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The Reaction between Activated Plasma Thromboplastin Antecedent and Diisopropylphosphofluoridate*

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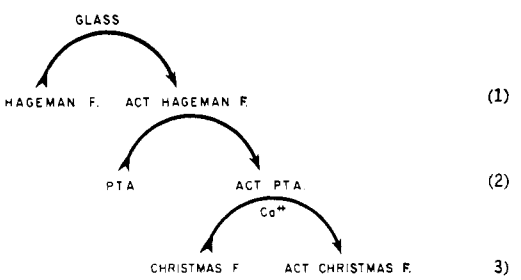
The reaction of activated plasma thromboplastin antecedent with P^{32} diisopropylphosphofluoridate has been investigated. The rate of the reaction rises steadily from *p*H 4 to 11; the reaction does not require calcium ions. In contrast, the reaction of activated plasma thromboplastin antecedent with its natural substrate, Christmas factor, has a *p*H optimum at 8.0 and requires calcium ions. Both reactions are inhibited by heparin. Total and partial acid hydrolysis of activated plasma thromboplastin antecedent labeled with diisopropylphosphofluoridate indicated that the diisopropylphosphofluoridate-binding site is the same as that in trypsin and thrombin, i.e., the hydroxyl group of serine in the peptide glycyl-aspartyl-seryl-glycine.

Hageman factor (factor XII), plasma thromboplastin antecedent (PTA, factor XI),[1] and Christmas factor (factor IX) are plasma proteins which participate in the early phases of blood clotting. In the presence of glass, Hageman factor is converted to an active enzyme. The activated Hageman factor then catalyzes the conversion of PTA to activated PTA. The activated PTA in turn catalyzes the conversion of Christmas factor to activated Christmas factor. The last reaction requires calcium ions and is inhibited by heparin or diisopropylphosphofluoridate (DFP). These reactions recently summarized by Ratnoff and Davie (1962) are shown in equations (1), (2), and (3).



The inhibition of reaction (3) by DFP suggests that DFP may be bound covalently to activated PTA in a manner similar to other DFP-sensitive enzymes. The present communication tests this possibility and deals with the various aspects of the reaction of activated PTA with DFP^{32} employing a highly purified enzyme preparation. The effects of *p*H, calcium ion, and heparin are reported and compared to their effects on the reaction of activated PTA with Christmas factor, its normal substrate. The site of binding of DFP to activated PTA was also examined and compared with the site of binding of DFP to other enzymes.

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[1] Abbreviations used in this work: PTA, plasma thromboplastin antecedent; DFP, diisopropylphosphofluoridate; DIP, diisopropylphosphoryl; BAEE, benzoyl-L-arginine ethyl ester; TAME, *p*-toluene-sulfonyl-L-arginine methyl ester; Tris, tris(hydroxymethyl)aminomethane; CM-cellulose, carboxymethyl-cellulose; EDTA, ethylenediaminetetraacetate; ATP, adenosine triphosphate.

MATERIALS AND METHODS

Heparin sodium, purified, dry powder, 145.7 USP units/mg, was a generous gift from Dr. Bernard P. Salafsky. It was originally provided by Eli Lilly and Co., Indianapolis, Ind.

Benzoyl-L-arginine ethyl ester (BAEE) was synthesized by the procedure of Bergmann *et al.* (1939).

p-Toluenesulfonyl-L-arginine methyl ester (TAME) was obtained from H. M. Chemical Co. Ltd., Santa Monica, Calif.

Trypsin (salt-free, 3-times recrystallized) and *soybean trypsin inhibitor* were obtained from Worthington Biochemical Corp., Freehold, N. J.

Thrombin was crude bovine topical thrombin, obtained from Parke, Davis and Co., Detroit, Mich.

Subtilisin was Nagarse crystalline bacterial protease, purchased from Biddle Sawyer Corp., N. Y.

DFP^{32} was obtained from the New England Nuclear Corp., Boston, Mass. It was supplied in solution in propylene glycol in a concentration of 1 mg/ml and contained 200 ± 50 μc/mg when shipped. The preparation was used without further purification.

Tris buffer was 0.15 M in tris(hydroxymethyl)aminomethane, and was adjusted to *p*H 7.5 by the addition of HCl.

Mixed buffer was 0.05 M in each of four components: acetic acid, maleic acid, Tris, and glycine; it was adjusted to various values in the range of *p*H 4.0–11.0 by addition of 1 N NaOH or 1 N HCl.

Acetate buffer contained 0.15 M acetic acid–sodium acetate, *p*H 5.2. *Phosphate-saline buffer* was 0.05 M sodium phosphate in 0.35 M NaCl at *p*H 6.3. The buffer used for paper electrophoresis contained 1 part pyridine, 10 parts glacial acetic acid, and 89 parts water (v/v) at *p*H 3.6. *Barbital-saline buffer* was prepared as previously described (Ratnoff *et al.*, 1961) and contained 0.025 M barbital and 0.125 M NaCl, *p*H 7.4.

Carboxymethyl-cellulose was prepared by the method of Ellis and Simpson (1956) under conditions which yield a product with a substitution of approximately 0.72 meq/g.

Protein concentration was determined by the biuret reaction (Gornall *et al.*, 1949). When following gradient-elution chromatography of activated PTA, protein concentration was estimated by determining the optical density of the effluent at 280 mμ (Warburg and Christian, 1941). A solution of partially purified activated PTA with a protein concentration of 0.1 mg/ml had an optical density of 0.667 at 280 mμ.

Inorganic phosphate was determined as the reduced phosphomolybdate complex (Fiske and Subbarow, 1925).

O-Phosphoserine was determined by the ninhydrin reaction (Troll and Cannon, 1953).

Radioactivity was determined in the Packard Tri-Carb liquid scintillation counter, using the scintillant previously described (So and Davie, 1963). *Protein-bound radioactivity* was determined by precipitation of the radioactive protein on filter paper disks (Mans and Novelli, 1961). The washes with hot trichloroacetic acid and hot ethanol-ether, used in the published procedure for cruder protein preparations, were omitted.

Esterase assays were performed by a titrimetric method (Schwert *et al.*, 1948) employing a Radiometer automatic titrator and a special reaction vessel (Dixon and Wade, 1958) at 25°. Each substrate used was in a final concentration of 1×10^{-2} M in the assay mixture, which contained 1×10^{-1} M KCl, 5×10^{-2} M $CaCl_2$, and 1×10^{-2} M Tris. The *p*H of the mixture was maintained at *p*H 7.9, for 5–10 minutes after adding the enzyme, by the automatic addition of 0.1 M NaOH. The protein concentration varied from 0.05 mg/ml for activated PTA to 0.007 mg/ml for trypsin.

Platelet-deficient citrated plasma was prepared from normal blood to which one-ninth volume of 0.13 M trisodium citrate had been added (Ratnoff *et al.*, 1961). This plasma, which had had no contact with glass, was used in all the experiments to be described, and was stored in small aliquots in siliconized plastic tubes at −20° until just prior to use.

Centrolex-P suspension was prepared by blending 100 mg of Centrolex-P ($CHCl_3$ extract of soybeans, Central Soya, Inc., Chicago, Ill.) with 100 ml of 0.15 M NaCl in a Waring Blendor for 2–3 minutes. The resulting suspension was divided into 2- to 5-ml aliquots and stored at −20° in plastic tubes.

Human oxalated serum was prepared by the method of Alexander (1955).

Barium sulfate eluates of the above human oxalated serum were prepared as previously described (Ratnoff and Davie, 1962) but with the following modification. Elution of the barium sulfate was performed only once, with a volume of 10% (w/v) trisodium citrate dihydrate equal to one-fifth the volume of the oxalated serum adsorbed. This eluate was mixed with sufficient 10^{-1} M DFP in isopropanol to bring the DFP concentration to 10^{-3} M. After incubation at room temperature for 1 hour, during which time any traces of activated PTA were destroyed, the solution was divided into 2- to 3-ml aliquots, which were stored at −20°. One or more aliquots were thawed 8–16 hours prior to use, dialyzed at 4° against 0.15 M NaCl, and centrifuged for 15 minutes at 35,000 × *g* to remove insoluble material.

This barium sulfate eluate was used without further purification as the source of Christmas factor in the present studies. The preparation, while depleted of prothrombin, contained significant amounts of Stuart factor and precursor of serum prothrombin conversion accelerator. These factors have previously been shown not to participate in the reaction under study (Ratnoff and Davie, 1962).

Partially purified activated PTA was prepared as previously described (Ratnoff and Davie, 1962). It was assayed by its ability to shorten the clotting time of normal platelet-deficient plasma in silicone tubes, using a procedure similar to that previously described for activated Christmas factor (Ratnoff and Davie, 1962). The following modifications were employed. Platelet deficient normal plasma was substituted for Christmas factor-deficient plasma; Centrolex-P was substituted for crude rabbit brain cephalin; and the calcium chloride was 0.025 M rather than 0.05 M. A standard calibration curve relating arbitrary units of activated PTA to clotting time on a log-log scale was set up each day. With this technique it is not possible to determine whether PTA which was not yet activated contaminated the preparation. The clotting factor had been previously identified as PTA employing PTA-deficient plasma and other deficient plasmas (Ratnoff and Davie, 1962).

Activated Christmas factor was also assayed by its ability to shorten the clotting time of normal platelet-deficient plasma in silicone tubes. Identification of the activated product as activated Christmas factor was previously established by Ratnoff and Davie (1962) employing Christmas factor–deficient plasma.

Further purification of activated PTA was accomplished by gradient-elution chromatography on CM-cellulose. A typical preparation is presented below; all operations were carried out at 0–4°.

Partially purified activated PTA prepared from 2 liters of human serum (Ratnoff and Davie, 1962) was dissolved in about 10 ml of distilled water and dialyzed overnight against 1 liter of acetate buffer. The resulting solution contained 13.5 ml with a protein concentration of 11.3 mg/ml. A 6.5-ml aliquot was applied to each of two CM-cellulose columns, which had previously been equilibrated with acetate buffer. Each of these columns had been made from 15 g of CM-

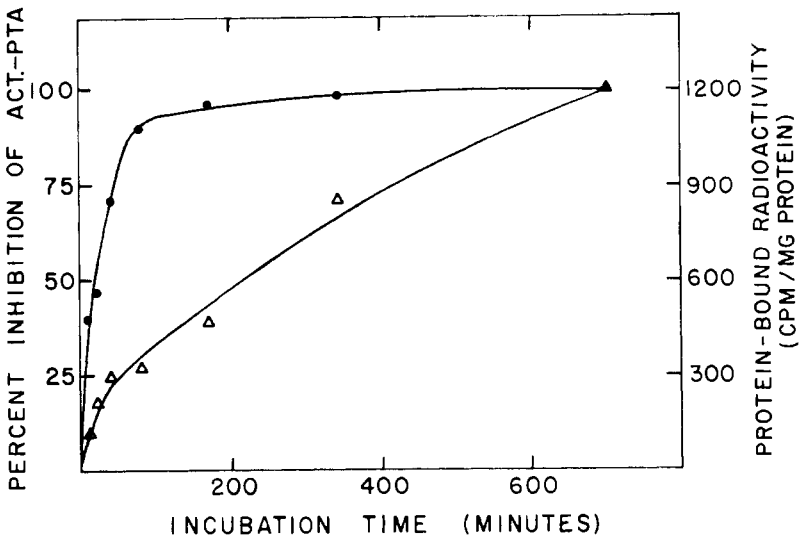

FIG. 1.—Time course of the reaction between DFP^{32} and partially purified activated PTA. Partially purified activated PTA (0.95 ml; 6.6 mg protein/ml in acetate buffer) was mixed with 50 μl of DFP^{32} and incubated at room temperature. The final concentration of DFP was 2.7×10^{-4} M. At various intervals samples were removed for assays of protein-bound radioactivity and samples were diluted and assayed for enzymatic activity. Radioactivity was expressed as counts per minute per mg of protein. Per cent inhibition of enzymatic activity was calculated assuming no inhibition at zero time. Both enzymatic assays and assays for radioactivity were run in duplicate. ●—●, per cent inhibition of enzyme; Δ—Δ, protein-bound radioactivity.

cellulose powder, which was washed three times by decantation with acetate buffer and packed into a column 1.6 × 50 cm. When the sample was completely applied each column was layered with 5 ml of acetate buffer, and the protein was eluted with a gradient solution established by placing 200 ml of acetate buffer in the mixing chamber and 200 ml of phosphate-saline buffer in the reservoir. In the procedure employed the volumes in the mixing chamber and reservoir were variable but proportional (Long, 1961). The eluting solution thus obtained was pumped through each column at a constant rate of 50 ml/hour; 5.0-ml fractions were collected every 6 minutes. The optical density of each fraction at 280 mμ was recorded; single assays for activated clotting-factor activity were performed immediately on a 1:10 dilution of each fraction in barbital-saline buffer, and the clotting times were recorded. The aliquot of the starting solution which had not been applied to the columns was then assayed for activated clotting-factor activity and a standard curve was constructed as previously described. From this standard curve arbitrary units/ml of activated PTA were calculated for each column fraction.

When the clotting assays were completed, the column fractions containing 10% or more of the total enzymatic activity were combined. The enzyme was immediately precipitated by addition of solid ammonium sulfate to 70% saturation. After the resulting suspension was stirred for 15 minutes, the precipitate was collected by centrifugation at 35,000 × *g* for 20 minutes. The supernatant was discarded and the precipitate was frozen at −20°. There was no detectable loss of activity on storage for several months as a frozen ammonium sulfate precipitate.

The ammonium sulfate precipitate was dissolved in acetate buffer and dialyzed against 1 liter of acetate buffer overnight just prior to use. This solution, which contained 1–3 mg protein/ml, was completely stable at 4° for a period of up to 4 weeks. Preliminary experiments indicated that the enzyme was unstable in solution at concentrations much lower than these, and was certainly less stable in the *p*H range of 7–8 at concentrations below 0.5 mg/ml.

This preparation was used for all the experiments in the present study, unless otherwise indicated. Its specificity was confirmed by testing its capacity to correct the defect of PTA-deficient plasma and its capacity to activate Christmas factor using the specific assays described previously (Ratnoff and Davie, 1962). It should be emphasized, however, that this preparation of activated PTA was still impure. Preliminary studies have recently shown that further purification can be achieved by column chromatography on hydroxyl apatite.

Preparation of DIP^{32}-activated PTA.—Activated PTA was dissolved in 5.0 ml of distilled water and dialyzed overnight at 4° against acetate buffer. The resulting solution, which had a protein concentration of 4.2 mg/ml, was incubated at room temperature with 50 μl of DFP^{32} solution. The final concentration of DFP was 5.4×10^{-5} M. The progress of the reaction was followed as described in sections I and II, and strict parallelism between counts incorporated and per cent inhibition was observed (see section II). At 450 minutes, when the inhibition was 96%, the reaction was terminated by chilling the solution to zero degrees and adding 2.9 g of solid ammonium sulfate to the remaining 4.5 ml of solution. This gave a final concentration of 70% saturation with ammonium sulfate. After standing 15 minutes at 0°, the suspension was centrifuged at 30,000 × *g* for 15 minutes to collect the precipitate. The supernatant was discarded and the precipitate was dissolved in 2.5 ml of distilled water. This solution was dialyzed for 3 days against three 1-liter batches of distilled water. The third dialysate had no detectable radioactivity after 16 hours of dialysis. The protein solution was removed from the dialysis tubing and centrifuged for 5 minutes at 3500 × *g* to remove a small amount of nonradioactive denatured protein which formed during dialysis. The supernatant, 2.5 ml in all, contained 102,500 cpm. This material, referred to as DIP^{32}-activated PTA, was used in both the subsequent degradation experiments.

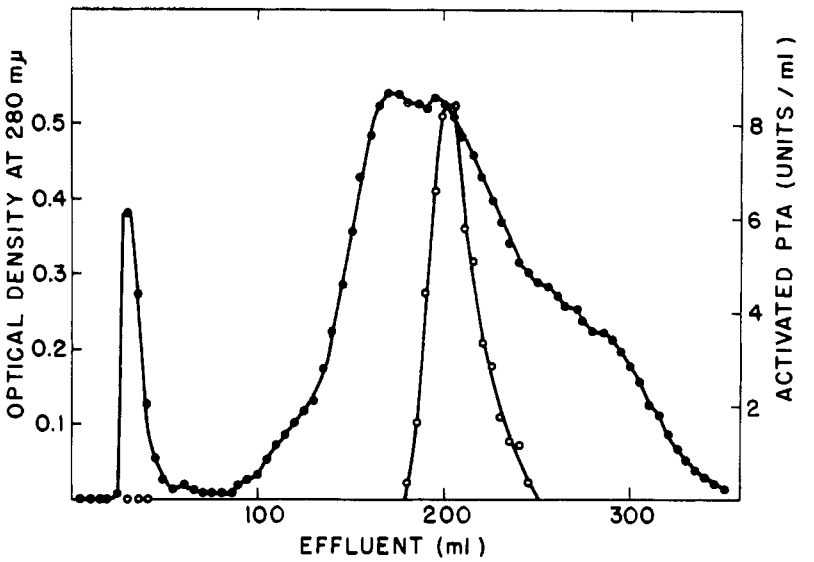

FIG. 2.—Gradient-elution chromatography of activated PTA on CM-cellulose. ●—●, protein; O—O, enzymatic activity. See Materials and Methods for details.

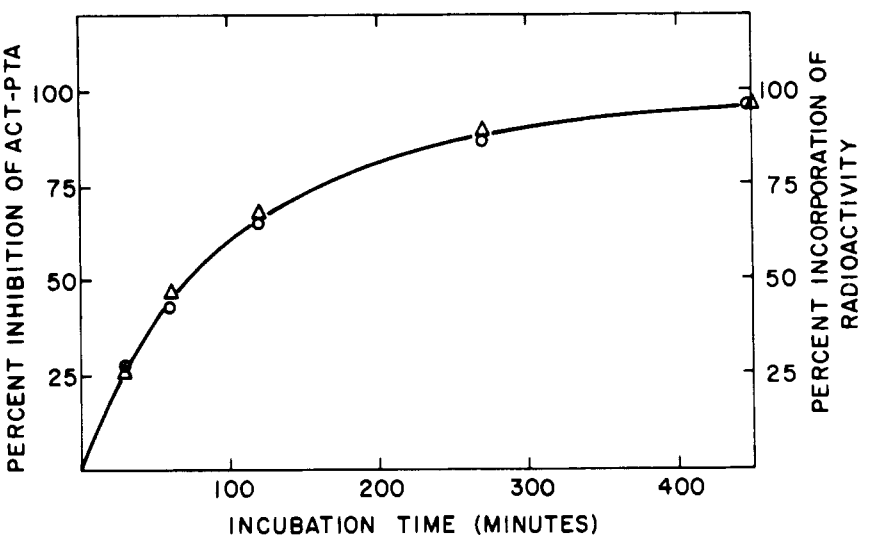

FIG. 3.—Time course of the reaction between DFP^{32} and further purified activated PTA. Further-purified activated PTA (0.99 ml; 4.2 mg protein/ml in acetate buffer) was mixed with 10 μl of DFP^{32} and incubated at room temperature. The final concentration of DFP was 5.4×10^{-5} M. Assays and calculations were performed as described under Fig. 1. O—O, per cent inhibition of enzyme; Δ—Δ, per cent incorporation of radioactivity.

DIP^{32}-trypsin, DIP^{32}-thrombin, and DIP^{32}-subtilisin were prepared from commercial preparations of the enzymes and DFP^{32} in the manner described for DIP^{32}-activated PTA.

P^{32}-phosphorylase a was prepared from ATP^{32} and phosphorylase *b* (Fischer *et al.*, 1959), and was generously provided by Dr. Edwin G. Krebs.

RESULTS

I. Time-Course of the Reaction between DFP^{32} and Partially Purified Activated PTA.—The reaction between partially purified activated PTA and DFP^{32} was followed with respect to time, in an attempt to ascertain whether all the radioactivity being bound to protein was actually being bound to activated PTA. Results of a typical experiment are shown in Figure 1.

If one assumes that the only binding of radioactivity was to activated PTA and that the covalent binding of the diisopropyl group to the enzyme resulted in complete loss of enzymatic activity, then the two curves should be parallel; they were not. In fact, it is evident from Figure 1 that additional binding of DFP^{32} to protein was taking place long after the enzyme was completely inhibited.

A control experiment with propylene glycol in place of DFP^{32} indicated that the activated PTA was completely stable under the present experimental conditions. Variation of reaction conditions, especially *p*H and DFP concentration, indicated that at higher *p*H values and higher DFP concentrations a nonspecific binding of radioactivity to proteins such as bovine plasma albumin was possible. Such a reaction, probably between DFP and the phenolic groups of tyrosine residues, has been described for bovine albumin and horse hemoglobin (Jandorf *et al.*, 1955). However, changing reaction conditions did not affect the basic findings as given above, and the conditions listed above were used in subsequent experiments with the exception that the DFP concentration was decreased one-fifth to minimize nonspecific binding.

II. Gradient-Elution Chromatography of Activated PTA.—Since DFP^{32} was binding to activated PTA as well as to other proteins, further purification was

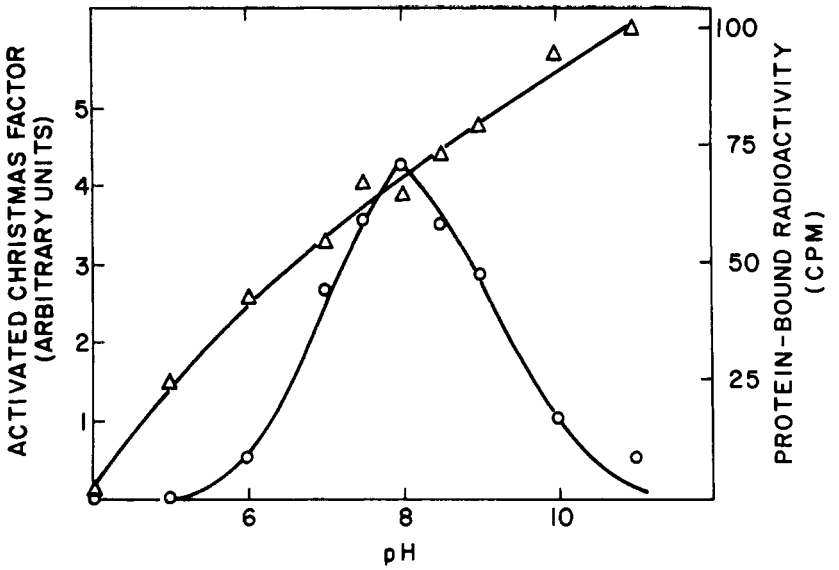

FIG. 4.—Effect of *p*H on the reaction between DFP^{32} and activated PTA and Christmas factor and activated PTA. (1) DFP^{32} and activated PTA: Activated PTA was dissolved in 1 ml of distilled water and dialyzed for 2 hours at 4° against 1 liter of 0.15 M NaCl. Twelve-hundredths ml of this solution (1.2 mg/ml) was pipetted into several tubes containing 0.10 ml of mixed buffer at various *p*H values; 10 μl of DFP^{32} was then added to each tube; the final concentration of DFP was 2.3×10^{-4} M. Fifty-μl aliquots taken in duplicate were assayed at zero time and at 30 minutes for protein-bound radioactivity. Duplicates were averaged, and zero-time values were subtracted from 30-minute values. Δ—Δ, radioactivity. (2) The activation of Christmas factor by activated PTA: Two-tenths ml Christmas factor (3.8 mg protein/ml), 0.1 ml activated PTA (0.22 mg protein/ml in 0.15 M NaCl), 0.1 ml mixed buffer, and 0.1 ml 0.025 M $CaCl_2$ were incubated in a siliconized test tube at 37°. At zero time and 5 minutes, 0.1-ml aliquots were removed and added to 0.9 ml of ice-cold barbital-saline buffer. The diluted samples were assayed immediately for activated Christmas factor. The amount of activated Christmas factor generated in 5 minutes was then calculated for the various *p*H values. O—O, activated Christmas factor.

necessary. Figure 2 shows the column chromatography of partially purified activated PTA on carboxymethylcellulose. This procedure consistently produced an additional purification of 2- to 4-fold with a yield of 60–80%.

A time curve for the reaction of DFP^{32} with further purified activated PTA is shown in Figure 3. In this case, per cent incorporation of radioactivity and per cent inhibition of enzyme were parallel. The contaminating protein which was binding DFP had been removed by gradient-elution chromatography on CM-cellulose, and apparently was not a potent activated clotting factor, since it was not detected by the enzyme assay as performed on 1:10 dilutions. The exact parallelism between enzyme inhibition and DFP binding provides strong evidence that the DFP^{32} is being bound only to activated PTA under these conditions. The possibility of a significant amount of DFP binding to a second protein at a rate identical to the inhibition of activated PTA seems unlikely. *For all subsequent experiments reported, the further-purified preparation of activated PTA was employed.*

The contaminating protein present in the partially purified activated PTA was found to be present in the first protein peak and is referred to subsequently as *fraction A*. (Figure 2). Although this fraction reacted with DFP^{32} at a rate slower than did activated PTA, it accounted for a major portion of the radioactivity bound to protein in the partially purified preparation of activated PTA.

III. Esterase Activity of Activated PTA.—The reaction of activated PTA with DFP suggested that this enzyme might also possess esterase activity. Table I shows the esterase activity of activated PTA when tested against TAME and BAEE. For comparison, three other DFP-sensitive enzymes, thrombin, trypsin, and fraction A are included. It can be seen that activated PTA has substantial esterase activity toward both TAME and BAEE. The ratio of the activities for the two substrates is different from that found for thrombin, trypsin, and fraction A. However, these experiments do not provide final proof for the identity of esterase activity and activated PTA since the enzyme preparation employed is not chromatographically homogenous.

TABLE I

ESTERASE ACTIVITY OF ACTIVATED PTA AND SEVERAL OTHER ENZYMES[a]

Enzyme	Esterase Activity		Ratio of Esterase Activity TAME: BAEE
	TAME (μmoles/min/mg)	BAEE (μmoles/min/mg)	
Activated PTA	0.78	0.10	7.8
Thrombin	2.3	0.98	2.4
Trypsin	142.5	25.5	5.6
Fraction A	2.6	1.7	1.5

[a] All four enzymes were made up in solutions of about the same protein concentration (3–5 mg/ml) and assayed for esterase activity in the automatic titrimeter, under identical conditions, using BAEE and TAME as substrates. Specific activities were calculated, and expressed as μmoles of ester hydrolyzed/min/mg.

IV. Effect of pH on the Reaction between DFP^{32} and Activated PTA and Christmas Factor and Activated PTA.—The *p*H dependence of the initial rate of the reaction between DFP^{32} and activated PTA is shown in Figure 4. It can be seen that the reaction with DFP^{32} increases at the higher *p*H values. This binding at the higher *p*H may be due in part to a nonspecific reaction with several residues in the enzyme such as

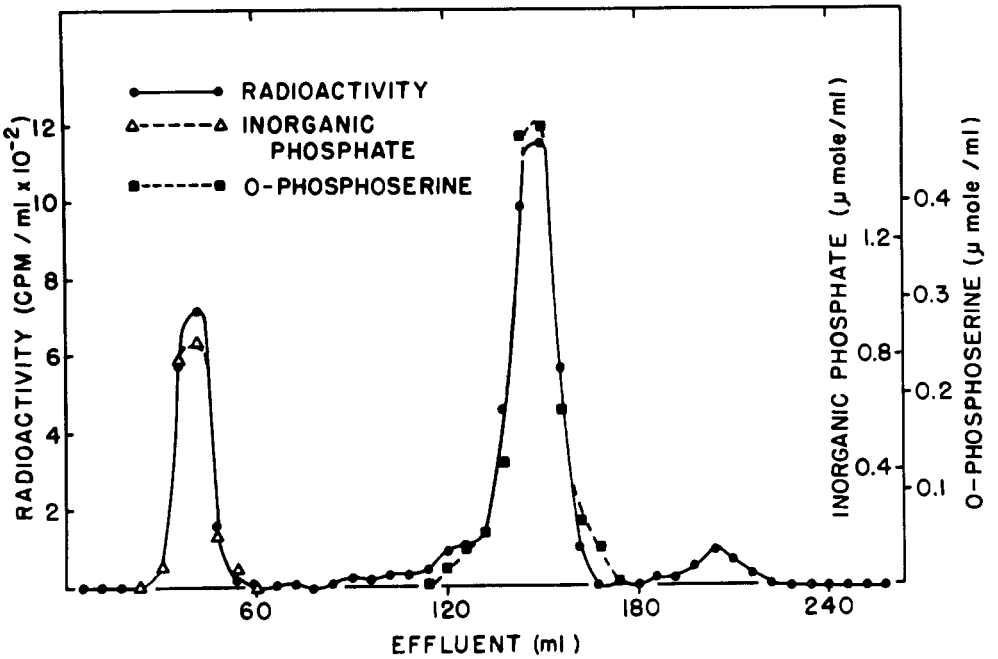

FIG. 5.—Dowex-50 chromatogram of a total acid hydrolysate of DIP^{32}-activated PTA.

serine and tyrosine. The *p*H dependence of the activation of Christmas factor by activated PTA is also shown in Figure 4. The latter experiments were carried out as previously described (Ratnoff and Davie, 1962) employing initial reaction rates. For Christmas factor, which is the physiological substrate for activated PTA, the *p*H optimum is 8.0.

V. Effect of Calcium and Heparin on the Reaction between DFP^{32} and Activated PTA.—Since calcium ion is required for the action of activated PTA on Christmas factor, and since heparin inhibits this reaction, an experiment was performed to test the effect of these substances on the incorporation of DFP^{32} into activated PTA. The results are presented in Table II. The experiment with EDTA was performed in order to exclude trace contamination with calcium ions; the concentration of EDTA employed completely inhibited the activation of Christmas factor by activated PTA, even in the presence of the normal amount of added $CaCl_2$. The results demonstrate that calcium ion is not necessary for the reaction between activated PTA and DFP^{32}. This suggests that the action of calcium is not at the active center of activated PTA, but rather that it affects the interaction between Christmas factor and activated PTA, perhaps by affecting the configuration of Christmas factor. It is also possible that calcium acts on the conversion of the enzyme-substrate complex to enzyme and product. In this case calcium would not be expected to have an effect on the reaction between DFP^{32} and activated PTA. Bergsagel (1955) has suggested that calcium ion is bound to activated Christmas factor and is an integral part of it. The present studies do not eliminate this possibility.

Heparin inhibits the reaction between activated PTA and DFP^{32}, although concentrations higher than those producing inhibition of the reaction with Christmas factor are required. This difference in concentration required could be due to the difference in *p*H between the two experiments, or to the difference in concentration of activated PTA. An influence of concentration of activated PTA on the effectiveness of heparin as an inhibitor has been noted (Kingdon and Davie, unpublished results). In this regard it is interesting to attempt to estimate the molar ratio of heparin and activated PTA in these experiments. Assuming that the data supplied by the manufacturer with the DFP^{32} were approximately correct, the average value of 118 cpm/50-μl aliquot in the three control experiments (Table II) represents 2.6 μμmoles of DFP^{32} bound to protein. This would be equivalent to 2.6 μμmoles of DFP-binding sites on activated PTA per 50-μl aliquot. Since these samples were withdrawn at 30 minutes, and since the reaction is 25–33% complete at 30 minutes (see Figure 3), this represents a total amount of DFP-binding sites in activated PTA of about 8–10 μμmoles/50 μl aliquot.

Assuming a molecular weight of 16,600 for heparin (Jensen *et al.*, 1948), a concentration of 0.25 unit/ml (1.7 μg/ml) represents 5 μμmoles of heparin/50 μl aliquot, and a concentration of 1.0 unit/ml represents

TABLE II

EFFECT OF HEPARIN AND CALCIUM ON THE REACTION BETWEEN DFP^{32} AND ACTIVATED PTA[a]

Final Concentration of Additive			Radioactivity (cpm/50 μl aliquot; 30 min)
Heparin (units/ml)	$CaCl_2$ (M)	EDTA (M)	
—	—	—	121
0.05	—	—	129
0.25	—	—	101
1.00	—	—	43
—	—	—	119
—	2×10^{-3}	—	108
—	5×10^{-3}	—	110
—	1×10^{-2}	—	115
—	—	—	115
—	—	1×10^{-2}	94

[a] Activated PTA was taken up in 1 ml of distilled water, and dialyzed overnight at 4° against 1 liter of acetate buffer; 0.12 ml of this solution (1.9 mg/ml) plus 0.10 ml of either acetate buffer alone, or buffer containing $CaCl_2$, heparin, or EDTA were pipetted into each of 10 small test tubes. To these mixtures was added 10 μl of DFP^{32}; the final concentration of DFP was 2.3×10^{-4} M. Fifty-μl aliquots taken in duplicate were assayed at zero time and at 30 minutes for protein-bound radioactivity. Incubation was at room temperature. The binding of radioactivity at zero time was less than 7 cpm for all samples.

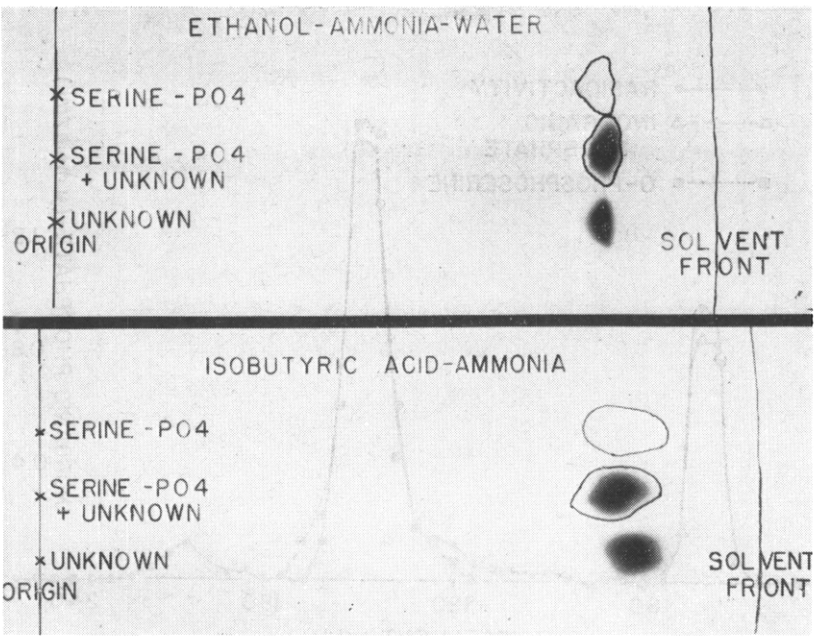

FIG. 6.—Paper chromatography of radioactive *O*-phosphoserine isolated from a total acid hydrolysate of DIP^{32}-activated PTA. The solvents employed were ethanol–concentrated ammonia–water (36:3:61) and isobutyric acid–1.0 M ammonia (3:5). The X-ray film obtained by radioautography was photographed directly after being lettered with India ink. The ink circles show the location on the paper of ninhydrin-positive material.

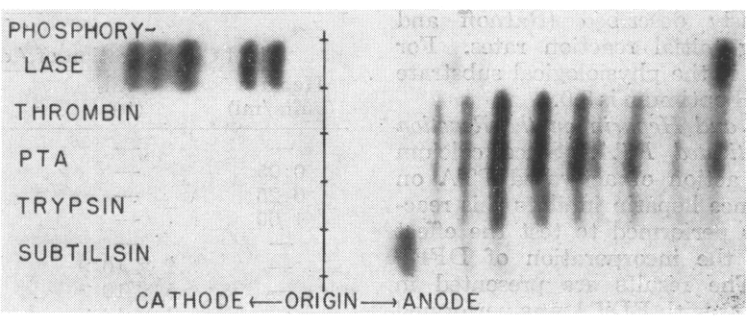

FIG. 7.—Paper electrophoresis of a partial acid hydrolysate of DIP^{32}-activated PTA and other P^{32}-labeled enzymes. An amount of each radioactive protein containing 8,000 cpm was subjected to hydrolysis in 6 N HCl at 100° for 25 minutes. After removal of HCl, the samples were placed *in toto* in 1-in. bands immediately adjacent to each other on Whatman No. 3 filter paper, and subjected to electrophoresis at *p*H 3.6, at 2 kv, for 65 minutes. The X-ray film obtained by radioautography was photographed directly, after being lettered with India ink.

20 μμmoles. Since the latter concentration of heparin significantly inhibited the reaction of DFP^{32} with 10 μμmoles of activated PTA, this suggests that heparin is bound strongly to activated PTA, perhaps in a 1:1 molar ratio. Recently, Laurent (1961) has calculated the molecular weight for several heparin fractions ranging from 7,600 to 11,800. Employing the lower molecular weight would indicate a ratio of 3 or 4 to 1 for heparin to activated PTA.

VI. The Isolation of O-Phosphoserine from Total Acid Hydrolysates of DIP^{32}-activated PTA.—Two ml of DIP^{32}-activated PTA, prepared as described under Materials and Methods, was subjected to total acid hydrolysis and Dowex-50 chromatography by the same procedure used by Schaffer *et al.* (1953) for chymotrypsin. Half the digest was chromatographed without carrier and half was chromatographed in the presence of 10 μmoles each of *O*-phosphoserine and inorganic phosphate. The results of the latter column are shown in Figure 5. Twenty-nine per cent of the applied radioactivity was recovered in the first peak, which corresponded to the carrier inorganic phosphate; 69% of the radioactivity was recovered in the second peak, which corresponded to the carrier *O*-phosphoserine. The recovery of carrier inorganic phosphate was 105%, and the recovery of carrier *O*-phosphoserine was 92%.

The pattern of radioactivity was the same in the case of the column without carrier. The radioactive fractions from the latter column which corresponded to *O*-phosphoserine were pooled and taken to dryness in a rotating evaporator. This material was then subjected to paper chromatography in four solvent systems, and was shown to be identical with *O*-phosphoserine in each case. Representative chromatograms for two solvent systems are presented in Figure 6.

This experiment demonstrated that the diisopropylphosphoryl group is bound to a serine residue in DIP^{32}-activated PTA.

VII. Partial Acid Hydrolysis of DIP^{32}-activated PTA and Other DIP^{32} Enzymes.—These experiments were performed essentially by the procedure of Hartley *et al.* (1959). A typical radioautogram of the electrophoretic pattern for the partial acid hydrolysates of several enzymes is shown in Figure 7. It can be seen that DIP^{32}-activated PTA yielded exactly the same family of peptides as did DIP^{32}-trypsin and DIP^{32}-

thrombin. DIP^{32}-subtilisin and P^{32}-phosphorylase *a* yielded different families of peptides. The latter two enzymes have been shown to differ in their P^{32}-binding site from the trypsin family of enzymes. These results indicate that activated PTA has the same DFP-binding site as that found in trypsin and thrombin, that is, gly-asp-ser-gly. The term "DFP-binding site" refers to the amino acid residues immediately adjacent to the reactive serine. Differences in amino acid sequence which are slightly more distant from the reactive serine on the peptide chain, and which might account for the differences in specificity of the enzyme, are not excluded by the technique employed.

Discussion

The crude activated PTA used in the present studies was found to be grossly contaminated with a second protein (fraction A) which readily bound DFP^{32}. In a time reaction, the binding of DFP^{32} was parallel to the inhibition of clotting activity only after the contaminating esterase was removed by further purification. This parallel in DFP binding and inhibition of clotting activity provides strong evidence that the DFP binding occurred only to the clotting enzyme. The inhibition of the DFP^{32}-binding reaction by heparin is also consistent with this conclusion. The problem of DFP^{32} purity, however, was not examined in the present experiments. Striking differences have been reported in the effectiveness of different commercial preparations of DFP as enzyme inhibitors (Ebata *et al.*, 1962, Gould *et al.*, 1963). Thus, it is possible that some inhibition or binding of radioactivity in the present experiments is due to minor contaminants present in the DFP^{32}.

The reaction of activated PTA with DFP and its apparent esterase activity strongly suggest that the physiological role of activated PTA in blood clotting may be the activation of Christmas factor by partial proteolysis with the liberation of peptide material. Similar mechanisms are well known for the activation of the digestive enzymes and for the conversion of fibrinogen to fibrin by thrombin.

The preliminary calculations relating DFP-binding sites on activated PTA to heparin concentration suggest further investigations. The inhibition by heparin of the reaction between activated PTA and DFP demonstrates that heparin masks the DFP-binding site of activated PTA. Thus, it appears probable that heparin plays a similar role in blocking the activation of Christmas factor by activated PTA. Experiments are now in progress to test this possibility.

In the present experiments the binding site of DFP^{32} was examined by the partial acid-hydrolysis technique introduced by Hartley *et al.* (1959). This technique was chosen for its sensitivity, which was necessary in working with activated PTA. One entire purified preparation of activated PTA from 2 liters of human serum contained only 1–3 mμmoles of enzyme, as judged by the amount of DFP^{32} bound. Thus, peptide fractionation and direct amino acid analysis by conventional procedures were not feasible.

The partial acid-hydrolysis technique has been shown to be sensitive enough to detect as subtle a difference as that between horse liver aliesterase (gly-glu-ser-ala) and chymotrypsin (gly-asp-ser-gly) (Sanger and Shaw, 1960). Since this technique was successful in demonstrating very small differences in the DFP-binding sites of these enzymes, as well as the similarities between trypsin and thrombin, it appears most probable that activated PTA has the same DFP-binding site as that found in trypsin (Dixon *et al.*, 1958) and thrombin (Gladner and Laki, 1958), that is, gly-asp-ser-gly. Confirmation of this finding, however, will require larger amounts of enzyme which will yield adequate amounts of peptides for direct amino acid analysis. The peptides containing a reactive serine group in a number of enzymes have recently been tabulated (Milstein and Sanger, 1961, Schwartz *et al.*, 1963).

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