SC 2242

On the occurrence of phosphorus in fibrinogen

We have earlier reported[1] on the occurrence of organically bound phosphorus in one of the peptides split off from human fibrinogen during its transformation to fibrin by thrombin. This phosphorus could be accounted for by the demonstration of an *O*-phosphoserine residue in the peptide. As regards the origin of the phosphorus it seemed likely that it was the fibrinogen molecule itself. Further work, presented here, has settled the question in favour of this alternative.

Fibrinogen (Fraction I-4) and fibrinopeptide were prepared and determined as described elsewhere[2–5]. Fibrinogen preparations (1–2 % in 0.3 M NaCl) were dialysed in the cold for 2–3 days against 0.3 M NaCl. The phosphopeptide preparations were passed through a Sephadex G-25 column equilibrated with water. The thrombin used had an activity of 280 N.I.H. units per mg (1 μg P/mg)[6,7]. For the dephosphorylation calf intestinal phosphatase[§] was used[8].

The total phosphorus content of human and bovine fibrinogen was found to be 9.4 μg/100 mg and 14.5 μg/100 mg, respectively (Table I). Fibrinogen was also isolated from the blood of severely ill haemophiliacs. In a patient afflicted with Factor-VIII deficiency the fibrinogen contained 7.8 μg P/100 mg and in a patient with Factor-IX deficiency 8.0 μg P/100 mg.

Next the fate of phosphorus during the clotting of fibrinogen was determined: 10 ml of 0.5–0.6 % fibrinogen in 0.3 M NaCl (adjusted to pH 7 with 0.1 N NaOH) was diluted with 10 ml of distilled water and clotted with 0.1 ml of bovine thrombin solution (100 N.I.H. units/ml). The incubation was at room temperature (26°) for between 1 and 21 h. The clots were collected on glass rods and transferred to tubes for phosphorus analyses[9]. Protein determinations were performed on the clot supernatants. After incubation with thrombin for 3 h or more, less than 6 % of the fibrinogen remained unclotted. The supernatants were then evaporated to a small volume *in vacuo* on a rotary-film evaporator and total phosphorus determined. Corrections for the phosphorus in the unclotted fibrinogen left in the supernatant were made (Table I).

From these experiments it may be concluded that the phosphorus of the human fibrinopeptide is derived directly from the fibrinogen. However only 25–40 % of the

TABLE I

PHOSPHORUS IN FIBRINOGEN, FIBRIN AND CLOT-SUPERNATANTS

	Number of determinations	*Phosphorus in protein (μg/100 mg)*		*Molar ratio phosphorus/protein**	
		Mean	*Range*	*Mean*	*Range*
Human fibrinogen	21 on 3 different batches	9.4	8–14	1.0	0.9–1.5
Human fibrin	17 on 2 different batches	5.5	5–7	0.6	0.5–0.7
Human clot-supernatant	11 on 1 batch	2.8**	2–4	—	—
Bovine fibrinogen	7 on 2 different batches	14.5	13–17	1.6	1.4–1.8
Bovine fibrin	3 on 1 batch	14.3	13–14	1.6	1.4–1.7

* Assumed mol. wt. 340000.

** Per 100 mg fibrinogen.

§ Generously supplied by Dr. L. ENGSTRÖM, Department of Physiological Chemistry, University of Uppsala, Uppsala (Sweden).

phosphorus of the fibrinogen was recovered in the clot supernatant, the rest being retained by the clot. This is in agreement with the observation that in preparative experiments the yield of phosphopeptide was correspondingly low. Thus 0.3–0.4 mole of peptide is released per mole fibrinogen[4]. This low yield may be due to an incomplete cleavage of the peptide or to it being trapped by the clot. In bovine fibrinogen the phosphorus was retained by the fibrin to the extent of 90% or more. This might be expected since bovine fibrinopeptides do not contain phosphorus.

Protein-bound phosphoric acid can be split off by phosphatases[10,11]. We have investigated fibrinogen and the phosphopeptide in this respect. The enzyme used was calf intestinal alkaline phosphatase (EC 3.1.3.1). The incubations were carried out at pH 5.6 and 9.0 at 37°. The reaction was followed by determinations of inorganic phosphate in the digest[12,13]. It was found that inorganic phosphate added to fibrinogen was recovered only to the extent of 70–80% and recovery experiments were therefore included in most runs, and the results corrected accordingly. At pH 5.6 the phosphatase splits off the bound phosphoric acid quantitatively from human and bovine fibrinogen. At the higher pH the reaction is slower, and may not reach completion (Fig. 1a). The phosphopeptide was also quantitatively dephosphorylated at pH 5.6 and 9.0 by alkaline phosphatase but in contrast to dephosphorylation of fibrinogen the rate of hydrolysis was (as one would expect) more rapid at pH 9.0 than at pH 5.6

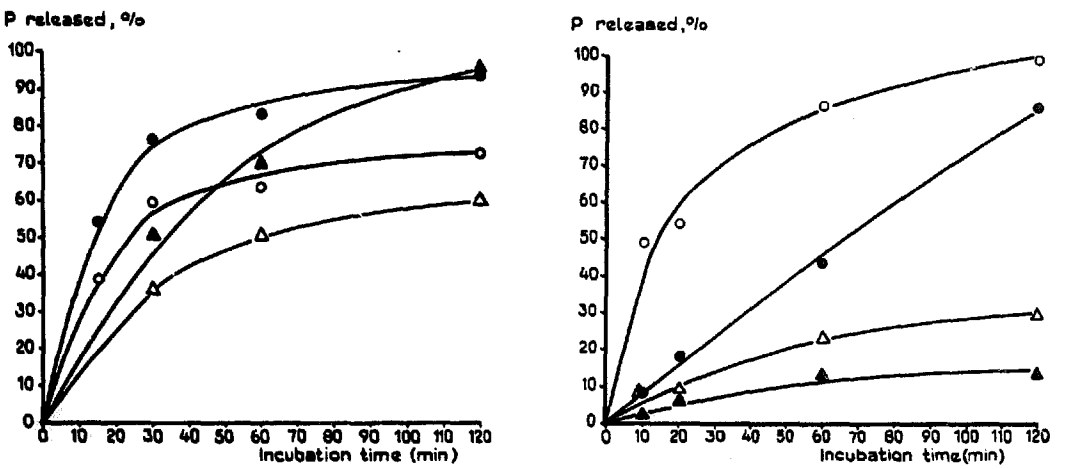

Fig. 1a. Dephosphorylation of human and bovine fibrinogen with calf intestinal alkaline phosphatase. Human fibrinogen. Expt. 1 (O—O): 2.85 ml fibrinogen (45.5 mg = 4.3 μg P) in 0.3 M NaCl + 0.15 ml 1.0 M Tris–acetate buffer (pH 9.0), 0.2 M with respect to $MgCl_2$. 4.2 μl phosphatase added, final concentration in the mixture being 2.8 μg/ml (*i.e.* 180 Portmann units/ml). Digestion at 37°. Inactivation of enzyme by adding 0.3 ml 0.2 M EDTA (disodium salt) and freezing in dry ice. Expt. 2 (●—●): 1 M acetate–acetic acid buffer (pH 5.6), 0.2 M with respect to $MgCl_2$, otherwise as above. Bovine fibrinogen. Expt. 3 (△—△): digestion at pH 9.0 of 48 mg (7.0 μg P) fibrinogen. Otherwise see Expt. 1. Expt. 4 (▲—▲): digestion at pH 5.6 of 48 mg (7.0 μg P) fibrinogen. Otherwise see Expt. 2.

Fig. 1b. Dephosphorylation of human fibrinogen and phosphopeptide with calf intestinal alkaline phosphatase. Human fibrinopeptide. Expt. 1 (O—O): 0.53 mg peptide (7.2 μg P) in 2.85 ml 0.3 M NaCl + 0.15 ml 1.0 M Tris–acetate buffer (pH 9.0), 0.2 M with respect to $MgCl_2$. 4.2 μl phosphatase added, final concentration in the mixture being 0.2 μg/ml (*i.e.* 13 Portmann units/ml). Digestion at 37°. Inactivation of enzyme by adding 0.3 ml 0.2 M EDTA (disodium salt). Expt. 2 (●—●): 1 M acetate–acetic acid buffer (pH 5.6), 0.2 M with respect to $MgCl_2$ was used, otherwise as above. Human fibrinogen. Expt. 3 (▲—▲): digestion at pH 9.0 of 55 mg (4.8 μg P) fibrinogen, enzyme concentration 13 Portmann units/ml. Otherwise see Expt. 1, Fig. 1a. Expt. 4 (△—△): digestion at pH 5.6 of 55 mg (4.8 μg P) fibrinogen, enzyme concentration 13 Portmann units/ml. Otherwise see Expt. 2, Fig. 1a. No corrections for yield of added phosphate has been made in these experiments.

(Fig. 1b). The type of bond in the phosphopeptide is an O–P monoester linkage. This may also be the only type of bond in the protein although other modes of linkage, *e.g.* phosphoamide and diester linkages, are not ruled out on the present evidence.

Phosphorus-free human fibrinogen on a larger scale was prepared as follows: 14 ml of 2.2% fibrinogen in 0.3 M NaCl + 0.75 ml buffer (pH 5.6) (see Fig. 1a) + 250 μl (about 0.5 mg) of alkaline phosphatase were incubated for 4 h at 37°. After dephosphorylation the fibrinogen was refractionated[2]. Two fractions were obtained; Fraction I-3 (discarded) and the main fibrinogen fraction, I-4. The latter was dialysed for 36 h against 0.3 M NaCl containing 0.02 M Tris–acetate buffer (pH 7.2). No phosphorus could be demonstrated in the preparation. A control preparation contained 8.7 μg P/100 mg.

The dephospho-fibrinogen sedimented at the same rate as native fibrinogen in the ultracentrifuge (Fig. 2). Thus the molecule had not undergone any major change in size or shape as a result of the dephosphorylation.

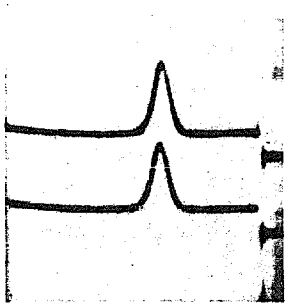

Fig. 2. Ultracentrifugation of dephosphorylated human fibrinogen. Ultracentrifuge (Spinco Model E, one wedge window cell) patterns of native (top) and dephosphorylated (bottom) human fibrinogen. Speed 42 040 rev./min, protein concentration 5 mg/ml in 0.3 M NaCl–0.02 M Tris–acetate (pH 7.2), temp. 22°. Exposure 80 min after attaining speed. Sedimentation from right to left.

The phosphorus-free human fibrinogen was clottable with bovine thrombin to the same extent as the untreated fibrinogen when the samples were incubated with thrombin (5 N.I.H. units/ml) for 2–3 h at room temperature. It thus appears that the phosphoric acid grouping in human fibrinogen is not a condition *sine qua non* for clotting. However, both when human and bovine thrombin was used for clotting a moderate but significant increase in the clotting time was observed for the dephosphorylated human fibrinogen.

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Stimulation of glucose oxidation without glycogen deposition by oxytocin and vasopressin

It has recently been shown that the posterior pituitary hormones oxytocin and vasopressin share some of the metabolic properties of insulin[1,2]. All three substances stimulate glucose uptake from the incubation medium, oxidation of labeled glucose to $^{14}CO_2$, and incorporation of the ^{14}C of labeled glucose into lipids. Using a number of synthetic oxytocin analogues MIRSKY AND PERISUTTI showed that the insulin-like action on fat appeared to parallel the oxytocic activity and that not all these substances possessed such activity. Because the stimulation of glycogen deposition may represent a more characteristic action of insulin, we have evaluated oxytocin and vasopressin for this activity while simultaneously measuring glucose oxidation. The results indicate that the action of the posterior pituitary peptides is in some way different from that of insulin, despite their other similarities.

Epididymal fat pads were obtained from *ad libitum* fed Sprague–Dawley rats weighing approx. 200 g and were incubated as previously described[1,2] with the following modifications. The arrangement of the three segments of each fat pad was such that the control and each concentration of the test substances used were incubated with one proximal segment, one middle segment, and one distal segment. This was necessary to avoid bias due to the greater activity of the proximal segments. All incubations were conducted in 25- or 30-ml flasks (uniform in any experiment) fitted with a removable center well and were carried out for 1 h at 37° in a Dubnoff shaking incubator. The labeled substrate D-[1-^{14}C]glucose (New England Nuclear Corp.) was added at an activity of 0.25 μC/ml and a concentration of 1 mg/ml in a total volume of 2 ml of Krebs–bicarbonate buffer. At the end of the incubation period 2.0 ml of 4% trichloroacetic acid were injected through the rubber cap into the incubation medium and 1 ml of "Hyamine" base (Packard Instrument Company) was injected into the center well. The flasks were then incubated for another hour at room temperature, after which the center well was removed and the "Hyamine" transferred into a counting vial containing scintillator (2,5-diphenyloxazole, 0.5%, 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene, 0.03%) in toluene and counted in a