The *N*-Terminal Amino Acids of Human Plasma Proteins

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ABSTRACT A study has been made of the *N*-terminal amino acid pattern of human plasma proteins under normal and pathological conditions. The normal pattern shows the following *N*-terminal amino acids in order of diminishing quantities: aspartic acid, glutamic acid, valine, alanine, tyrosine, leucines, and glycine. In healthy individuals this pattern is qualitatively stable with moderate quantitative differences between individuals. On the other hand radical quantitative changes in the pattern have been observed under pathological conditions.

Present classification of plasma proteins rests almost entirely on physicochemical properties; *i.e.*, electrophoretic mobility and sedimentation rate. This is a natural consequence of the fact that until recently only the physicochemical methods were sufficiently perfected to offer a useful means of classification. However, the advent of powerful methods for the study of the primary protein structure has opened up new possibilities. Thus it is now entirely feasible to identify a protein through a certain structural feature, *e.g.* a terminal amino acid sequence, or less rigorously, a terminal amino acid(s). This approach is not restricted to individual proteins since it should also be possible to characterize a mixture of proteins by the quantitative composition of, for example, their *N*-terminal amino acids. The purpose of the present communication is to demonstrate the application of this principle to a natural mixture of proteins, namely the plasma proteins. For the *N*-terminal determinations we have used the phenyl*iso*thiocyanate method (1) since it is well suited for quantitative evaluation.

A preliminary account of this work has been published (2). Simultaneously another group of workers has produced a preliminary report on the same subject (3).

EXPERIMENTAL

Materials

Commercial grade solvents were rectified as follows: Pyridine was dried over KOH and distilled (b.p. 114–116°). Benzene (6 volumes) was shaken repeatedly with concentrated H_2SO_4 (1 volume), washed with distilled water, dried over KOH, and distilled (b.p. 79–80°). Methylethyl ketone was refluxed with solid $KMnO_4$, dried over anhydrous K_2CO_3, and distilled (b.p. 79–80°). Ethyl acetate (1 volume) was washed with 5 per cent (w/v) aqueous Na_2CO_3 (1 volume), then with saturated $CaCl_2$ solution, dried over anhydrous K_2CO_3, and distilled (b.p. 77°). Ethylene chloride (6 volumes) was shaken twice with concentrated H_2SO_4 (1 volume), washed consecutively with distilled water, 5 per cent (w/v) aqueous Na_2CO_3, distilled water, and finally dried over anhydrous K_2CO_3 and distilled (b.p. 83–84°). Ethanol, 96 per cent, was once distilled and free of ultraviolet absorption. Acetic acid was of the A.R. grade (The British Drug Houses Ltd.). Water was glass-distilled.

Phenyl*iso*thiocyanate was distilled once *in vacuo* (b.p. 95°/12 mm) and stored in the cold. After long storage it turned slightly yellowish, and was then redistilled.

N-Allylpiperidine was synthesized according to Menshutkin (5).

The syntheses of the PTH-amino acids[1] have been described earlier (4).

Methods

BLOOD SAMPLING Twenty-four ml samples were drawn from the cubital vein and mixed in the syringe with 6 ml of 3.8 per cent (w/v) sodium citrate. The cells were centrifuged off at 3,500 RPM in an angle centrifuge, and the plasma immediately frozen and stored at −30° until used. The cell volume was determined by the hematocrit.

PREPARATION OF THE PTC-PROTEINS The coupling of the plasma proteins with phenyl*iso*thiocyanate was carried out in aqueous pyridine at pH 9. A buffer with good capacity in this region was sought in order to obviate the need for pH adjustments during the reaction. It was found that the buffer system *N*-allylpiperidine–acetic acid fulfilled this requirement.

A stock solution of the buffer was made up in the following way. A 0.2 M solution of *N*-allylpiperidine in pyridine was prepared. An aliquot of this solution was mixed with an equal volume of plasma, and the amount of acetic acid required to bring the pH to 9.0 was determined. A corresponding amount of acetic acid was then added to the *N*-allylpiperidine solution.

Six ml of plasma was transferred to a 50 ml centrifuge tube (Pyrex glass), mixed with an equal volume of buffer solution, and 0.2 ml of phenyl*iso*thiocyanate added. The tube was covered with aluminium foil, and left in a water bath at 40° for 1 hour.

[1] The following abbreviations have been used; PTH for 3-phenyl-2-thiohydantoin, and PTC for phenylthiocarbamyl.

The phenyl*iso*thiocyanate was gradually brought into solution by occasional gentle rotations of the tube.

The reaction mixture was subsequently extracted three times with 12 ml of benzene and the extracts discarded. Troublesome emulsions were broken by centrifugation. The pH of the remaining aqueous phase was adjusted to 4.7 by the addition of *N*-acetic acid. Then followed three consecutive extractions with 30 ml of methylethyl ketone with centrifugation after each extraction and disposal of the extracts. The semidry protein precipitate was now stirred up in 5 ml of water, and this suspension again extracted with methylethyl ketone as described. This resuspension and extraction procedure was repeated once more. The precipitate was finally extracted with 12 ml of ethanol, and centrifuged and the extract discarded. The residue was dried, first in a gentle stream of air and finally for 0.5 hour *in vacuo.*

HYDROLYSIS OF THE PTC-PROTEINS AND EXTRACTION OF THE PTHS The preparation resulting from the preceding operation was ground into a fine powder with a glass rod. Five ml of N-HCl were added, and the tube left in an oil bath at 100° for 1 hour. A "cold finger" was used to minimize evaporation.

The hydrolysate was extracted three times with 6 ml of ethyl acetate. The extracts were combined in a widemouthed test tube, washed with an equal volume of distilled water, and evaporated with slight heating (not above 40°) in an air jet to 2 to 3 ml. The solution was transferred to a 4 ml tube with a tapered tip and evaporated to dryness without heat.

ESTIMATION OF THE PTHS BY PAPER CHROMATOGRAPHY This technique has been described earlier (6), and only its adaptation to the present purpose will be described.

Only the solvent systems D and F were used. Chromatography in solvent system F was performed in the following way. The sample to be analyzed was dissolved in 200 μl of acetic acid, and quantities of 5, 20, and 40 μl were applied to the paper. The reference solution contained: 0.9 mg PTH-aspartic acid, 1.1 mg PTH-glutamic acid, 0.4 mg PTH-tyrosine, 0.3 mg PTH-glycine, and 0.5 mg PTH-alanine per 1 ml of acetic acid; 20 μl of this solution was applied. One lane was left empty for the blank.

For chromatography in solvent system D the remainder of the sample solution was evaporated to dryness in an air jet, redissolved in 40 μl of ethylene chloride, and 15 μl of this solution applied to the paper. The reason for this change of solvent is that the acetic acid disturbs the chromatography by dissolving out the formamide impregnation at the point of application. The reference solution contained: 0.7 mg PTH-glycine, 0.8 mg PTH-alanine, 1.0 mg PTH-valine, and 0.5 mg PTH-leucine per 1 ml of ethylene chloride; 15 μl of this solution was applied. An empty lane served as a blank.

After completion of the chromatography the papers were dried at room temperature in a stream of air. Using the fluorescent screen (6) as a guide the spots were cut out together with areas of corresponding size and level from the blank lanes. The cut out pieces were eluted with 1.5 ml of 70 per cent aqueous ethanol overnight.

Spectrophotometric readings were made at the wave length 269 mμ. All extinctions were corrected for the background absorption of the paper. The unknowns were also corrected for chromatographic losses. The correction factor, C_1, was the quotient between the calculated and the observed extinction of the corresponding reference

spot. The expected extinction was calculated from the molar extinction of the appropriate PTH (7). In general these corrections were small in system F, somewhat larger in system D, and particularly for PTH-leucines, when chromatographic losses of 30 per cent and more were common.

PTH-alanine, and occasionally PTH-glycine, could be determined in both solvent systems. However, the PTH-glycine spot in system D was usually not sufficiently well separated from the ultraviolet-absorbing material at the origin to allow a reliable determination. As a rule the two independent determinations were in excellent agreement, and the results have been averaged.

The chosen set of unknown and reference samples allowed in most instances the evaluation of an "unknown" spot against a reference spot of approximately the same intensity and this served to improve accuracy. The accuracy is estimated to be within ± 10 per cent for quantities above 10 μg in a spot, but below that level it decreases because of the relative increase in importance of the blank correction. The figures for PTH-tyrosine, PTH-leucines and PTH-glycine in normal plasma are therefore probably not more accurate than ± 15 per cent.

A final correction, C_2, was applied for the dilution of the plasma by the citrate solution used as the anticoagulant, according to the formula:

$$C_2 = 1 + \frac{100}{100 - D} \cdot \frac{B}{A}$$

where A is the volume of blood sample, B, the volume of citrate solution, and D, the percentage cell volume.

SPECTROPHOTOMETRIC MEASUREMENTS These were made on a Beckman DK-2 quartz spectrophotometer provided with quartz absorption cells with a path length of 1 cm and a holding volume of 1.5 ml.

PH MEASUREMENTS These were made with a glass electrode.

RESULTS

The results of the *N*-terminal determination of pooled plasma are presented in Table I. These figures can be taken to represent the average *N*-terminal amino acid pattern in a healthy population. Determinations have also been made on plasma from healthy individuals in order to obtain a measure of the normal variation. These individuals were chosen from both sexes, and varied in age from 20 to 59 years (Table I).

A potential source of error in the determination of *N*-terminal amino acids in plasma is the occurrence of free amino acids in plasma, and much attention has been given to the problem of excluding interference from this source. The procedure finally adopted was an exhaustive extraction of the PTC-proteins with methylethyl ketone. Loading experiments, in which free amino acids were added to blood plasma, showed that a sixfold increase of the normal plasma level of free amino acids did not affect the results (Table II). The

TABLE I

N-TERMINAL AMINO ACID PATTERN IN PLASMA PROTEINS OF HEALTHY INDIVIDUALS

Age and sex	Concentration of plasma proteins	Concentration of *N*-terminal amino acid (μg PTH per ml of plasma, per cent of mean within parentheses)						
		PTH-aspartic acid	PTH-glutamic acid	PTH-valine	PTH-alanine	PTH-tyrosine	PTH-leucines	PTH-glycine
	gm per 100 ml.							
57 yrs. ♀	7.8	122 (92)	53 (90)	9.8 (99)	10.0 (103)	5.6 (83)	6.7 (111)	4.2 (94)
59 yrs. ♀	8.5	134 (101)	48 (82)	9.6 (97)	10.6 (109)	7.7 (114)	4.9 (81)	4.7 (105)
25 yrs. ♂	8.8	146 (110)	72 (122)	10.8 (109)	10.4 (108)	9.7 (144)	5.6 (94)	5.5 (122)
25 yrs. ♂	7.8	118 (89)	54 (92)	12.1 (122)	7.3 (76)	5.4 (79)	9.3 (155)	4.3 (95)
21 yrs. ♀	8.7	142 (107)	53 (90)	8.7 (88)	9.2 (95)	6.9 (102)	5.8 (97)	5.1 (113)
21 yrs. ♀	7.6	122 (92)	49 (83)	9.2 (93)	8.8 (91)	5.4 (79)	6.3 (105)	3.7 (82)
20 yrs. ♀	8.0	162 (122)	82 (139)	13.6 (138)	12.4 (128)	8.5 (126)	7.6 (126)	5.2 (115)
25 yrs. ♂	7.2	128 (96)	59 (100)	6.0 (61)	9.4 (97)	4.6 (68)	1.8 (30)	3.7 (82)
59 yrs. ♂	7.7	122 (92)	59 (100)	9.2 (93)	9.1 (94)	7.2 (106)	5.8 (96)	4.2 (93)
Mean	8.0	133	59	9.9	9.7	6.8	6.0	4.5
Pooled plasma (33 individuals)		143	59	14.5	12.8	7.9	7.8	4.4

figures for the normal plasma level are those of Stein and Moore (8). This level is believed to vary little under physiological and pathological conditions, and our procedure should therefore offer a reasonable safety margin against contamination from free amino acids.

The simplified version of the phenyl*iso*thiocyanate method employed in this investigation did not allow the determination of certain *N*-terminal amino acids. Thus, in the acid hydrolysis of the PTC-proteins the PTH derivatives of serine, threonine, cysteine, and tryptophan are destroyed, and the derivatives of asparagine and glutamine are converted to PTH-aspartic

TABLE II

THE EFFECT OF ADDING FREE AMINO ACIDS TO PLASMA ON *N*-TERMINAL DETERMINATIONS

PTH-amino acid	Concentration of *N*-terminal amino acid	
	Unloaded	Loaded*
	μg PTH/ml	*μg PTH/ml*
Aspartic acid	110	106
Glutamic acid	49	50
Valine	10.1	12.5
Alanine	10.4	10.5
Tyrosine	5.4	6.7
Leucines	11.2	10.1
Glycine	4.6	5.7

* The load of free amino acids per milliliter of plasma was 26 μg asparagine; 490 μg glutamine; 149 μg valine; 42 μg phenylalanine; 177 μg alanine; 56 μg tyrosine; 128 μg leucine; 75 μg glycine.

acid and PTH-glutamic acid. Further, no attempt was made to determine arginine and histidine, although this might have been possible. Uncertainties also arise from the fact that certain pairs of PTH-amino acids partly overlap in the paper chromatograms and small amounts of one amino acid may therefore have escaped notice in the presence of a large amount of the other. This applies to the pairs valine-phenylalanine and leucine-isoleucine.

Our figures are probably somewhat too low, as no correction for losses during hydrolysis has been made. Such correction is difficult to make, but since PTHs are quite stable to dilute aqueous acids, with the exceptions mentioned above, it is certain to be small.

DISCUSSION

The *N*-terminal amino acids already reported for various plasma protein fractions (Table III) are all represented in the over-all pattern with the exception of serine and threonine. The latter amino acids are not included in the present determination for reasons already discussed.

Our results and those of Sjöquist *et al.* (3) are generally found to be in good agreement when recalculated to a common basis. A notable exception however, is *N*-terminal glutamic acid, where our figure is two to three times higher. No explanation for this discrepancy can be offered at present. On the other hand, the absence of *N*-terminal tyrosine in the analysis of Sjöquist and coworkers is probably explained by the fact that they used blood serum, since it is known that tyrosine is *N*-terminal in human fibrinogen (20, 21).

The pathological *N*-terminal amino acid patterns (Fig. 1) do not allow much comment at present. However, it should be obvious that the *N*-terminal pattern is a very sensitive index of changes in the plasma protein composition

TABLE III

N-TERMINAL AMINO ACIDS IN HUMAN PLASMA PROTEIN FRACTIONS

Plasma protein fraction	*N*-terminal amino acid	Reference
Albumin	Aspartic acid	9–11
Lipoprotein		12–14
D = 1.149	Aspartic acid	
D = 1.093	Aspartic acid	
D = 1.029	Glutamic acid	
D = 1.002–1.006	Serine and threonine	
Transferrin	Valine	15
Ba-α_2-glycoprotein	Alanine and threonine	16
γ-Globulin	Aspartic acid, glutamic acid, and serine	17–19
Fibrinogen	Tyrosine and alanine	20, 21
Haptoglobin	Valine and isoleucine	22

in disease. Diagnostic and other implications of these observations remain to be investigated.

Finally a comment should be made on the principle of characterizing plasma proteins through details of their primary structures. Its main merit lies in the fact that it utilizes an invariant property of the protein molecule. This is in contrast to the physicochemical characterization where the magnitude of the parameters measured is dependent on experimental variables; *i.e.*, hydrogen ion concentration, ionic strength, temperature, and so on. Further, secondary modifications of the protein molecule, *e.g.* through polymerization or adsorption of non-protein material, are less likely to involve the structural detail used for the characterization. Generic relationships between plasma proteins should therefore be more easily recognizable.

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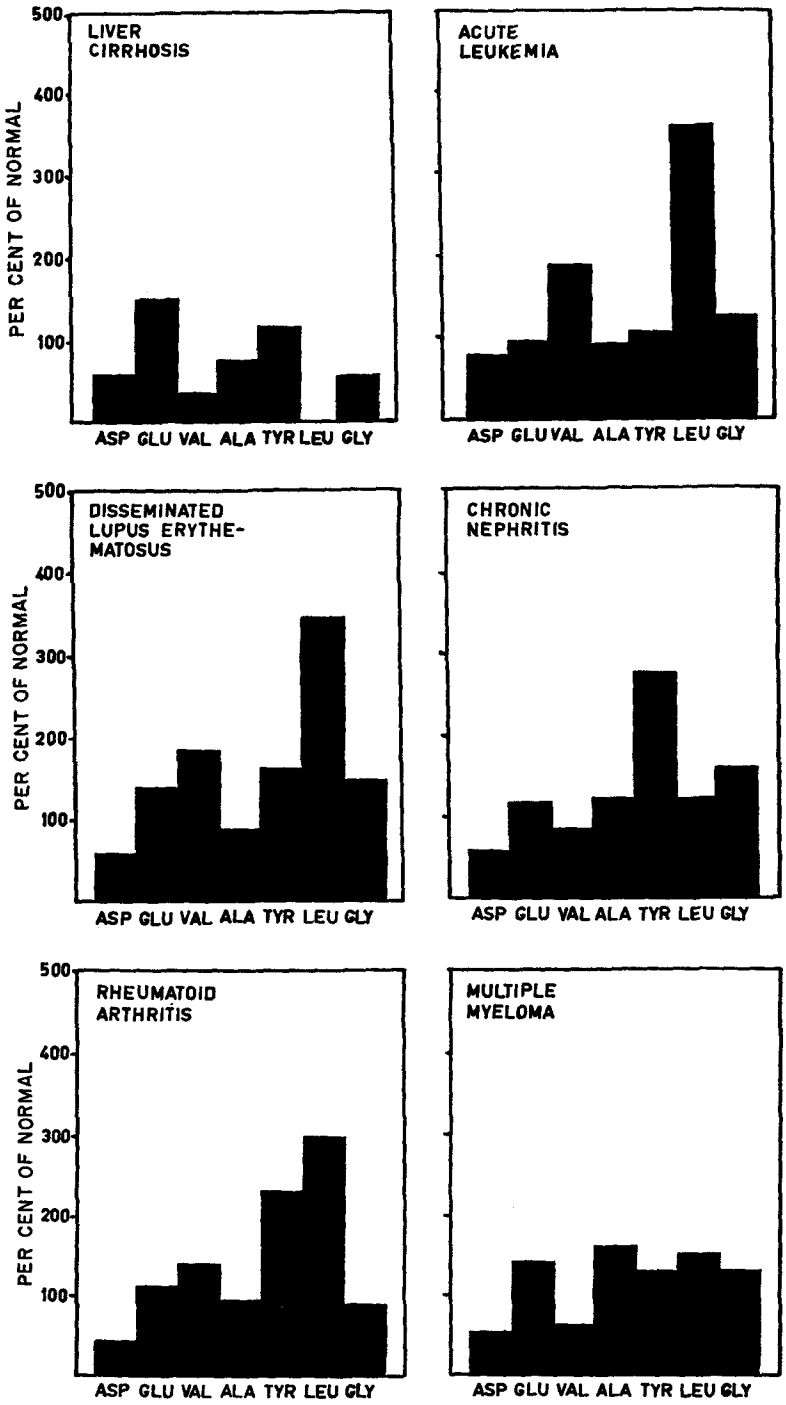

FIGURE 1. *N*-terminal amino acid pattern of plasma proteins in individuals in various pathological conditions.

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