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APPLICATION OF RADIOISOTOPIC YIELD TO THE QUANTITATION OF THE N-TERMINAL AMINO ACIDS OF FIBRINOGEN

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SUMMARY

The N-terminal amino acids of bovine and human fibrinogen have been quantitated with an isotopic-yield procedure using the phenylthiohydantoins of [^{14}C]amino acids as internal standards. Losses of N-terminal amino acids in the EDMAN–SJÖQUIST procedures subsequent to the isolation of the phenylthiocarbamyl protein were evaluated for each determination. The average N-terminal amino acid content found for a series of three bovine fibrinogen samples was 1.6 moles glutamic acid and 1.9 moles tyrosine per mole of fibrinogen while the average content found for a series of six human fibrinogen samples was: alanine, 1.6; tyrosine; 1.9 and aspartic acid, 0.9 mole per mole fibrinogen. Further work is necessary to establish that aspartic acid is an N-terminal residue of native human fibrinogen. The results of this study are compared with the results reported by other investigators and the values commonly accepted for the N-terminal amino acids of bovine and human fibrinogen.

INTRODUCTION

Accurate quantitation of the N-terminal amino acids of complex proteins is a difficult procedure. Two general methods are used predominantly. The older method, based upon SANGER's original technique[1,2], involves the reaction of the protein with FDNB followed by acid hydrolysis, identification and measurement of the DNP-amino acids isolated by chromatography. The second method, first described by EDMAN[3] and further improved by SJÖQUIST[4,5] and by ERIKSSON AND SJÖQUIST[6], involves the reaction of the protein with PITC forming the PTC-protein which when treated with acid yields phenylthiohydantoin derivatives of the N-terminal amino acids.

The major obstacle in quantitating N-terminal amino acids by these techniques is the estimation of the losses occurring during hydrolysis of the DNP-proteins or cyclization of the PTC-proteins, and during the extraction, chromatography, and elution steps. The usual technique for the determination of correction factors involves

Abbreviations: PITC, phenylisothiocyanate; PTC-, phenylthiocarbamyl-; PTH, phenylthiohydantoin.

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addition of known amounts of amino acids to a measured quantity of protein (or addition of appropriate amino acid derivative to the DNP- or PTC-protein) and calculation of the amounts recovered after subtracting the amount found in a second determination on the protein alone[7-10]. Since a difference between two determinations is involved, errors will result if an absolute loss is inherent in the procedure. The necessity of using an average recovery factor is an additional shortcoming. Differences in the procedures used for samples of protein with and without added amino acid may give rise to additional errors. BLOMBÄCK AND YAMASHINA[8] apparently omitted an acetone washing of the PTC-fibrinogen when amino acids had been added prior to reaction of the fibrinogen with PITC.

LAVER[11] has described a very sensitive microprocedure employing [^{35}S]PITC for use with small samples. The method, however, does not permit an evaluation of the recovery factors.

The use of DNP derivatives of [^{14}C]amino acids as internal tracers in the SANGER procedure had been investigated previously[12]. Overall recoveries were erratic and frequently very low. In addition, photolytic decomposition of the compounds liberated CO_2 and unidentified volatile acids[13]. A similar procedure was then developed for use with the PTH method.

This communication describes the application of the isotopic yield procedure to the determination of the N-terminal amino acids of bovine and human fibrinogen.

MATERIALS AND METHODS

Radioactive amino acids were purchased from Nuclear-Chicago Corp., the New England Nuclear Corp., and the Isotope Specialties Co. The specific activities of these compounds varied from 1 to 12 μC/μmole. The compounds were labeled in the positions indicated in data for individual experiments. PITC (Distillation Products Corp.) was redistilled *in vacuo*. Triethylamine and pyridine were refluxed with phthalic anhydride and distilled as recommended by SJÖQUIST[4].

Reference PTH compounds were synthesized using the micro-synthetic method of SJÖQUIST[4]. Solutions of these compounds were prepared in 90% acetic acid as recommended by ERIKSSON AND SJÖQUIST[6]. The PTH's, particularly of aspartic and glutamic acids, are more stable in the acetic acid solvent than in ethanol. All phenylthiohydantoins were checked frequently for radiopurity by paper chromatography and scanning on a recording scanner (Actigraph II, Nuclear-Chicago Corp.). Impurities were quantitated by weighing the paper areas under the peaks.

The chromatographic systems used were those described by SJÖQUIST[5] and an additional solvent (*n*-heptane–1,2-dichloroethane–75% formic acid (1:1:2) described by WALLEN AND SJÖHOLM[14] for the separation of the PTH derivatives of methionine, lysine, and alanine.

Quenching of the chromatograms was visualized by preparation of a photograph using ultraviolet light (Mineral Light, Model SL 2537, Ultraviolet Products, South Pasadena, Calif. (U.S.A.)) and commercial photostat papers (Gevaert).

The procedure of BLOMBÄCK AND YAMASHINA[8] was used for the preparation of PTC-proteins and cyclization of the N-terminal PTC-residues. The acetone used for washing the PTC-protein was distilled from $KMnO_4$ as needed.

Bovine and human fibrinogen were purified from Fraction I using LAKI's method[15], or, on occasion, the method of BLOMBÄCK AND BLOMBÄCK[16].

Determination of recovery factors

The radioactivity of PTH derivatives of the [^{14}C]amino acids was determined by plating 1–5 μl of a solution onto a stainless steel planchet. The samples were counted either with a Geiger–Müller thin-window detector, or with a gas-flow detector (Model D-47, Nuclear Chicago Corp.).

It should be emphasized that careful control of the drying of planchets is necessary to avoid some loss of activity. Temperatures only slightly above room temperature should be used, and the planchets should be counted within a few hours after drying. An appreciable loss of activity, particularly from the phenylthiohydantoins of [1-^{14}C]alanine, [1-^{14}C]valine, and [1-^{14}C]glycine, is noted if the planchets are overheated or allowed to stand for several days. Loss of activity from PTH-[$^{14}C_9$]tyrosine appears to be negligible.

The nature of the radioactivity loss appears to be complex and is probably due to factors in addition to volatility of the parent compounds. It has been observed that loss of activity is greater initially in air than is the case when planchets are kept under nitrogen. Rechromatography of the phenylthiohydantoin of alanine from planchets heated to 100° shows an almost complete loss of the original peak and the appearance of several large impurity peaks.

It is recommended that windows cut from Mylar sheets (obtained from Nuclear-Chicago Corp.) be used for gas-flow counting. Such windows may be washed with solvents or replaced when desirable at very low cost.

All radioactivity values were corrected to a standard count for a ^{14}C reference planchet. Known radioactive tracer-amounts of not more than three PTH derivatives of [^{14}C]amino acids were added from micropipettes just prior to cyclization of the PTC derivatives with 1 N HCl. Such a procedure evaluates losses subsequent to the isolation of the PTC-protein derivatives.

The extracted PTH-amino acids were taken to dryness and transferred to 0.5 ml glass-stoppered tubes using carefully measured volumes of 90% acetic acid. Solutions were generally prepared so as to represent 15 mg of the original protein/30 μl of solution. When not in use, solutions were kept at —20° and while pipetting were kept in ice.

Radioactivity scans were made to observe correspondence of quenching with radioactive areas. Areas corresponding to the quenching and radioactive bands were eluted with 2 ml of 95 % ethanol. Aliquots were counted for calculation of ^{14}C recovery and for measurement of the absorbancy at 269 mμ. An average molar extinction value of 16000 based on individual values given by SJÖQUIST[4] was used for all PTH's except the ε-PTC-derivative of lysine-PTH for which a value of 29000 (*loco citato*) was employed.

When low specific activity PTH compounds are used, it is necessary to apply small corrections for the contribution of the recovered added compounds to the absorbance of the isolated phenylthiohydantoins.

The recovery factor is calculated as follows:

$$\text{Recovery factor} = \frac{\text{Counts recovered in eluate from peak corresponding to pure compound}}{\text{Counts added to amount of sample represented by aliquot counted and corrected for any radioactive impurities in the original PTH standard}}$$

Calculation of N-terminal residues per mole of protein

The calculation is as follows:

$$\text{Moles N-terminal residue per mole of protein} = \frac{A_{269\,m\mu}}{\alpha_M} \times \frac{1}{\text{Fraction of total sample applied to chromatogram}} \times \frac{\text{Milliliters of solvent used to elute PTH from chromatogram}}{1000 \times \text{recovery factor}} \times \frac{\text{Molecular weight of protein}}{\text{Gram protein labeled with PITC}},$$

where: $A_{269}\,m\mu$, observed absorbance at 269 mμ in a 1-cm cell; α_M, molar extinction coefficient of the PTH added.

An error in the aliquot applied to the chromatogram will yield a corresponding error in the determined recovery factor but will not affect the result for the number of moles of N-terminal acid per mole of protein. This situation results from the fact that an error in aliquot size will yield compensating errors in the recovery factor and in the measured absorbance. Since the former appears in the denominator and the latter in the numerator, a cancellation of the errors results.

Any errors in measurement of the original amount of protein to be labeled or losses in the preparation of the PTC-protein are not evaluated by the procedure in its present form. If the recovery factor is of no interest, the fractional amount of sample applied is of no consequence and the calculation may be reduced to the following form:

$$\text{Moles N-terminal acid per mole of protein} = \frac{A_{269\,m\mu}}{\alpha_M} \times \frac{\text{Milliliters of solvent used to elute PTH from chromatogram}}{1000} \times \frac{\text{Total counts added prior to cyclization}}{\text{Counts recovered in aliquot chromatographed}} \times \frac{\text{Molecular weight of protein}}{\text{Gram protein labeled with PITC}}$$

RESULTS

The necessity for quantitating the radiopurity of the phenylthiohydantoins used as internal standards is illustrated by the data of Fig. 1. Small amounts of radioactive contaminants invariably are found. The original amino acids were shown to be free of contaminants by chromatography in a *n*-butanol–acetic acid–water (4:1:5) system followed by scanning with the Actigraph II.

The maximum impurity in the series of PTH standards shown in Fig. 1 is found in chromatogram D (PTH of [1-^{14}C]alanine) and represents 10.8% of the total radioactivity. Quantitation of small amounts of impurities in nonradioactive phenylthiohydantoins by absorbance measurements generally is unreliable.

Quantitation of the N-terminal residues of fibrinogen

Application of the tracer procedure to the determination of the N-terminal acids of bovine fibrinogen is illustrated by the data of Fig. 2 and Table I. In the experiment of Fig. 2 the phenylthiohydantoins of [1-^{14}C]glutamic acid, [$^{14}C_9$]tyrosine, and [2-^{14}C]-glycine were added as internal standards. The average values for the data of Table I are: glutamic acid, 1.6 and tyrosine, 1.9 moles/mole of fibrinogen. Traces of glycine and aspartic acid are occasionally noted.

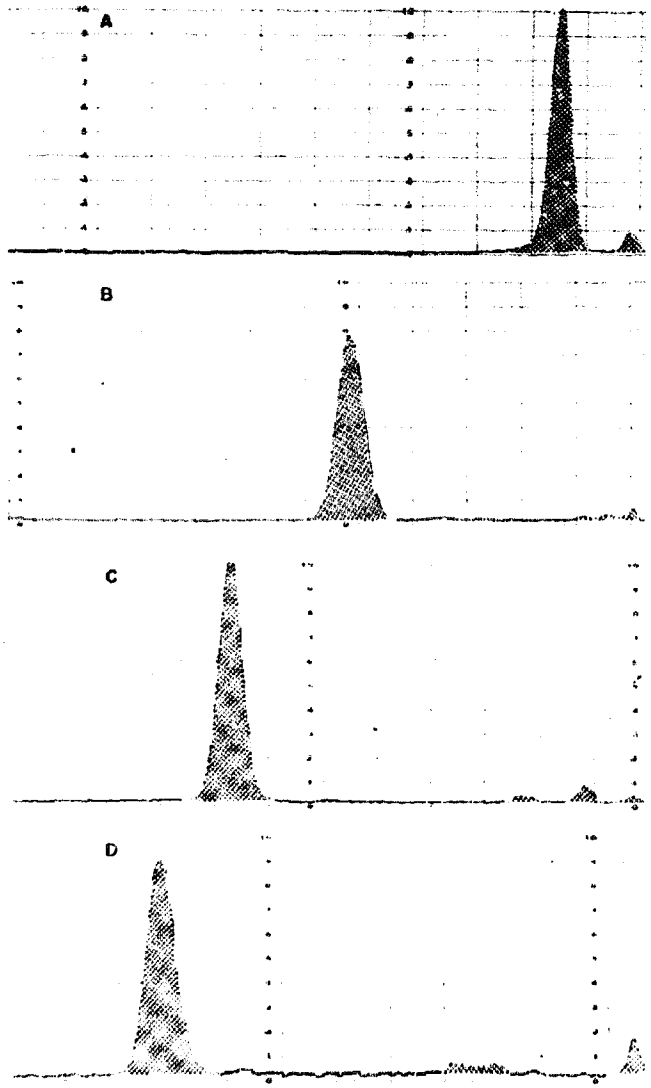

Fig. 1. Typical chromatograms of the phenylthiohydantoins synthesized from [^{14}C]amino acids by the procedure of SJÖQUIST[4]. Developed with solvent System III of SJÖQUIST[5]. A, PTH of [$^{14}C_4$]aspartic acid (impurities = 5.2%); B, PTH of [$^{14}C_9$]tyrosine (impurities = 3.4%); C, PTH of [2-^{14}C]glycine (impurities = 0.6%); D, PTH of [1-^{14}C]alanine (impurities = 10.8%).

Application of the procedure to human fibrinogen is illustrated by the data of Fig. 3 and Table II. The quenching area marked (?) shown in Fig. 3 does not appear to be an amino acid derivative since the absorbance ratio at 245 to 269 mμ is greater than 2 whereas the phenylthiohydantoins of the amino acids have ratios of about 0.4 (see ref. 4). This spot appears to be the result of inadequate washing with acetone at the PTC stage. Table II lists a series of experiments with human fibrinogen. The average values for this series are: tyrosine, 1.9; alanine, 1.6; and aspartic acid, 0.9 moles per 340000 g fibrinogen.

Amino acids yielding anomalous results

As applied to fibrinogen or enzymic degradation products of fibrinogen, anomalous results have been obtained with N-terminal lysine, serine, and threonine.

Addition of ε-PTC–PTH-[$^{14}C_6$]lysine to human PTC-fibrinogen followed by cycli-

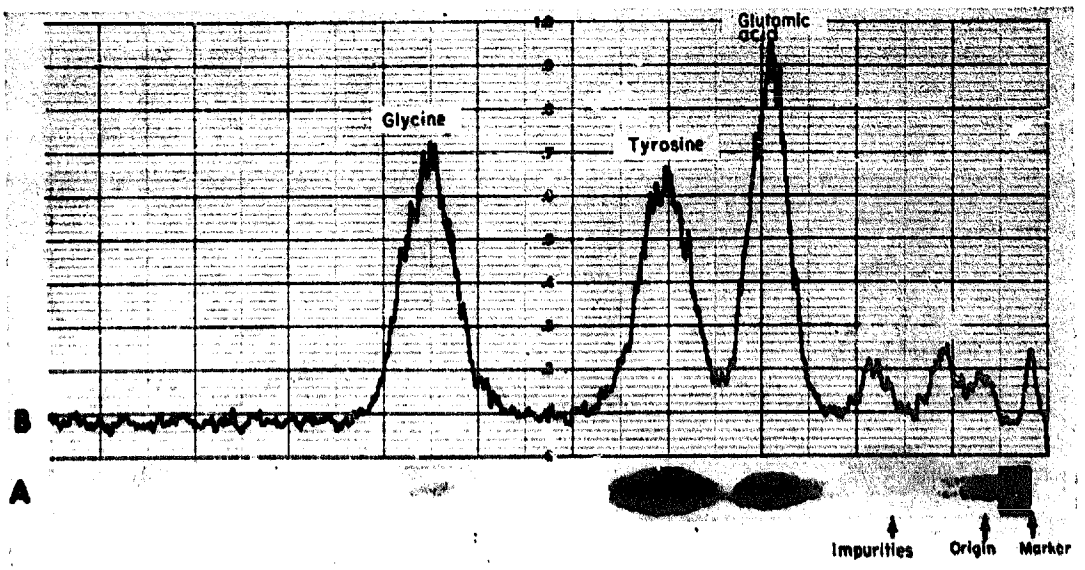

Fig. 2. Chromatogram of the N-terminal amino acids of bovine fibrinogen with radioactive phenylthiohydantoins added as internal standards. Fibrinogen, 94 % clottable, purified by the method of LAKI[18]. Standards added: PTH of [1-^{14}C]glutamic acid, 11.5 μC/μmole; PTH of [$^{14}C_9$]tyrosine, 11.5 μC/μmole; PTH of [2-^{14}C]glycine, 4.0 μC/μmole. A, Ultraviolet light photograph of chromatogram; B, Actigraph scan of chromatogram, full scale deflection = 500 counts/min. Slit width, 1/8 in; rate, 6 in/h; time constant, 40 sec.

TABLE I

QUANTITATION OF THE N-TERMINAL AMINO ACIDS ON A SERIES OF BOVINE FIBRINOGENS

Sample	*Clottability (%)*	*N-terminal amino acid (moles/340 000 g fibrinogen)*			
		Glutamic	*Tyrosine*	*Glycine*	*Aspartic*
1*	94	1.6 (61)**	1.5 (71)	0.2 (54)	0
		1.6 (70)	1.5 (85)	0.2 (54)	0
2	97.5	1.8 (65)	2.0 (65)	—	0
		1.8 (70)	2.4 (62)	—	0
3	95.5	1.4 (60)	2.0 (56)	—	0.4 (50)***

* This is the same sample shown in Fig. 2.
** Figures in parentheses are recovery values.
*** Assumed recovery (based on results from other samples).

zation led to almost complete loss of radioactivity in the lysine region. If, in addition, cold carrier ε-PTC–PTH-lysine was added, the bulk of isotope coincided with the quenching area in the lysine region and only a small radioactive peak with greatly increased R_F was noted. Fig. 4 illustrates the result obtained on adding ε-PTC–PTH-[$^{14}C_6$]lysine to a sample of human PTC-fibrinogen. Before chromatography of the sample of Fig. 4C, a trace of ε-PTC–PTH-[$^{14}C_6$]lysine was spotted at the origin. The larger peak of lower R_F indicates the position of ε-PTC–PTH-lysine while the smaller peak of greater R_F is due to a decomposition product formed from ε-PTC–PTH-lysine

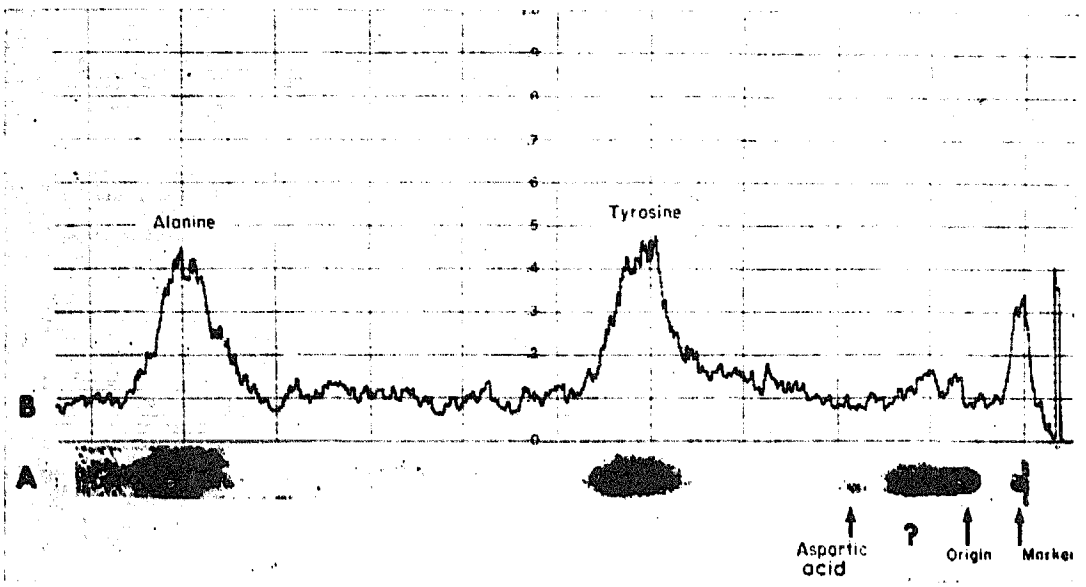

Fig. 3. Chromatogram of the N-terminal amino acids of human fibrinogen with radioactive phenylthiohydantoins added as internal standards. Fibrinogen, 92% clottable, purified by the method of LAKI[15] from plasma Fraction I (American Red Cross and E. R. Squibb and Sons). Standards added: PTH of [$^{14}C_9$]tyrosine, 11.5 μC/μmole; PTH of [1-^{14}C]alanine, 1.16 μC/μmole. A, Ultraviolet light photograph of chromatogram; B, actigraph scan of chromatogram. Full scale deflection, 500 counts/min; slit width, 1/8 in; rate, 6 in/h; time constant, 40 sec.

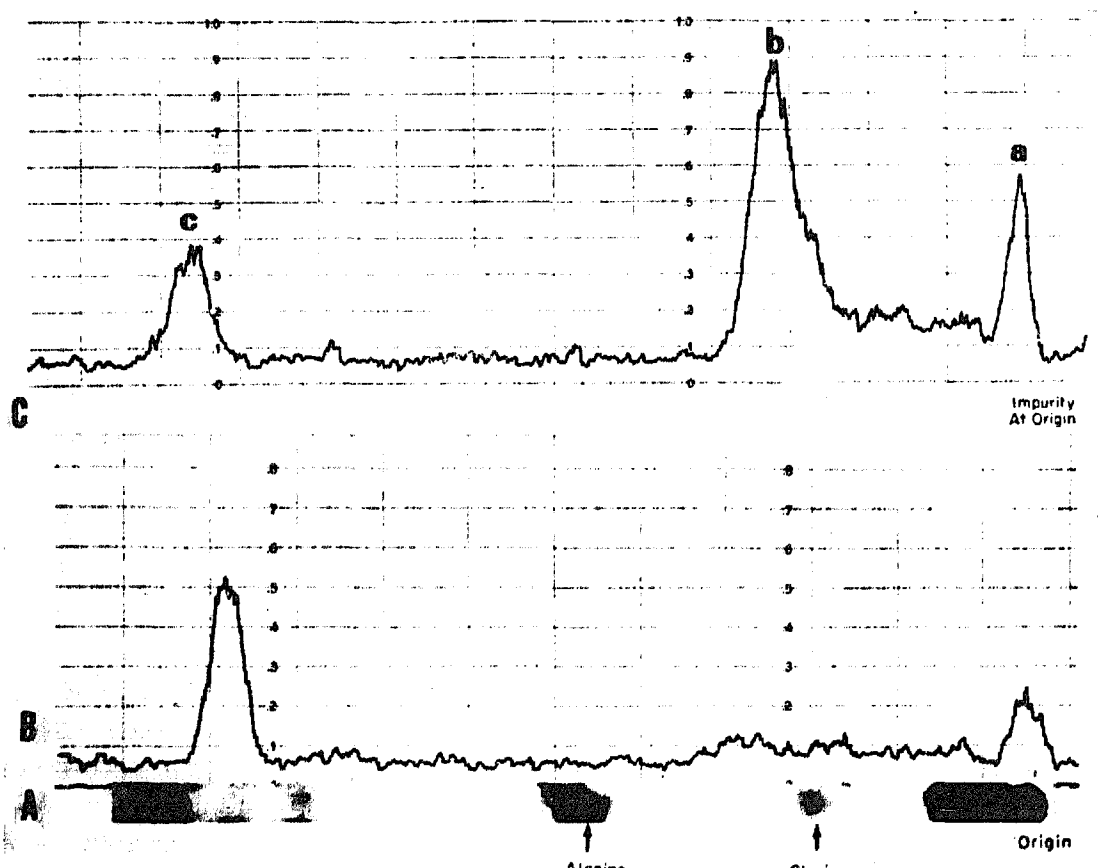

Fig. 4. (A) Ultraviolet light photograph of a chromatogram of the phenylthiohydantoins from human PTC-fibrinogen to which ε-PTC–PTH-[$^{14}C_6$]lysine was added prior to cyclization. (B) Actigraph scan of A. The almost complete decomposition of ε-PTC–PTH-[$^{14}C_6$]lysine added prior to cyclization is evident by comparison with scan C which represents a parallel chromatogram of the sample to which ε-PTC–PTH-[$^{14}C_6$]lysine was also added at the origin prior to chromatography. Solvent system of WALLEN AND SJÖHOLM was used[14].

TABLE II

QUANTITATION OF THE N-TERMINAL AMINO ACIDS ON A SERIES OF HUMAN FIBRINOGEN SAMPLES

Sample	Clottability (%)	N-terminal amino acid (moles end group/mole protein)			
		Tyrosine	Alanine	Glycine	Aspartic
1*	92	1.6 (82)**	1.3 (55)		0.8 (50)***
2	93	2.0 (64)	1.7 (69)		—§§
3	96	1.9 (87)	1.8 (58)		0.7 (51)
4	95	2.1 (57)	1.4 (76)		1.2 (37)
5	96	2.4 (81)	2.2 (62)	0.3 (66) §	—§§
6	98	1.6 (65)	1.6 (55)		0.9 (50)***

* Each result for this sample is the average of triplicates.
** Figures in parentheses are recovery values.
*** Assumed recovery (based on results from other samples).
§ Recovery figure taken from a chromatogram run in parallel.
§§ Quenching area visible but not quantitated.

added prior to cyclization. Fig. 4B illustrates a chromatogram of the same sample without addition of authentic tracer just prior to chromatography.

Variable amounts of the PTH of threonine are converted to dehydrothreonine after cyclization in 1 N HCl (BLOMBÄCK AND YAMASHINA[8]). These authors estimate

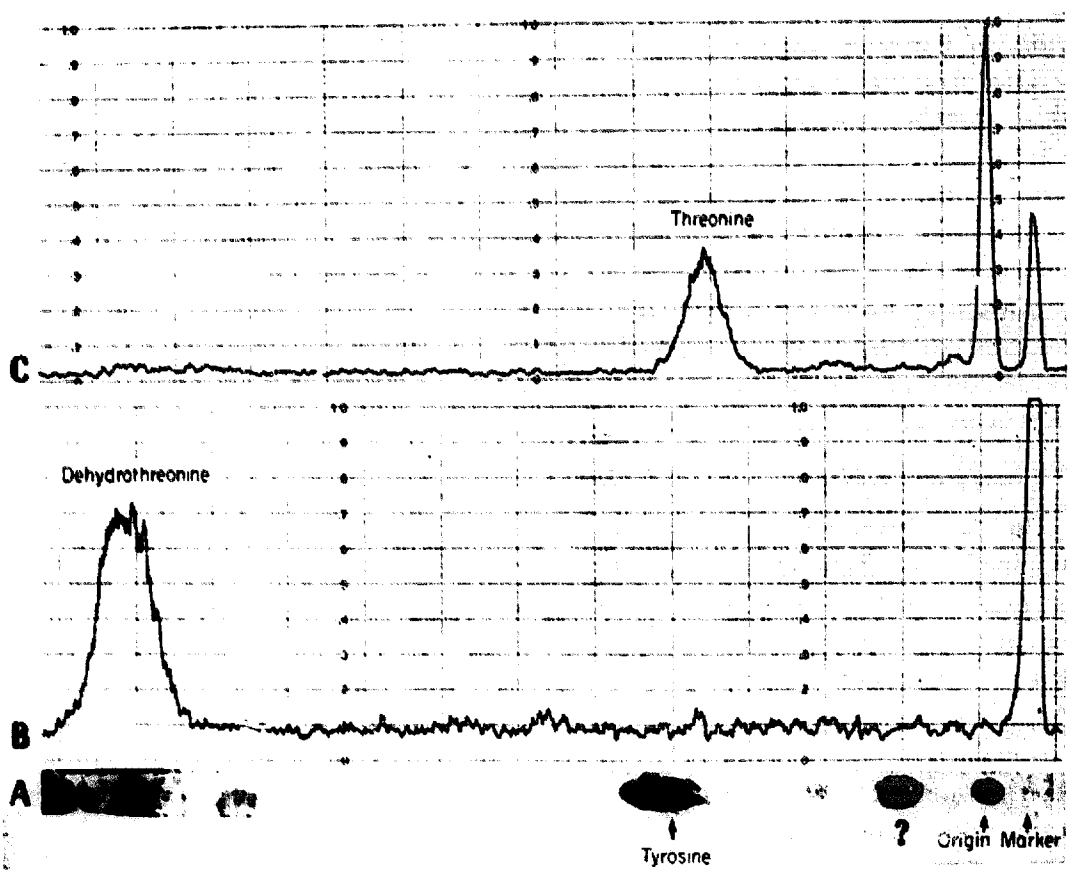

Fig. 5. (A) Ultraviolet light photograph of the N-terminal residues of equine fibrinogen (purified from a Fraction I obtained from Pentex), 75% clottable. Solvent system III (see ref. 5) was used. (B) Actigraph scan of A. PTH of [$^{14}C_4$]threonine was added prior to cyclization of the PTC-fibrinogen. (C) Actigraph scan of a chromatogram of authentic PTH of [$^{14}C_4$]threonine.

threonine as the sum of the phenylthiohydantoins of threonine and dehydrothreonine. Fig. 5 demonstrates the dehydration of the radioactive PTH derivative of threonine added prior to cyclization of equine PTC-fibrinogen. Except for the activity at the origin (which occurs in all the chromatograms irrespective of which radioactive amino acid derivative is used*) all of the radioactivity corresponds to that of the dehydro-compound. The PTH of dehydrothreonine may be confused with the PTH of methionine in SJÖQUIST's solvent System III, but not in System II.

DISCUSSION

Methodology

Quantitation of the N-terminal residues of a complex protein such as fibrinogen is a tedious and difficult task. The road must be traveled to appreciate its roughness. The difficulties in accurate quantitation of N-terminal residues seldom are emphasized adequately in the literature. BISERTE *et al.*[9] have discussed the variabilities and problems which are more generally the rule rather than the exception.

Application of the isotopic yield procedure to N-terminal residue determinations is believed to be theoretically sound and to yield the most reliable data obtainable to date. The method provides an opportunity to isolate errors arising from several sources.

Several possibilities for improvement remain. As presently used, the procedure does not evaluate losses occurring during the coupling of the protein with PITC and during the washing of the PTC-protein.

The principal remaining source of error is the measurement of the corrected optical absorbance. Failure to obtain a valid correction for the "blank" (taken from an empty lane adjacent to the sample) may be serious when the absorbance of the sample is only 2–3-fold greater than the absorbance of the blank. Areas eluted from regions of R_F lower than a given phenylthiohydantoin frequently have a higher absorbance than the corresponding area from the control strip.

If careful control of the plating, drying, and counting of the planchets is maintained, the small but definite loss of activity does not significantly affect the results. It is troublesome to the extent that the slight contamination of the detector must be removed occasionally.

The N-terminal acids of bovine and human fibrinogen

Bovine fibrinogen has N-terminal tyrosine, glutamic acid[8,17,18] and acetylthreonine[19]. The data from the first three laboratories have been calculated for a molecular weight of 340 000 for fibrinogen and summarized by BLOMBÄCK AND YAMASHINA[8]. The results for tyrosine vary from 1.5 to 2.3 and for glutamic acid from 0.7 to 1.9 moles/mole of fibrinogen. BLOMBÄCK AND YAMASHINA conclude that bovine fibrinogen contains two moles each of N-terminal tyrosine and glutamic acid. Two additional chains previously believed to be cyclic are now known to terminate in acetylthreonine. The results shown in Table I are in agreement with the higher range of values reported previously.

* It is of interest to note that LAVER[11] using [^{35}S]labeled phenylthiohydantoins observed radioactivity at the origin in all chromatograms while in our studies the phenylthiohydantoins of [^{14}C]labeled amino acids also yield radioactivity at the origin indicating the presence of both S and C from the amino acid in these decomposition products.

BLOMBÄCK AND YAMASHINA[8] have reported only one analysis for the N-terminal residues of human fibrinogen: tyrosine, 0.492 and alanine, 0.391 moles/10^5 g. Calculated to a molecular weight of 340000 the values are: tyrosine, 1.7 and alanine, 1.3 moles/mole fibrinogen. These authors have suggested that human fibrinogen contains two moles each of tyrosine and alanine. The tyrosine and alanine values of Table II are substantially in agreement with the reported values.

LORAND AND MIDDLEBROOK[20] reported values obtained with the DNP procedure and human fibrinogen which when calculated for a molecular weight of 340000 are: tyrosine, 1.5 and alanine, 1.1 moles/mole fibrinogen.

Aspartic acid has not been reported as N-terminal in human fibrinogen. We have invariably observed this acid, however. Several possible explanations can be advanced. (a) The masked group on one of the fibrinopeptides may be aspartic acid with the masking group easily labilized under certain conditions. (b) A proteolysis due to a contaminating enzyme may readily release aspartic acid during manipulation procedures. Aspartic acid appears early and in substantial amounts when fibrinogen is digested by plasmin[21,22]. Plasminogen contaminates all purified fibrinogen unless additional extraction procedures are used[23]. Evidence[12] for the presence of plasmin degradation products of fibrinogen in fibrinogen fractions is frequently observable by N-terminal assays.

Further work will be required to explain the appearance of aspartic acid as an N-terminal residue in human fibrinogen preparations.

Differences between the values obtained for N-terminal residues and whole numbers may be due to several factors: (a) losses not evaluated by the procedure, (b) absorbance errors due to non-correspondence between the true and measured blanks (see p. 706), and (c) uncertainty as to the molecular weight of fibrinogen (for example, see FANTL AND WARD[24]).

Amino acids exhibiting anomalous behavior

Lysine has been reported to be liberated during the proteolysis of bovine fibrinogen by plasmin[22]. During the course of our investigations on plasmin degradation of fibrinogen[21], we have had occasion to study the behavior of ε-PTC–PTH-[$^{14}C_6$]lysine in the presence of PTC-fibrinogen.

It is particularly evident in the case of lysine that nearly all of a phenylthiohydantoin may be destroyed when only traces are present initially. The result of this is a greater likelihood of failure to detect trace contaminants and to underestimate the amount of impurities when an internal standard procedure is not used.

It is well-known that the PTH of threonine and the PTH of serine readily undergo dehydration during the cyclization procedure. In studies on abnormal fibrinogen fractions using the DNP procedure, we have consistently observed[12] the presence of serine and threonine. These acids have not been observed on the same samples when the PTH procedure is used. While threonine is readily detectable in a sample such as equine fibrinogen possessing N-terminal threonine, we have been unable to detect threonine in samples in which the DNP procedure unambiguously detects small amounts.

In a similar manner, serine is detected in fibrinogen degradation products by the DNP-procedure, but is not detected by the PTH procedure. The decomposition of the PTH of serine appears more complex than that of threonine since several radio-

active peaks are detected after chromatography. The report[25] that the PTH of serine can be rapidly and almost completely released from PTC-seryl peptides in 3 N HCl at 40° without appreciable dehydration has yet to be checked with the fibrinogen derivatives.

The method reported in this paper was developed for use in the study of the structure of fibrinogen and fibrinogen derivatives. Although only several assays have been performed on fibrin, the latter appears to offer less trouble than does fibrinogen. Because of the numerous problems inherent in the study of fibrinogen and because each protein frequently offers unique and characteristic features, we had no occasion to apply the procedure to simple proteins. The ease with which reproducibility is obtained with fibrinogen leads us to believe that application to other proteins would also be advantageous.

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