A Study of the Preparation and Anticoagulant Properties of Some Phosphorylated Proteins¹

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Interest in blood anticoagulants has increased since 1946 when the American Heart Association appointed a committee to evaluate the use of anticoagulants in the treatment of coronary thrombosis, which causes about 200,000 deaths annually. A report of this committee showed that the death rate of individuals treated with anticoagulants was significantly lower than for a control group which did not receive such treatment.

The normal anticoagulant present in the blood is heparin. This compound as well as dicoumarol are used clinically in the treatment of coronary thrombosis, but both have distinct limitations. Heparin is expensive, is not very effective unless given intravenously and its anticoagulant effect lasts for only a few hours necessitating continual injections. Dicoumarol can be given orally but there are several serious disadvantages in its use, such as delayed action, persistence of its action for several days which can be relieved only by transfusion, and capillary dilation which may result in undesirable bleeding. Because of the limitations of heparin and dicoumarol there has been extensive search for an inexpensive, nontoxic substance with anticoagulant action equal to heparin. Thus far no such anticoagulant has been found. Extensive testing of many compounds has indicated that some of the requirements of an anticoagulant are that it must be water soluble, have a high molecular weight and contain combined sulfuric acid or other acid groups of similar strength.

Certain sulfated proteins prepared by the methods of Reitz and coworkers (8) (9) using concentrated sulfuric acid and pyridine-chlorosulfonic acid were found by Reitz and Cords (7) to have anticoagulant activity equal to approximately 1/10 that of heparin. These sulfated derivatives were found to be relatively non-toxic so that it would be possible to use them clinically. Thus encouraged, it was decided to investigate the possible anticoagulant activities of phosphorylated proteins.

Preparation of Derivatives

Materials to be phosphorylated were first ground to pass through a 200 mesh sieve. The phosphorylation procedure was similar to that of Ferrel, et al. (1). The phosphorylating reagent was prepared by introducing 94 g. of phosphorus pentoxide into 125 g. of 85 per cent orthophosphoric acid and stirring until all of the oxide had dissolved. Phosphorylation was accomplished by slowly dusting 1 g. of the material into 100 g. of the cooled reagent and stirring until a smooth dispersion was obtained. The dispersion was then placed in a dessicator over phosphorus pentoxide and allowed to react at room temperature for 72 hours. Following the reaction period the very viscous liquid was diluted with cold

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distilled water and poured over cracked ice. This solution was neutralized to pH 7.0 with 10 normal sodium hydroxide, adding cracked ice to maintain the temperature below 5° C. Toluene was added to prevent bacterial action and the solution dialyzed in cellophane tubing against distilled water until no phosphate ion could be detected in the dialysate. This required from three to five days. After concentration of the solution in the dialysis tubes by hanging them in a current of warm air, they were dialyzed for five days against 10 per cent sodium chloride to remove loosely bound phosphorus. Following this the sodium chloride was removed by dialyzing against distilled water until the dialysate gave a negative test for chloride ion. At this stage of the preparation an insoluble portion was usually observed. This was removed by centrifugation and dried by washing with alcohol and ether. The soluble portion was concentrated to a small volume and lyophilized.

Very low yields were obtained when soybean protein, blood fibrin and lactalbumin were phosphorylated by the exact procedure of Ferrel. The values shown in Table I for these proteins were obtained by using only

TABLE I							
Yield, Composition and Anticoagulant Activity of							
Phosphorylated Materials							

Material	Nitrogen content % (a)	Moles of phosphate P per 10 ⁴ g. of orig. protein (b) (a)	Yield insoluble phos. deriv. %	Yield soluble phos. deriv. % (a)	Phosphate P. in sol. fraction % (a)	Anticoag. activity ACU/mg.
Phos. Zein	. 10.9	13.5	4.23	105.0	2.9	1.7
Orig. Zein	. 15.5			• • • •	• • •	
Phos. Gliadin	. 13.2	12.5	1.09	50.0	3.1	1.3
Orig. Gliadin	. 16.7	• • • •	• • • •	••••	•••	• • •
Albumin	. 12.1	13.3	0.54	34.0	3.5	0.7
Orig. Bovine Albumin	. 14.4		• • • •		•••	
Phos. Soybean Protein	. 9.6	21.0	13.00	29.8	4.5	1.0
Orig. Soybean	190					
Drog Dlood Fibri	. 10.0 	140	••••	40.9		1.0
Pros. Blood Fibri	0.0	14.9	5.04	40.0	2.4	1.0
Urig. Blood Fibri	n 16.2	••••	• • • •		••••	• • •
Phos. Lactalbum	n 12.8	9.3	6.44	27.2	2.7	1.3
Orig. Lactalbumi	n 13.4	• • • •			•••	•••
Phos. Casein (c)	. 15.6	26.3	28.50	28.6	7.6	1.7
Orig. Casein (d)	. 16.7	• • • •				• • •
Phos. Pectin	• •••	••••	16.2	96.0	3.5	1.3
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(a) Calculated on a moisture free basis

(b) Moles of phosphate phosphorus per 10⁴ g. of protein =

 $\frac{100 \times \text{per cent P in phos. deriv.} \times \text{per cent N in orig. protein}}{31 \times \text{per cent N in phos. deriv.}}$

(c) Prepared using phosphoric acid and urea

(d) Original casein contained 1.14 per cent P

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90 g. of phosphorylating reagent to one g. of protein. This change reduced the large amount of sodium phosphate formed during neutralization, shortened the time required for dialysis and possibly decreased the amount of protein hydrolysis, thus increasing the yield.

When the preparative work was almost completed a patent by Olcott and Mohammad (5) came to our attention. It described the phosphorylation of proteins by the use of phosphoric acid and urea. This method employed higher temperatures than Ferrel's method but much less phosphoric acid was required and the reaction time was reduced from 72 hours to 30 minutes. The phosphorylated casein listed in Table I was prepared by mixing 10 g. of the protein with 3 ml. of water for thorough wetting, then adding 3 g. of urea followed by only 2.5 ml. of phosphoric acid. The thick gel that resulted was heated at 140° C. for 30 minutes, then diluted with water, poured over cracked ice and neutralized to pH 7.5. Isolation of the product in this case required less time as much smaller quantities of salts needed to be removed by dialysis. The values for casein shown in Table I indicate that more phosphorus is introduced by this method but that the yield of insoluble material is more than twice as high as that obtained by the other method.

Analysis of Derivatives

Phosphorus content of the products was determined by the method of Fiske and Subbarow (2). Samples were digested with sulfuric acid, decolorized with hydrogen peroxide, diluted and boiled to decompose pyrophosphate and any residual hydrogen peroxide. The blue color that formed on addition of ammonium molybdate and 1,2,4-aminonaphtholsulfonic acid to known aliquots was allowed to develop 10 minutes and read in an Evelyn photoelectric colorimeter using a 660 millimicron filter.

The total nitrogen content of the derivatives was determined by a micro-Kjeldahl method described by Hawk, Oser and Summerson (3). Digestion of the phosphorylated derivatives was carried out by heating three hours with 5 ml. of ten normal sulfuric acid followed by decolorization with hydrogen peroxide. Use of catalysts was avoided because they interfered with subsequent phosphorus determinations.

The percent of nitrogen and phosphorus in the soluble derivatives is shown in Table 1. Phosphorus content of the soluble derivatives was also calculated on the basis of moles of phosphorus per 10^4 g. of the original protein. These values are more significant than the percentage figures alone in assessing the amount of phosphorus introduced since the nitrogen content of both the original and the phosphorylated derivatives is included in their calculation.

Assay of Anticoagulant Activity

The heparin assay method of Quick (6) was used because of its simplicity, convenience and because it had been used for similar studies (7). This method is based on the assumption that heparin forms an antithrombin with a component of the plasma and that the amount of the antithrombin can be quantitatively measured by determining the quantity of thrombin it will neutralize. The assumption that phosphorylated proteins act in the same manner as heparin seems justified since anticoagulants of similar structure have been shown to act by this mechanism. Thrombin Topical, manufactured by Parke-Davis, was used instead of the thrombin ordinarily prepared from bovine plasma. For purposes of comparison one anticoagulant unit (ACU) is defined as that amount of moisture free material which when added to freshly oxalated plasma will neutralize 0.1 ml. of full strength thrombin solution in 10 minutes at 37° C. A standard heparin solution containing 0.5 mg. per ml. was assayed to contain 100 ACU per mg. The results of the determinations of the anticoagulant activities of seven phosphorylated proteins as well as a carbohydrate, pectin, are assembled in Table I. It will be noted that none of the derivatives has an anticoagulant activity greater than about two per cent that of heparin. Corresponding soluble sulfated derivatives were found by Reitz and Cords (7) to have about ten per cent the anticoagulant activity of heparin. No adequate explanation has been found as to why the phosphorylated derivatives should have so much less anticoagulant activity than the corresponding sulfated derivatives. Similar results, however, were reported by Karrer et al. (4), who found that derivatives prepared by the phosphorylation of certain polysaccharides and gelatin had very low anticoagulant activity. They offered no explanation for the low activity. Possibly the presence of a phosphatase in plasma or the fact that a considerable portion of the phosphorus in the derivatives is present as metaphosphate may in part explain the results.

Summary

Phosphorylated derivatives of seven proteins and a carbohydrate were prepared. A phosphorylating reagent employing urea and phosphoric acid was found to be superior to one consisting of phosphoric acid containing excess phosphorus pentoxide. The anticoagulant activities of these derivatives was found to be about two per cent that of heparin.

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