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Molecular Weight of Human Fibrinogen Derived from Phosphorus Determinations

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1. Fibrin clots obtained from diluted human plasma with bovine thrombin often contain amounts of phospholipids that cannot be diminished by further plasma dilution. 2. The 'cold insoluble residue' obtained during fibrinogen preparation has a higher phosphorus content than the purified fibrinogen. 3. Evidence showed that adsorption of phospholipids or phosphorus-containing fibrinopeptides on purified fibrinogen or fibrin was unlikely. 4. *O*-Phosphorylserine was detected in acid hydrolysates of human fibrin. 5. On the basis of phosphorus determinations the average molecular weight of human fibrinogen cannot be less than 342000 (304000–383000) for a group of ten donors, and 265000 for two other persons, assuming 1 phosphorus atom/molecule and incomplete splitting of the phosphorus-containing fibrinopeptide. Complete splitting of the phosphopeptide would require molecular weights twice as high. 6. Fibrinolysis was a possible cause of lower phosphorus contents found in isolated fibrinogen and fibrin from a donor who showed apprehension during blood collection and in a fibrinogen preparation that had been submitted to prolonged dialysis.

The presence of phosphorus in fibrin was first reported by Mulder (1838). The phosphorus content of mammalian fibrinogens and fibrins has been determined by Dmochowski & Krajewski (1961), Fantl & Ward (1962), Blombäck, Blombäck & Searle (1963) and Krajewski & Dmochowski (1963). Since fibrinogen can be separated from plasma in high purity a minimum molecular weight can be calculated from the phosphorus content. Further work on human fibrinogen and fibrin under various experimental conditions is presented below.

EXPERIMENTAL

Materials. Venous blood was obtained from regular blood donors and from patients who had received 1 mc of inorganic [^{32}P]phosphate intravenously on 3 successive days, blood being taken 3 days after the last injection. In all cases 110ml. of 0·102M-trisodium citrate was mixed with 390ml. of blood. Centrifugation was first carried out at 2000*g* at 4° for 20min., and to remove the remaining platelets the plasma was centrifuged at 35000*g* at 4° for 30min. Processing of the plasma was commenced on the same day. Fibrinogen was isolated in most cases by the ether precipitation technique of Kekwick, Mackay, Nance & Record (1955), the only alteration being that in the final precipitation step 0·2M-Na_2HPO_4 was replaced by 0·1M-Na_2CO_3. In some cases the insoluble residue remaining from the extraction of fraction F.1W with citrate buffer, pH6·18 and *I*0·3, at 0–2° was retained for analysis. This is referred to below as the 'cold insoluble residue'. Occasionally the ethanol precipitation method of Blombäck & Blombäck (1956) was used. In some preparations 6-amino-*n*-hexanoic acid, an inhibitor of fibrinolysis, was added to plasma.

Although only reagents of analytical grade were used it was found necessary to check that NaCl was phosphorus-free, since one brand added 1μg. of phosphorus to fibrin clots prepared in 500ml. of 0·15M-NaCl.

Bovine thrombin (topical: Parke, Davis and Co. Ltd., Detroit, Mich., U.S.A.) was purified by dissolving and dialysing against distilled water for 1 day at 2°. The sediment was discarded after centrifugation at 35000*g* at 4°. This preparation is referred to below as partially purified. It contained approx. 2·5μg. of phosphorus/500 units of thrombin. For further purification it was freeze-dried and fractionated in a column of Amberlite IRC-50 according to the procedure of Rasmussen (1955). Inorganic phosphate was removed by dialysing against distilled water, freeze-drying and filtering through a column of Sephadex G-25 in 0·1M-NaCl. The preparation contained 0–0·3μg. of phosphorus/500 units of thrombin.

Determination of phosphorus. Phosphorus was determined by the method of Allen (1940), with the addition of extra acid as described by Fantl & Ward (1962). When the NaCl concentration was greater than 0·6M the technique of Berenblum & Chain (1938) was used.

Measurement of radioactivity. For the measurement of radioactivity the materials were dissolved in 0·5ml. of N-NaOH and evaporated to dryness in stainless-steel planchets. Counting was carried out in a gasflow counter (model D–47; Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.).

Determination of protein and clottability of fibrinogen. Protein in fibrinogen and fibrin was determined from the

extinction at 282mμ in N-NaOH and correction for non-specific absorption was made as described by Goodwin & Morton (1946). The clottability of fibrinogen was determined in 0·15M-NaCl; thrombin was added at room temperature to give a clotting time of 15–30sec. After 1hr. fibrin was collected on a glass rod, squeezed free of fluid and washed with 0·15M-NaCl until protein-free. Fibrin was weighed if the amount was greater than 10mg.; the clots were prepared as above, washed with 0·15M-NaCl and with distilled water and extracted repeatedly with boiling ethanol–diethyl ether (3:1, v/v). Fibrin was dried at 105° to constant weight over magnesium perchlorate. The following characteristics were used for the calculations. Human fibrin in N-NaOH has an $E^{1\%}_{1\,cm.}$ value at 282mμ of 17·0 (mean of determinations performed at least in duplicate, and usually in triplicate, on samples from ten persons; variation ±0·44). Human fibrin dissolved in 8M-urea, pH8·07, had an $E^{1\%}_{1\,cm.}$ value at 282mμ of 15·3. The ultraviolet absorption of bovine fibrinogen is 2% lower than that of fibrin at 282mμ (Blombäck, 1958). On this basis the $E^{1\%}_{1\,cm.}$ value of human fibrinogen in N-NaOH was calculated from that of fibrin to be 16·7. The same value was applied to total protein. Percentage clottability was calculated as 100 × weight of fibrin/weight of protein. The yield of fibrin was accepted to be 97% of fibrinogen (Lorand, 1952).

Chromatography. Chromatography of fibrin hydrolysates for phosphorus compounds was carried out according to the procedure of Heald (1958). The hydrolysate was fractionated on a column of Dowex 50W (X4; 100–200 mesh). Fractions were eluted with 0·05N-HCl and analysed for inorganic phosphorus and, after ashing, for organic phosphorus.

RESULTS

Results of determinations of phosphorus in human fibrinogen and fibrin are given in Table 1, which shows that 1g.atom of phosphorus was present in 342000g. of fibrinogen (range 304000–383000) and in 603000g. of fibrin (range 509000–696000). In two further preparations with a clottability of 93–95%, 1g.atom of phosphorus was present in 265000g. of fibrinogen and in 450000g. of fibrin. Table 1 also shows that the phosphorus content of fibrin was not influenced by the phosphorus concentration of the thrombin preparation.

During the blood collection the above donors were quite calm, but in a case where signs of stress were evident during blood collection the separated fibrinogen contained 1g.atom of phosphorus/425000g. Partial fibrinolysis may have been responsible for this result.

Fibrinogen was also isolated from plasma of patients who had received [³²P]phosphate injections. Radioactivity was measured, with the results given in Table 2, which shows that on average 53% of the radioactivity of the isolated fibrinogen was present in fibrin and 39% was recovered in the clot liquor.

Fibrin has the property of adsorbing other compounds. In the belief that dilution would decrease adsorption the phosphorus content of fibrin ob-

Table 1. *Phosphorus content of isolated human fibrinogen and fibrin*

Fibrinogens were prepared according to the method of Kekwick *et al.* (1955), except Tr, which was prepared by the method of Blombäck & Blombäck (1956). Suffixes 1 and 2 indicate preparations from the same donor at different times. Cm was prepared by further purification of a commercial preparation. The fibrinogen solutions were precipitated by 10% (w/v) trichloroacetic acid at 2° and phosphorus was determined in the insoluble sediment. In the first seven preparations purified thrombin was used for clotting; in the next five, partially purified thrombin was used.

Preparation	Percentage clottability of fibrinogen preparation	10^{-3} × Wt. containing 31g. of P (g.)	
		Fibrinogen	Fibrin
Ke	97	310	509
An*	90	363	612
Co–1	100	317	—
Co–2	92	327	696
Me–1	95	350	621
Me–2†	94	304	521
Wi†	96	343	673
Cm	95	383	673
Tr	96	360	—
Ma	97	363	553
Pa	93	334	588
Wa	87	351	588

* Reprecipitated twice in the presence of 0·1M-6-amino-*n*-hexanoic acid as described by Mosesson & Finlayson (1963).

† 6-Amino-*n*-hexanoic acid was added to the blood during collection to give a concentration of 0·035M in the plasma; a concentration of 0·01M was maintained throughout the isolation.

Table 2. *Radioactivity of human fibrinogen, fibrin and clot liquor*

The radioactivity of fibrin is calculated on the basis 97mg. of fibrin being produced from 100mg. of fibrinogen. The radioactivity of the clot liquor is calculated/100mg. of fibrinogen.

Preparation	Percentage clottability of fibrinogen preparation	Radioactivity (% of that of fibrinogen)	
		Fibrin	Clot liquor
De	94	53	—
Sw	93	51	—
Th	93	59, 56*	38
Wi	96	49	40

* Refers to 22·7mg. of fibrin dissolved in 1ml. of 1% (w/v) monochloroacetic acid and reprecipitated by the addition of 9ml. of water and 10ml. of 20% (w/v) trichloroacetic acid.

Table 3. *Phosphorus content of fibrin prepared from concentrated and dilute fibrinogen solutions*

The fibrinogen preparation was 85% clottable and was dissolved in 0·15M-NaCl. In column 3 clots were washed with 0·15M-NaCl, dissolved in 1ml. of 1% (w/v) monochloroacetic acid and reprecipitated by the addition of water and trichloroacetic acid (final concn. 10%, w/v); the sediment was centrifuged down.

Concn. of fibrinogen in reaction mixture (mg./100ml.)	P content (μg./100mg. of fibrin)	
	Fibrin washed with 0·15M-NaCl and water	Fibrin dissolved and re-precipitated
1290	7·10	6·88
134	7·05	6·84

Table 4. *Phosphorus content of plasma fibrin clots*

Pre-paration	Concn. of fibrinogen in reaction mixture (mg./100ml.)	10^{-3} × Wt. of fibrin containing 31g. of P (g.)		Ratio (2):(1)
		(1) Washed with 0·15M-NaCl and water	(2) Extracted with ethanol–ether (3:1, v/v)	
To	8·7	278	429	1·54
Ca	13·3	482	655	1·36
We	11·0	480	535	1·11
We	3·5	441	502	1·14
Ma	7·8	393	536	1·36
Ma	2·5	385	499	1·30

tained by clotting at different fibrinogen concentrations was determined. Results in Table 3 indicate, first, that fibrin clots prepared from either concentrated or dilute solutions contained approximately the same amount of phosphorus and, secondly, that this was not significantly altered by solution of fibrin in monochloroacetic acid and precipitation by dilution and addition of trichloroacetic acid.

Table 4 gives results obtained in fibrin clots formed at various dilutions of plasma. The phosphorus determinations were carried out on the clots after extraction in boiling ethanol–diethyl ether (3:1, v/v). The phosphorus-containing compounds were co-precipitated with fibrin and this could not be abolished by even higher dilution. The ethanol–ether-soluble material consisted of compounds characteristic of lipoprotein components. This was established by thin-layer chromatography. Phosphatidylcholine, neutral lipids, free fatty acids and traces of phosphatidylethanolamine were identified.

The form in which phosphorus was bound in fibrin prepared from purified fibrinogen was determined according to the procedure of Heald (1958). The peak of the fraction containing organic phosphorus coincided with that obtained with an authentic specimen of *O*-phosphorylserine treated in a similar manner.

Table 5. *Phosphorus content of 'cold insoluble residue' and fibrin obtained therefrom*

The 'cold insoluble residue' was dissolved in 0·15M-NaCl–0·01M-6-amino-*n*-hexanoic acid. The precipitate from 10% (w/v) trichloroacetic acid, and the fibrin formed with purified thrombin, were both extracted with boiling ethanol–ether (3:1, v/v) before determining phosphorus.

Preparation	Percentage clottability	10^{-3} × Wt. containing 31g. of P (g.)	
		'Cold insoluble residue'	Fibrin
Me–2	74	240	356
Wi	76	253	415

Fractionation procedures yielded only 20–30% of plasma fibrinogen. Further, there are some indications of inhomogeneity of human fibrinogen (Caspary & Kekwick, 1957; Mosesson & Finlayson, 1963). For these reasons, in addition to purified fibrinogen, the 'cold insoluble residue' was analysed with the results given in Table 5, which show that the phosphorus contents of the 'cold insoluble residue' and the fibrin clot obtained from it were 1·27–1·62 times those of the corresponding materials listed in Table 1.

DISCUSSION

The heterogeneity of human fibrinogen has been considered by Caspary & Kekwick (1957). Mosesson & Finlayson (1963) separated by column chromatography two major types of human fibrinogen that differed in electrophoretic mobility but were identical in all other characteristics studied, including phosphorus content. The phosphorus content of plasma fibrin clots, 'cold insoluble residue', purified fibrinogens and fibrins derived from them have been compared in the present work. Although at appropriate plasma dilutions fibrin gave constant phosphorus values, on further dilution adsorption of lipoproteins was still demonstrable. Morrison (1947) observed that lipoproteins can be adsorbed by fibrin prepared from isolated fibrinogen. The 'cold insoluble residue' contained 75% of clottable protein. The phosphorus content of this fraction was 1·27–1·35 times that of purified fibrinogen, and this might indicate heterogeneity of plasma fibrinogen or co-precipitation of other plasma proteins containing phosphorus. With fibrinogen of high purity any phosphorus-containing contaminant must be of low molecular weight to affect significantly the phosphorus content. No evidence of

appreciable adsorption of the phosphopeptide released during clotting (Blombäck, Blombäck, Edman & Hessel, 1962) was observed. It is therefore concluded that phosphorus is a structural component of human fibrin. Hydrolysis experiments on fibrin obtained by clotting purified human fibrinogen indicated that phosphorus was bound as *O*-phosphorylserine.

One g.atom of phosphorus was present in 304000–383000g. (mean 342000g.) of fibrinogen prepared from the plasma of each of ten persons, and in two other instances 1g.atom of phosphorus was present in 265000g. of fibrinogen. This could support the existence of different molecular species of fibrinogen in human plasma. Both the chemical determination of phosphorus and the radioactivity of ^{32}P-labelled human fibrinogen and fibrin gave ratios of phosphorus in fibrinogen to that in fibrin 1·6–2·2, which were not altered by treatment with monochloroacetic acid and ethanol–ether extraction. These measures make the adsorption of water-soluble phosphorus compounds unlikely and indicate the absence of phospholipids in purified fibrinogen.

It is possible that the phosphorus content of both fibrinogen and fibrin could be decreased by proteolytic enzymes that may contaminate the preparations. Lower values were observed in a fibrinogen preparation that was dialysed for more than 2 days against 0·1 M-ammonium acetate, and also in a fibrinogen preparation that was isolated from the plasma of a donor who showed signs of stress during blood collection. These conditions can induce fibrinolysis.

In experiments in which 6-amino-*n*-hexanoic acid was present throughout the isolation procedure no difference in phosphorus content was obtained from those experiments in which it was absent (Table 1), indicating that fibrinolysis did not normally occur during the preparation.

The evidence presented points to an average molecular weight of human fibrinogen in the ten donors of Table 1 that cannot be lower than 342000. However, since 1g.atom of phosphorus was present in an average of 603000g. of fibrin it is possible that thrombin splits off the phosphopeptide incompletely or that fibrinogen contains 2 phosphorus atoms/molecule, with a molecular weight of 684000 from which thrombin splits the above phosphopeptide completely.

Values obtained for the molecular weight of human fibrinogen by physical techniques vary, as shown by the following published figures: 670000 (Holmberg, 1944; recalculated by Edsall, Foster & Scheinberg, 1947); 580000 and 400000 (Oncley, Scatchard & Brown, 1947); 340000, and 130000 at low concentration (Caspary & Kekwick, 1954, 1957); 269000 (Sowinski, Freling & Koenig, 1960).

In contrast with the physical techniques the present results require no assumptions other than that the analytical procedures were satisfactory.

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