been shown that the aglomerular renal tubule is permeable to a highly complex molecule such as tetracycline.

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Received May 29, 1963. P.S.E.B.M., 1963, v114.

Isolation of Human Plasma Prothrombin of High Specific Activity by Gel Filtration.* (28738)

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In previous studies(1), ultracentrifugal analyses of preparations of human plasma prothrombin of high specific activity indicated that the homogeneity of the protein as judged from the schlieren pattern was dependent upon the pH and ionic strength of the solvent. In addition, sedimentation velocity studies in a partition cell demonstrated that the activity of the zymogen sedimented at a rate greater than that of the majority of the protein. Since there was no distinct component in the schlieren pattern which could be attributed to the prothrombin activity, it was concluded that the specific activity of this zymogen must be considerably higher than previously suspected.

The present communication describes attempts further to fractionate these preparations by gel filtration, using solvent conditions in which the difference in molecular dimensions between the active zymogen and non-zymogen protein would be maximal and hence afford the most suitable conditions for fractionation and purification. The results obtained support previous speculations that human plasma prothrombin has a specific activity at least twice that of the bovine species.

Materials and methods. Prothrombin was isolated from freshly collected human ACD plasma by a procedure previously described (1). Prothrombin activity was measured by the 2-stage method of Ware and Seegers(2), using Lot 3B NIH thrombin as reference. Protein concentration was determined by the method of Daughaday *et al*(3), as well as by a micro-modification of the biuret method of Henry *et al*(4). Specific activity measurements are expressed in terms of NIH units/mg protein.

Sephadex G-75 (medium), G-100 and G-200 gel columns were prepared as outlined by Flodin(5). Column dimensions were 1.3 × 30 or 1.3 × 60 cm. As suggested by Flodin (5), effluent volumes are expressed as the elution volume (V_e); this is the volume of eluant required to move the peak concentration from the top to the bottom of the gel bed. This necessitated a correction for the volume contained in the drip point of the

* This investigation was supported in part by USPHS Research Grant from Nat. Heart Inst.

column, recording cuvette and tubing connecting the column to an automatic GME constant volume fraction collector. Flow rates were averaged over the entire elution pattern which was automatically scanned at 280 mμ and recorded on a Texas Instruments Recti/Riter with a chart speed of 6 in/hr. All column gel filtration experiments were performed at room temperature and 1 ml fractions of effluent were collected. Buffers employed were similar to those used previously (1) in which two-thirds of the ionic strength was contributed by NaCl and one-third by the buffer salt. In some experiments in which fractions emerging from the column were collected and lyophilized, 0.1 M NH_4HCO_3, pH 7.8 and 0.15 M ammonium acetate buffer, pH 6.0, were used because they are volatile. Routinely, 2 ml of a 5-20 mg/ml sample was filtered through the column using as eluant the same buffer with which the column had been equilibrated. Recoveries of prothrombin activity were calculated from the summation of all tubes collected and recovery of protein was checked by running several hold-up volumes of 0.1 N NaOH through the column.

Sedimentation velocity analyses were performed, as described previously(1), in a Spinco model E ultracentrifuge, except that 4 degree sector cells were used. Values of S_{20w} are expressed in Svedbergs corrected to water at 20°C. Solvent systems consisted of the same buffers as those used for the gel filtration experiments. In all instances, measurements were made on 1% protein solutions.

In some experiments crystalline bovine serum albumin (Armour #W69204) was subjected to gel filtration and sedimentation velocity analysis for purposes of comparison with the prothrombin preparations.

Results. In preliminary experiments, prothrombin preparations were filtered through columns of Sephadex G-75, G-100 and G-200, which had been equilibrated with buffers varying from pH 5.0 to 9.0, $\mu = 0.05$ to 0.15, to determine which, if any, gel resin afforded the greatest fractionation of components. The preparations emerged from G-75 and G-100 columns as broad single protein components. The elution volumes of the protein and prothrombin activity were almost identical, although the elution pattern of the activity was skewed toward the leading edge of the protein peak. Calculations indicated an approximate 10-15% increase of specific activity of the effluents and suggested that the preparations were heterogeneous under the conditions used. Similar results, but with a greater degree of purification, have been obtained by Mammen and Ramien(6) using bovine prothrombin preparations filtered on columns of Sephadex G-75 at pH 7.0 to 8.0.

Columns of G-200 equilibrated with buffers of pH 7.0 to 8.0 reveal 2 components (Fig. 1). The first component ($V_e = 12$ ml) comprises 5 to 10% of the original preparation and presumably is the rapidly sedimenting fraction seen in the schlieren pattern when these products are examined in the ultracentrifuge(1). The second protein peak ($V_e = 19$ ml) is broad with considerable trailing, and although it has the prothrombin activity associated with it, calculations of specific activity indicate that it contains nonprothrombin protein.

At pH 6.0 (Fig. 2), this major component splits into 2 fractions ($V_e = 20$ ml and 23 ml respectively), the activity being associated with the protein migrating more rapidly ($V_e = 20$ ml). Although the peak of the activity pattern ($V_e = 20$ ml) is far from completely separated from the slower moving component, the material has a specific activity of from 2 to 2.5 times the original unfiltered preparation (Table I). The degree of separation of the two components and the specific activity of the peak tubes is dependent to some extent on flow rate and column length (Table I, Exp. 2 to 4). There appears to be a minimum flow rate below which greater separation of components is not achieved (Table I, Exp. 5). The pattern of separation is identical, whether preparations of 20 mg/ml or 5 mg/ml are placed on the column or whether samples of the same preparation in solution are placed on the column 2 days apart after standing at room temperature. Preparations with lower specific activity separate on the

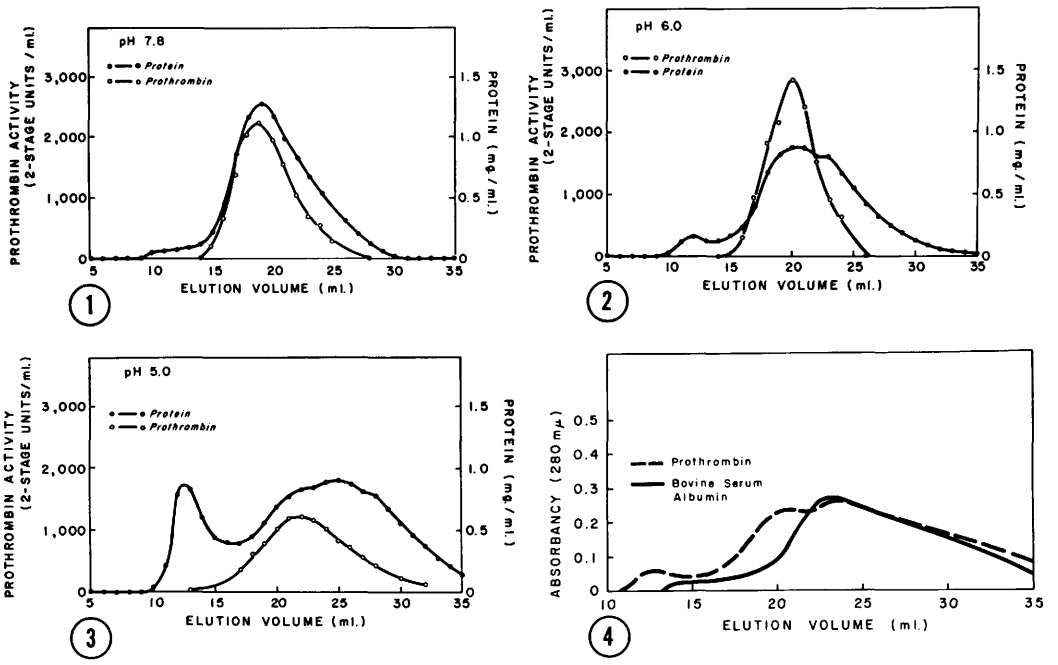

FIG. 1. Sephadex G-200 fractionation of a prothrombin sample at pH 7.8. A total of 14,580 N.I.H. units in 2 ml applied to 1.3 × 30 cm column. Initial specfic activity = 1300 N.I.H. units/mg protein. Sample #115.

FIG. 2. Sephadex G-200 fractionation of a prothrombin sample at pH 6.0. A total of 14,580 N.I.H. units in 2 ml applied to 1.3 × 30 cm column. Initial specific activity = 1300 N.I.H. units/mg protein. Sample #115.

FIG. 3. Sephadex G-200 fractionation of a prothrombin sample at pH 5.0. A total of 19,880 N.I.H. units in 2 ml applied to 1.3 × 30 cm column. Initial specfic activity = 1201 N.I.H. units/mg protein. Sample #176.

FIG. 4. Comparison of Sephadex G-200 fractionation of a human prothrombin preparation and crystalline bovine serum albumin at pH 6.0. Prothrombin sample #115.

column to the same or slightly greater extent than higher purity products (Table I, Exp. 6). In 2 experiments using columns equilibrated with ammonium acetate buffer, pH 6.0, the tubes representing the peaks of each of these components were pooled, lyophilized and refiltered on a second column. The elution volumes of each of the components were identical to the first column and no further separation of the peaks occurred.

At pH 5.0 (Fig. 3), there is some suggestion that these preparations might separate into 4 or more distinct peaks; however, the elution volumes of these peaks overlap to a considerable extent and in 2 experiments only 45 and 55% of the original prothrombin

TABLE I. Increase in Specific Activity of Some Human Prothrombin Preparations Following Gel Filtration at pH 6.0.

Exp No.	Preparation No.	Column dimensions (cm)	Flow rate (ml/hr)	Recovery of activity (%)	Specific activity (N.I.H. units/ml) Before*	After†
1	115	1.3 × 30	4	107	1300	3139
2	113	1.3 × 30	15	96	1370	2500
3	113	1.3 × 30	10	100	1370	2800
4	113	1.3 × 60	4	93	1370	3510
5	125	1.3 × 60	2	105	1462	2740
6	311	1.3 × 60	4	90	553	1820

* Specific activity of original preparation.
† Specific activity of tube at peak of activity curve.

activity could be recovered from the column. Hence, it is possible that such elution patterns under these conditions might represent degradation products of prothrombin, since 100% recovery of the protein was obtained.

Since it appeared that the best separation of components in this mixture was achieved at pH 6.0, certain aspects of these findings were explored further. In Fig. 4, the elution pattern of one prothrombin preparation is compared to that of crystalline bovine serum albumin under the same column conditions. By sedimentation velocity analysis at pH 6.0, this albumin preparation consisted of 10% of a component of $S_{20w} = 5.8$ and 90% of a component of $S_{20w} = 4.1$. These are presumably the dimer and monomer forms respectively. From the prothrombin analysis, it is seen that the major non-prothrombin component has an elution volume ($V_e = 24$ ml) almost identical to the albumin monomer ($V_e = 23$ ml) while the protein associated with the prothrombin activity has an elution volume ($V_e = 20$ ml) between that of the monomer ($V_e = 23$ ml) and dimer ($V_e = 16$ ml) of albumin. The elution volumes of the prothrombin components thus correlate quite favorably with their S_{20w} values by sedimentation velocity analysis(1) as well as with the S_{20w} values of the monomer and dimer forms of bovine serum albumin.

Discussion. Although we have been unable to completely separate prothrombin from the rest of the nonzymogen protein in these preparations, gel filtration offers an additional important analytical tool with which to assess the purity of these or any other prothrombin products. Previous studies have indicated that the original preparations are highly purified when examined by several physicochemical and immunochemical techniques(1). Under certain conditions, sedimentation velocity and diffusion measurements have indicated a high degree of homogeneity. It has been only with sedimentation measurements of activity and density gradient centrifugation that heterogeneity has been detected. Since the latter methods are extremely difficult and not readily available in most laboratories, gel filtration offers an extremely easy and practical analytical tool for evaluation of the purity of these preparations.

Fifteen preparations have been analyzed by column gel filtration. Five of these have been examined under column conditions considered most suitable to obtain material of the highest specific activity. The average of the highest specific activities obtained in these 5 experiments was 3200 ± 400 NIH units/mg of protein. Assuming that one NIH unit is equivalent to 1.25 Iowa units(7), this would correspond to a value of 4000 ± 500 Iowa units/mg protein, which is almost twice that of the best products obtained from the bovine species(8). These results support previous speculations in this regard(9), although it should be pointed out that considerable overlapping with non-prothrombin protein occurs in these elution patterns and the specific activity may be considerably higher than that recorded here.

Of particular interest is the fact that the highly active prothrombin isolated from these gel columns has been assayed by the Ware and Seegers 2-stage procedure unmodified by addition of clotting factors in the form of serum. In fact, when assayed by the modified 2-stage procedure of Goldstein *et al* (10), with added serum, the values were lower. Relatively small amounts of certain coagulation factors have been found in the original preparations (See(1) for details and discussion.) and presumably are located in certain fractions emerging from the column. The one-stage assays used to determine the presence of such factors in these products, however, give questionable results under these conditions because the prothrombin samples assayed contain 10- to 100-fold more prothrombin than is considered optimal for the tests.

Summary. Human plasma prothrombin has been subjected to gel filtration analysis and products having a specific activity at least twice that of bovine prothrombin have been obtained. Thus far, columns of Sephadex G-200 equilibrated with phosphate buffer pH 6.0, $\mu = 0.15$, at flow rates of about 4 ml/hr have achieved the best results. It is con-

cluded that gel filtration offers an extremely useful tool for assessing the degree of heterogeneity of these or similar products.

The expert technical assistance of Mr. John De Groot, the ultracentrifuge facilities of Prof. John W. Mehl, and the suggestions of Prof. Arnold G. Ware in preparing this manuscript are gratefully acknowledged.

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Received June 24, 1963. P.S.E.B.M., 1963, v114.

Renal Artery Response to Epinephrine.* (28739)

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Significant changes in the I^{131} radiorenogram occurred in both hypertensive patients and normal control subjects in association with signs and symptoms of apprehension. With relief of the subject's anxiety the renogram changes were no longer present. These renogram effects were considered secondary to an acute decrease in renal blood flow which was probably induced by a neurohumoral mechanism(1). This series of experiments was performed in an attempt to clarify the origin of this response.

Methods. Group A. Sixteen normal mongrel dogs weighing 12 to 14 kg were anesthetized with pentobarbital and standard control radiorenograms utilizing I^{131} Ortho-iodohippuric acid performed as previously reported(2). Seventy to 85 μg per kilogram of epinephrine (Parke, Davis and Co. ampoule #88, which does not contain any nor-epinephrine) were injected into the jugular vein and in one minute another renogram was recorded. A third renogram was recorded 30 minutes later.

This same procedure was followed in 3 dogs utilizing doses of epinephrine ranging from 7 to 75 μg per kilogram.

Group B. Thirteen dogs were anesthetized as above, and a #7 polyethylene catheter was placed in the aorta just above the origin of the renal arteries *via* a femoral artery cutdown. A control angiogram of the renal arteries and lower aorta was run with 12 ml of 75% hypaque injected at 200 lb pressure with an automatic injector. Serial films were obtained using a Schonander automatic film changer. The sequence of films varied from one per second to 6 per second for different periods. Seventy-five to 85 μg per kilogram of epinephrine were injected into the jugular vein and a repeat angiogram was run one minute later. Utilizing a similar technique, cineradiographic studies of the renal arteries and lower aorta were made in 10 dogs both before and after adrenaline injection.

Results. Group A. Epinephrine injection of 75 to 85 μg per kilogram was uniformly associated with a change in the radiorenogram consisting of a slowed ascent of the vascular (OA) and secretory (AB) and slowed descent of the excretory (BC) components. This is illustrated in Fig. 1. The smaller epinephrine doses caused less apparent but still significant changes in the reno-

* Supported by grants from Nat. Heart Inst., N.I.H., Bethesda, Md., and Duke University Research Council, Durham, N. C.