The N-Terminal Residues of Bovine Plasma Clot

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The discovery of the non-identity of fibrin and plasma clot obtained by clotting whole plasma in the presence of calcium ions (Robbins, 1944; Lorand, 1948; Laki & Lorand, 1948) served to clear up much of the confusion which surrounded the phenomenon of blood clotting. The determination of the N-terminal residues of fibrinogen and fibrin (Bailey, Bettelheim, Lorand & Middlebrook, 1951; Lorand & Middlebrook, 1952) suggested that a similar examination of plasma clot might throw light on the difference between it and fibrin.

MATERIALS

Thrombin. Hoffmann-La Roche's thrombin was used.

Fibrinogen. Citrated bovine plasma was fractionated by precipitation with 0.25-saturated $(\text{NH}_4)_2\text{SO}_4$ according to Lorand & Middlebrook (1952, section I), except that 0.38% (w/v) sodium citrate was used instead of 0.2% (w/v) sodium oxalate.

Fibrin. This was prepared from the fibrinogen by clotting with a minute amount of thrombin and purifying by precipitation from 30% (w/v) urea solution (Lorand & Middlebrook, 1952).

Plasma clot. Citrated bovine plasma was diluted with 4 vol. of water, then 1 vol. of 0.25M CaCl_2 was added with stirring. The solution was allowed to stand and after approx. 5 min. clotting started. This was allowed to proceed for a further 45 min., then the clot was broken up into small pieces which rapidly contracted and settled to the bottom of the beaker. This precipitate was thoroughly washed for 4 days at 5° with several lots of 0.9% (w/v) NaCl until the washing liquor was free from protein, as judged by precipitation with trichloroacetic acid solution. The plasma clot was then washed with distilled water at 5° until free from chloride ions for two days.

A further sample of plasma clot was swollen in 30% (w/v) urea solution after the NaCl treatment and the solution, maintained at 5°, was replaced 5 times over a period of 3 days. The urea was removed by further washing with distilled water. Both samples of plasma clot were washed in redistilled acetone to remove any fatty material, and finally dried over P_2O_5 .

Buffer. The pH 5.0 buffer contained 1.42 g. Na_2HPO_4 and 13.80 g. NaH_2PO_4 , H_2O in 100 ml. solution. All reagents were of analytical grade. Eastman's 1-fluoro-2:4-dinitrobenzene (FDNB) was used.

METHODS

Treatment with FDNB. The proteins were treated with FDNB under conditions recommended by Sanger (1945) for 24 hr. except that one-fifth the amount of NaHCO_3 was used. The treated proteins were washed with water and ethanol then immersed for approx. 2 hr. in a solution of the disodium salt of ethylenediaminetetra-acetic acid. Finally they were washed in glass-distilled water for 5 hr. and dried.

The diffusate from the plasma clot was treated similarly, but hydrolysed without washing.

Hydrolysis and separation of dinitrophenylamino acids (DNP-amino acids). About 500 mg. of the treated proteins were hydrolysed under reflux in 50 ml. constant-boiling HCl (prepared by distillation). The same flask and boiling-stick were always used, in conjunction with one electrical hot-plate set at the 'low' position. These precautions, together with the treatment with disodium ethylenediaminetetra-acetate and washing with glass-distilled water were believed to contribute to greater reproducibility of the results and less destruction of the DNP-amino acids. The times of hydrolysis ranged from 4 to 24 hr.

For chromatography, a different batch of silica gel was used from that used for the determination of the N-terminal residues of fibrin (Lorand & Middlebrook, 1952), and the author found that the properties of the batches differed considerably, even when using buffered columns. On the new gel, DNP-amino acids moved extremely slowly on a pH 6.0 column using ether as the mobile solvent. Therefore the following method of separation was used.

The hydrolysate was extracted 5 times with equal volumes of peroxide-free ether and the pooled ether solutions were evaporated to dryness in a flask. The residue was dissolved in a small amount of CHCl_3 and run on a 4 g. column containing 4 ml. pH 5.0 buffer, with CHCl_3 . The fast-moving decomposition products, including 2:4-dinitroaniline and 2:4-dinitrophenol, were removed but all the DNP-amino acids remained at the top of the column. The flask was rinsed with a small amount of ether, which was transferred to the column, and the mobile solvent was changed to ether. A strong band ($R=1.0$) was obtained, together with extremely weak bands, which were subsequently found to be due to DNP-aspartic acid, DNP-glutamic acid and DNP-serine. The strong band was run on a 2 g. pH 5.0 column with 1% (v/v) ethanol in chloroform. Three bands were obtained: $R=0.26$, found to be *ON*-bis-DNP-tyrosine; $R=0.09$, DNP-glycine; and a weak band which remained at the top of the column, was due to *N*-DNP-tyrosine (a decomposition product) and was removed by 3% (v/v) ethanol in chloroform.

The material in the acid phase was estimated by drying down a portion of the ether-extracted hydrolysate, then

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taking it up in 66% (v/v) methyl ethyl ketone in ether and passing it through a silica-gel column with water as the stationary phase. Only one band could be observed on this column, corresponding to *N*^ε-DNP-lysine.

Estimation of DNP-amino acids. The separated DNP-amino acids were made up to 25 ml. or 50 ml. in the appropriate solvent and estimated by means of a Beckman ultraviolet spectrophotometer (Lorand & Middlebrook, 1952). All calculations were based on the dry weight of original untreated protein.

Rates of decomposition of DNP-amino acids. The method used for determining the rates of decomposition of the DNP-amino acids (Table 1) was exactly the same as that used by Lorand & Middlebrook (1952).

In contrast to the previous statement (Lorand & Middlebrook, 1952) the yields of DNP-glycine, corrected for loss by decomposition, were not found to be lower after 6 hr. or 4 hr. hydrolysis. However, the yield of bis-DNP-tyrosine was found to be lower, after 4 hr. (1.4 moles/450 000 g. protein; cf. Table 2).

Protein content of DNP-proteins. The weight of the treated protein after washing and drying was compared with the dry weight of the original protein. The ratio of these two weights was approximately 1.15:1 in all cases. When the treated samples were washed with water for six days the ratio was 1.10:1.

Table 1. *Hydrolytic breakdown of DNP-amino acids by refluxing in constant-boiling HCl*

Amino acid derivative	Time of hydrolysis (hr.)	Measured* between (hr.)	Amount unchanged (%)
DNP-glycine	4	4-8	69.3
	4	8-12	72.7
	4	12-16	69.7
	8	4-12	50.4
	8	8-16	50.8
	12	4-16	35.2
bis-DNP-tyrosine	4	8-12	97.1
	4	12-16	95.0
	8	8-16	92.1
<i>N</i> ^ε -DNP-lysine	24	24-48	94.5

* Two samples of treated protein were hydrolysed for different times. The DNP-amino acids were separated and the percentage decomposition between these time periods was calculated.

Dialysis. Approx. 2 g. samples of fibrin and plasma clot were dissolved or swollen in solutions of monochloroacetic acid, so that the final concentration of the acid was 1% (w/v). These were dialysed in cellophan tubes, first against 1% (w/v) monochloroacetic acid, then against distilled water for 4 days at 5°. The diffusate from the plasma clot was extracted with ether, then evaporated to dryness *in vacuo*.

RESULTS

Plasma clot and fibrin. These proteins gave essentially the same results, and both contained the same ratio and concentration of *N*-terminal residues of glycine and tyrosine (Table 2). This is of interest, in view of the fact that Morrison (1947) showed that plasma clot could coprecipitate with other plasma proteins. Since both the ratio and concentration of the *N*-terminal residues in plasma clot are identical with those of fibrin, it is probable that the prolonged washing with 0.9% NaCl has removed the bulk of these adsorbed proteins. The small amounts of other DNP-amino acids obtained were presumably due to traces of foreign proteins or peptides.

Both proteins contained the same number of free ε-amino groups of lysine (Table 4).

Urea-washed and dialysed plasma clot and fibrin. By treatment with dispersing agents such as urea and dialysis with monochloroacetic acid, one *N*-terminal glyceryl residue/molecule (average mol. wt. = 450 000) of plasma clot was removed. Preliminary experiments suggest that fibrin is unchanged on dialysis with monochloroacetic acid (Table 2).

The diffusate. In confirmation of the loss of one *N*-terminal glyceryl residue from plasma clot, the diffusate was treated with FDNB, hydrolysed and found to contain a high concentration of DNP-glycine together with traces of other DNP-amino acids.

Prolonged washing of DNP-protein. As it was noticed that the wash liquor was always coloured yellow, in spite of many changes of water, additional samples of DNP-protein were repeatedly washed

Table 2. *Estimated number of moles of DNP-amino acids from 450 000 g. of protein, after correction for hydrolytic breakdown (time of hydrolysis (hr.) in brackets)*

	DNP-aspartic acid	DNP-glycine	Bis-DNP-tyrosine
Fibrin	Traces	4.3 (4)	2.0 (8)
Fibrin (dialysed)	Traces	3.7 (4)	—
		3.8 (8)	2.0 (8)
Plasma clot	Traces	3.9 (4)	—
		4.1 (8)	2.3 (8)
		3.7 (8)	2.2 (8)
Plasma clot (dialysed)	—	3.3 (8)	1.9 (8)
Plasma clot (urea-washed)	Traces	3.1 (4)	2.2 (4+4)*
	0.003 (8)	3.0 (8)	2.0 (8)
		3.2 (12)	2.0 (12)

* 4 hr. hydrolysis followed by further 4 hr. hydrolysis of peptide material.

Table 3. *Effect of prolonged washing of DNP-protein on the bis-DNP-tyrosine content of fibrin and plasma clot*

	Time of washing (hr.)	Moles bis-DNP-tyrosine/450 000 g. of protein
DNP-plasma clot	0	2.0
	6	2.1
	144	0.8
DNP-fibrin	6	2.0
	144	0.9

Table 4. *Lysine content derived from the N^ε-DNP-lysine estimations after 24 hr. hydrolysis*

	Moles lysine/450 000 g. of protein
Fibrinogen	195
	196
Fibrin	206
Plasma clot (urea-washed)	195
	198

with several lots of distilled water for 6 days. The final washings were still yellow, suggesting a slow evolution of 2:4-dinitrophenol from the *O*-DNP group of bis-DNP-tyrosine. This was confirmed by determination of the bis-DNP-tyrosine content of the hydrolysate, which was low, with a corresponding increase in the amount of *N*-DNP-tyrosine (Table 3). A sample of FDNB-treated plasma clot was hydrolysed, therefore, without washing to see if 6 hr. washing had any effect on the bis-DNP-tyrosine content. No effect was found, but a small amount of *N*-DNP-tyrosine was obtained even in this case. This was attributed to the slight decomposition of the bis-DNP-tyrosine during acid hydrolysis, which is normally corrected for. The length of time of washing had no effect on the estimation of the *N*-terminal glycyI residues.

DISCUSSION

Nanninga (1946) and Edsall, Foster & Scheinberg (1947) obtained a value of 450 000 for the molecular weight of bovine fibrinogen. Recently, Katz, Gutfreund, Shulman & Ferry (1952) and Shulman (1953) calculated this to be 340 000. Presumably, these would be maximal values for the molecular weights of fibrin and plasma clot. The present determinations are more in accord with the first value, for both fibrin and plasma clot.

The previous finding (Lorand & Middlebrook, 1952) that the molecule of fibrin of weight 450 000 has two *N*-terminal tyrosyl and four *N*-terminal glycyI residues was confirmed. This suggests that the molecule possesses six open peptide chains, not

necessarily of equal length. Although plasma clot, presumed to have a similar molecular weight, is precipitated with two *N*-terminal tyrosyl and four *N*-terminal glycyI residues, it seems that the molecule has really two tyrosyl and only three glycyI residues. Thus the molecule possesses five open peptide chains. The sixth chain, possessing an *N*-terminal glycyI residue (a product of the conversion of fibrinogen into fibrin by thrombin) (Lorand & Middlebrook, 1952), is strongly adsorbed on the plasma-clot molecule, but can be removed by 30 % (w/v) urea or monochloroacetic acid solution. The molecular weight of this chain is sufficiently small to allow diffusion through a cellophan membrane. It was detected in the diffusate by reaction with FDNB and separation of DNP-glycine after acid hydrolysis.

Within the limits of experimental error, fibrin and plasma clot contain the same number of free ϵ -amino groups of lysine, as revealed by the FDNB method.

SUMMARY

1. Bovine fibrin and plasma clot were treated by the dinitrophenyl method and the *N*-terminal residues determined.
2. Like the fibrin molecule, that of plasma clot has two *N*-terminal tyrosyl residues, but three instead of four *N*-terminal glycyI residues.
3. Plasma clot is precipitated with four chains having *N*-terminal glycyI residues, but one of these can be removed by urea or monochloroacetic acid solution.
4. Fibrin and plasma clot contain the same number of free ϵ -amino groups of lysine.

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