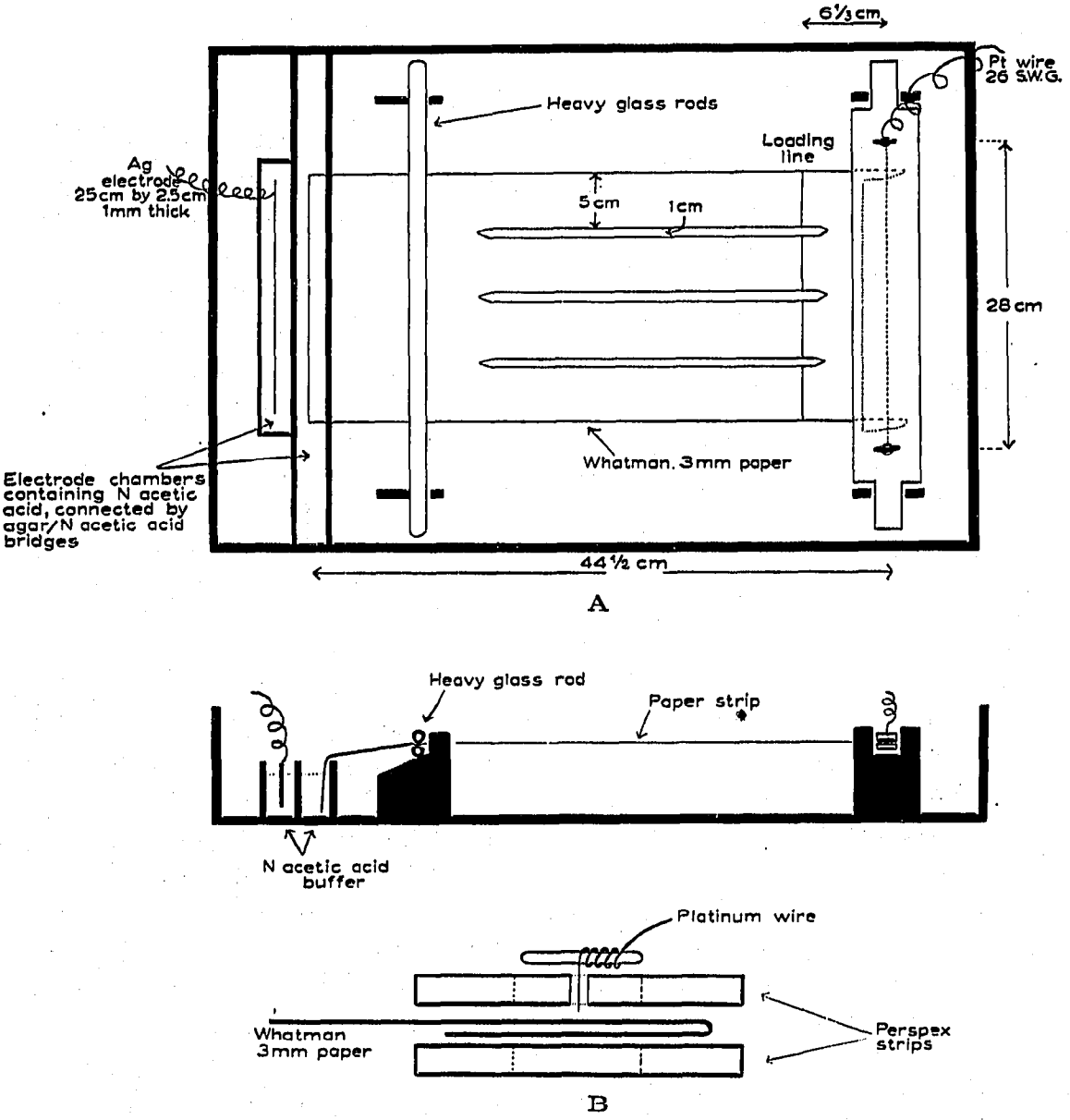

Fig. 1. Layout of the apparatus and paper for electrophoresis. (A) The apparatus, drawn to scale, is home built from perspex sheets. The thick paper is held at one end between two wide perspex strips (B) which are supported in two perspex holders, slotted vertically, and clamped at the other end between two heavy glass rods, resting on a perspex incline. This system holds the paper taut, and pools of electrolyte cannot accumulate. The liquid compartments are filled with *N* acetic acid, and connected by two agar–*N* acetic acid bridges not shown in plan. (B) An expanded plan of the platinum wire electrode.

The sheets are hung in a suitable glass tank, placed in the dark and the amino acids in the various groups are separated by descending chromatography, employing as solvent a mixture of *n*-butanol–ethanol–ethyl acetate–water and glacial acetic acid (see Table I). This solvent gives good separations, with very compact spots, especially if the amino acids are loaded on stripwise as described. The small amount of acetic acid present prevents "bearding" of valine, leucine and glutamine. The *o*-coumaric acid contamination in the extract has an R_F value of 0.89, and therefore travels near the solvent front. Table I shows the overall separations achieved.

TABLE I

THE FREE AMINO ACIDS OF NORMAL HUMAN SERUM

Separations achieved by electrophoresis in *N* acetic acid followed by paper chromatography.

Electrophoresis	*Time of development h*	*Paper chromatography*				
		Solvent:	*butanol* –	*ethanol* –	*ethyl acetate* –	*water* – *glacial acetic acid*
		Bands 1–5	*60 ml*	*15 ml*	*10 ml*	*20 ml* *2 ml*
		Band 6	*40 ml*	*15 ml*	*10 ml*	*20 ml* *20 ml*
Band 1	40	Asp and Hypro				
Band 2	40	CySH + Cy_2S_2, Asp-NH_2, Glu-NH_2 + Cit, Glu, Thr, Pro, Tyr, Met, Phe				
Band 3	24	Ser, Val, Leu + Ileu				
Band 4	40	Ala				
Band 5	40	Gly				
Band 6	72–96	Orn, Lys + His, Arg				

Increasing R_F value →

At the appropriate times (Table I) the sheets are removed, dried in a stream of cold air, and then sprayed evenly with a solution consisting of 100 ml of 95 % ethanol plus 2 ml of lactic acid and 2 g of ninhydrin. The paper is air dried for 10 min at room temperature in a gentle air current, and then heated in an oven at 105° for 4 min. The coloured spots are cut out and dropped into test tubes containing 7.8 ml of 90 % ethanol and 0.2 ml of 1 % aqueous cadmium sulphate and allowed to stand at room temperature for 10–20 min. The pink solutions are then determined in the Spekker with the Ilford green filter (No. 604). Aliquots of a standard leucine solution (5 mg/ml), ranging from 2.5–50 μg are spotted on Whatman No. 1 filter paper, using an Agla micrometer syringe, and developed simultaneously with the paper chromatograms. The larger quantities of leucine are distributed in small spots, and under these conditions straight line graphs are obtained up to 50 μg of leucine. The individual amino acids can be calculated in terms of leucine, which is quite satisfactory for comparative work within the laboratory. The absolute values can be obtained by constructing standard curves of the various amino acids, or by establishing a table of factors required for converting leucine equivalents to the appropriate amino acid. The amino acids may also be estimated by extracting the blue spots with 70 % alcohol and rapidly reading against a yellow filter (Ilford No. 606) but the previously described technique is preferred as the colorimeter readings are increased by 60–70 %, and the pink colour is stable overnight.

This technique does not measure small quantities of hydroxyproline or proline, but it is possible to increase the sensitivity of the reaction with proline by a similar estimation in acid solution.

The yellow proline spot obtained by the routine technique of spraying and heating is excised and dropped into 8 ml of a solution of calcium chloride in acetic acid (2.5 ml of 20% $CaCl_2$ + 97.5 ml of glacial acetic acid), then heated in a boiling waterbath for 5 min. The bright pink solution is rapidly cooled, and read with the Ilford 604 filter. The colours are stable for 15 min in daylight and at least 1 h in the dark. Reproducible straight line standard curves are obtained with 0-45 μg of proline.

In the developmental stage of the method the separated amino acids were located by the fluorescence technique followed by excision and estimation by the excellent quantitative method of TROLL AND CANNAN[24]. The results, however, proved to be unreliable due to very high variable paper blanks. This background interference could be completely removed by treatment with $N/10$ NaOH[25] or by $N/20$ Na_2CO_3 but we have observed losses of amino acids by this system.

RESULTS

Early experiments with mixtures of relatively few amino acids suggested that the speed of movement during electrophoresis altered slightly with the complexity of the mixture being separated, and location of the individual compounds was eventually achieved by running duplicate aliquots of serum extract, to one of which had been added a known amino acid.

Electrophoresis of serum extracts under the conditions described, normally gives six bands, five of which can be detected by the fluorescence method. Table I is a summary of the separations achieved by electrophoresis followed by paper chromatography. The acids in band 1, *i.e.* hydroxyproline, and aspartic acid, are usually present in concentrations too low to be visualised by fluorescence in the thick 3 MM paper. It is our practice to excise band 2 together with a 2 cm wide strip above it, thus combining bands 1 and 2.

This mixture contains thirteen amino acids, occurring in nine distinct spots, *i.e.* a diffuse double spot of cystine and cysteine, aspartic acid and asparagine, a deeply staining mixture of glutamine and citrulline, followed by the single amino acids glutamic acid, threonine, proline and tyrosine. Aminobutyric acid and methionine occasionally separate but are normally estimated together. Phenylalanine is the fastest moving compound in this group.

Band 3 contains serine, valine, and a fast moving double spot of leucine and isoleucine, which is not sufficiently separated for the estimation of each component. Occasionally this band also contains traces of glutamine and threonine from band 2, but these occur as individual spots before and after serine respectively.

Bands 4 and 5 contain no compounds other than alanine and glycine, and may conveniently be combined for paper chromatography. The basic amino acids which occur in band 6 move very slowly and require at least 72 h development for separation

into three spots, *i.e.* ornithine, lysine and histidine as a poorly separated double spot, and arginine.

The colorimetric estimation of amino acids

Under the standard conditions described, it was found that the slope of the leucine standard curves did not vary more than ± 3 % over a period of several months. The slopes were invariably straight lines, as were the standard curves of twelve other amino acids studied. The absorption curves of the unstable blue colours produced

TABLE II

REPLICATION EXPERIMENTS

Estimations were carried out in quadruplicate on each of four separate specimens of pooled serum. Range of results, expressed as μg of leucine.

Amino acid	*Expt. 1*	*Expt. 2*	*Expt. 3*	*Expt. 4*
Asp-NH_2	—	—	0.8– 1.0	—
Glu-NH_2 + Cit	49.0–53.6	55.3–61.4	44.5–51.3	50.4–56.4
Glu	19.3–21.4	28.2–32.3	23.4–26.6	—
Thr	4.8– 5.8	5.8– 8.4	6.4– 7.6	5.4– 7.6
Pro	2.5– 2.8	0.7– 1.2	1.4– 1.9	0.3– 0.5
Tyr	2.9– 3.6	3.7– 4.0	2.6– 3.6	—
Met	—	—	—	1.9– 2.7
Phe	1.6– 1.9	0.5– 0.7	1.8– 1.9	0.3– 0.9
Ser	13.0–13.9	18.8–22.3	18.1–19.8	14.7–17.0
Val	22.2–27.2	34.8–38.4	31.2–32.0	30.4–35.0
Leu	14.5–16.6	18.5–21.7	16.9–17.9	17.6–19.0
Ala	46.0–48.2	37.7–40.6	—	41.2–46.0
Gly	15.9–18.8	14.9–16.1	—	17.0–17.6

TABLE III

RECOVERY OF AMINO ACIDS

The recovery of amino acids was tested by (a) estimating duplicate aliquots of serum to one of which had been added a known quantity of amino acid and (b) analysing "serum-like" synthetic mixtures of amino acids.

Amino acid	*Amino acid added μg*	*(a) Serum % recovery*	*(b) Synthetic mixtures % recovery*
Glu-NH_2	20	113	
Cit	25	102	
Thr	10	74	93
Pro	20	76	85
Met	5		41
Phe	20	84	107
Ser	7	91	110
Val	20	90	93
Leu	20		88
Ileu	25	79	
Ala	30	101	122
Gly	20		124
Orn	9		77
Arg	10	82	53
Mean		89	83

by alcohol extraction gave rather broad maxima between 570–580 mμ, but the pink colours with added cadmium sulphate possessed sharp absorption curves with maxima between 510–515 mμ except that for phenylalanine which was at 500 mμ. The increased stability and sensitivity of the latter colour reaction is of considerable advantage in estimating the trace quantities of some amino acids present in serum.

The estimation of amino acids in synthetic mixtures and in serum

The reproducibility of the method has been tested by running quadruplicate estimations on each of four separate pooled specimens of serum. The experimentally determined range of values for each amino acid, expressed as μg of leucine, are shown in Table II which clearly demonstrates that consistent replication may be obtained even below the 5 μg level.

Overall recovery experiments were then carried out in duplicate using either

TABLE IV

THE FREE AMINO ACIDS OF NORMAL FASTING HUMAN SERUM

The values given for each subject are the means for at least two specimens, drawn on different days.

Amino acid	*Amino acid in serum, mg/100 ml*							*Mean (range)*
	Subject C	*Subject R*	*Subject G*	*Subject Q*	*Subject P*	*Subject B*	*Subject M*	
CySH + Cy_2S_2	0.6	1.7	0.8	1.2	0.7	1.2	1.1	1.0 (0.6– 1.7)
Asp + Asp-NH_2	1.4	1.7	3.0	1.9	2.7	2.3	2.7	2.2 (1.4– 3.0)
Glu-NH_2 + Cit	8.7	11.5	10.6	8.9	8.9	7.6	7.8	9.1 (7.6–11.5)
Glu	0.6	0.8	0.7	1.0	1.1	1.1	1.4	1.0 (0.6– 1.4)
Threo	1.4	1.6	1.3	1.4	1.8	1.5	2.1	1.6 (1.3– 2.1)
Pro	3.2	4.1	2.3	3.4	2.7	3.0	3.7	3.2 (2.3– 4.1)
Tyr	1.3	2.3	1.3	2.0	1.6	1.4	1.4	1.6 (1.3– 2.3)
Met	0.6	1.1	1.0	1.1	1.4	0.8	0.9	1.0 (0.6– 1.4)
Phe	0.8	1.1	0.8	1.3	1.1	1.1	1.1	1.1 (0.8– 1.3)
Ser	1.0	1.3	1.0	1.1	1.4	1.0	1.7	1.2 (0.9– 1.7)
Val	2.5	3.1	2.5	3.2	2.8	2.7	2.6	2.6 (2.5– 3.2)
Leu + Ileu	2.6	3.7	2.3	3.5	3.1	2.8	3.0	3.0 (2.3– 3.7)
Gly	1.7	2.1	1.6	2.0	2.3	1.9	2.5	2.0 (1.6– 2.5)
Ala	3.2	3.8	3.4	3.6	4.3	4.1	4.2	3.8 (3.2– 4.3)
Orn	1.0	1.1	0.7	1.5	1.2	1.7	1.6	1.3 (0.7– 1.7)
Lys + His	3.9	3.0	3.6	5.0	4.9	4.6	4.9	4.3 (3.0– 5.0)
Arg	1.5	1.7	1.9	2.6	2.0	1.6	2.3	1.9 (1.5– 2.6)

pure amino acids added to serum, or a mixture of amino acids in proportions resembling those in serum. The recoveries obtained, Table III, were usually between 80 % to 110 %, mean 86 %, and a subsequent quantitative study of the three main steps in the technique suggested that the major losses occurred prior to electrophoresis.

Initially the colorimetric readings from an analysis were calculated as leucine equivalents, but in later estimations, the specimens of serum were worked up in parallel with a synthetic serum-like mixture of amino acids, and the concentration of amino acid in the unknown calculated by simple proportion from the colorimetric readings. This procedure was considered to be justified by reason of the close replication obtainable and the straight line standard curves given by the different amino acids, and also has the advantage of internally correcting for any systematic analytical errors.

The serum levels of a small number of normal males were then investigated. Two fasting specimens of blood were taken from each subject, generally with an interval of two or more days, and the serum stored at —10° until required for analysis. The results of analysis showed that the individual pattern is remarkably constant over a small number of days.

The average pattern of values obtained for each of the seven subjects has been plotted in Table IV, which clearly shows that for these normal males there is little variation from the mean by the various amino acids. A similar consistency in values for each amino acid between individual subjects was shown by MOORE AND STEIN[2].

TABLE V

THE FREE AMINO ACIDS OF NORMAL SERUM OR PLASMA

The values obtained by the method described are compared, where possible, with values quoted in the literature.

Amino acid	*Amino acid in plasma or serum, mg/100 ml*				
	Present work (a)	MOORE AND STEIN[2] (b)	McMENAMY *et al.*[26] (c)	BORDEN *et al.*[27] (d)	SALISBURY *et al.*[28] (e)
CySH + Cy_2S_2	1.0	1.2			
Asp + Asp-NH_2	2.2	0.6			
Glu-NH_2 + Cit	9.1	8.8	6.6		
Glu	1.0	0.7	0.3		1.2
Thr	1.6	1.4	1.6	1.3	1.9
Pro	3.2	2.4	1.6	2.9	0.7
Tyr	1.6	1.0	1.3	1.0	
Phe	1.1	0.8	0.9	1.1	1.1
Ser	1.2	1.1			1.3
Val	2.6	2.9	2.0		2.5
Leu + Ileu	3.0	2.6	1.8		2.3
Gly	2.0	1.5			2.2
Arg	1.9	1.5	1.2	1.5	0.5

(a) Electrophoresis followed by paper chromatography.
(b) Chromatography on ion exchange columns.
(c) Paper chromatography.
(d) Microbiological technique.
(e) Microbiological technique.

Table V compares the average values obtained in this study with those published by other authors. There is a reasonable agreement on the whole, but for the large discrepancy between our value for aspartic acid plus asparagine, and that of MOORE AND STEIN[2]; this is, however, due to calculating the mixed spot in equivalents of aspartic acid which gives considerably less colour per molecule than asparagine in the modified ninhydrin reaction.

SUMMARY

1. A method is decribed for the estimation of a number of free amino acids in serum.

2. The acids are extracted by Zeocarb 225H, and are then fractionated into six groups by electrophoresis at 1350 V in *N* acetic acid buffer. The various groups are located by a fluorescence technique employing *o*-coumaric acid in acetone, and are then separated into individual amino acids by descending paper chromatography. The solvent used is a mixture of butanol, ethanol, ethyl acetate, water and acetic acid. The final colorimetric estimation is made by spraying the paper with lactic acid and ninhydrin followed by elution with alcoholic cadmium sulphate. This technique produces a stable pink colour which is more sensitive than the normal ninhydrin blue.

3. Replication is close, with a mean recovery of about 86 %.

4. Fasting levels are given for seven males, and a comparison with other published values is made.

REFERENCES

1 J. I. ROUTH, *Rheumatism*, 12 (1956) 85.
2 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 915.
3 P. BOULANGER, G. BISERTE AND J. COURTOT, *Bull. soc. chim. biol.*, 34 (1952) 366.
4 T. ASTRUP, G. CARLSTROM AND A. STAGE, *Acta Physiol. Scand.*, 24 (1951) 202.
5 J. C. M. SHELDON-PETERS AND J. M. BARRY, *Biochem. J.*, 63 (1956) 676.
6 N. R. JONES, *Biochem. J.*, 60 (1955) 81.
7 G. HUNTER, *J. Clin. Pathol.*, 10 (1957) 161.
8 S. GORDON AND G. L. NARDI, *J. Lab. Clin. Med.*, 43 (1954) 827.
9 J. AWAPARA AND Y. SATO, *Clin. Chim. Acta*, 1 (1956) 75.
10 V. JIRGL, *Clin. Chem.*, 3 (1957) 154.
11 W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 192 (1951) 293.
12 E. V. FLOCK, F. C. MANN AND J. L. BOLLMAN, *J. Biol. Chem.*, 192 (1951) 293.
13 E. V. MCCOLLUM AND A. A. RIDER, *Arch. Biochem. Biophys.*, 40 (1952) 20.
14 R. R. REDFIELD, *Biochim. Biophys. Acta*, 10 (1953) 344.
15 M. E. CARSTEN, *J. Am. Chem. Soc.*, 74 (1952) 5954.
16 K. A. PIEZ, E. B. TOOPER AND L. S. FOSDICK, *J. Biol. Chem.*, 194 (1952) 669.
17 W. A. COCHRANE, W. W. PAYNE, M. J. SIMPKISS AND L. I. WOOLF, *J. Clin. Invest.*, 35 (1956) 411.
18 E. L. DURRUM, *J. Colloid Sci.*, 6 (1951) 274.
19 E. R. COOK AND M. LUSCOMBE, *Nature*, 180 (1957) 708.
20 E. KAWERAU AND T. WIELAND, *Nature*, 168 (1951) 77.
21 H. J. MCDONALD, *Ionography*, Year Book Publ. Inc., Chicago, 1954, p. 62.
22 T. WIELAND AND E. FISCHER, *Naturwiss.*, 35 (1948) 29.
23 K. H. SLOTTA AND J. PRIMOSIGH, *Mem. Inst. Butantan (São Paulo)*, 24 (1952) 85.
24 W. TROLL AND R. K. CANNAN, *J. Biol. Chem.*, 200 (1953) 803.
25 L. FOWDEN, *Biochem. J.*, 48 (1951) 327.
26 R. H. MCMENAMY, C. C. LUND AND J. L. ONCLEY, *J. Clin. Invest.*, 36 (1957) 1672.
27 A. L. BORDEN, E. C. BRODIE, E. B. WALLRAFF, W. P. HOLBROOK, D. F. HILL, C. A. L. STEPHENS, R. B. JOHNSON AND A. R. KEMMERER, *J. Clin. Invest.*, 31 (1952) 377.
28 P. F. SALISBURY, M. S. DUNN AND E. A. PERCY, *J. Clin. Invest.*, 36 (1957) 1227.