viously not possible to specify where this conjugated metabolite was formed, the presence of the 3β-sulfoxy-5-ene grouping in this compound as well as in pregnenolone sulfate, 17α-hydroxypregnenolone sulfate, and dehydroisoandrosterone sulfate merits attention.

The possible metabolic role that steroid sulfates may play in biosynthetic processes has already been discussed in our previous paper (Calvin *et al.*, 1963).

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Purification and Properties of Bovine Factor V: A Change of Molecular Size During Blood Coagulation*

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Received September 9, 1963

The preparation of purified factor V from bovine plasma and bovine serum was achieved through chromatography on Sephadex G-200. Evidence was presented in support of a molecular weight and activity difference between factors V from the two sources. Factor V from plasma was eluted quantitatively as a single peak with a distribution coefficient of 0.05, while factor V from serum was eluted in two separate fractions, one with a distribution coefficient of 0.05 and the other with a distribution coefficient of 0.18. This latter component of activity was present to a significantly greater amount in two-day-old serum than in fresh serum. Density-gradient centrifugation confirmed that the factor V activity from serum with a distribution coefficient of 0.18 had a smaller molecular weight than plasma factor V. Factor V with a distribution coefficient of 0.18 and an apparently smaller molecular weight could also be obtained from purified plasma factor V by treatment with thrombin. This latter technique provided a facile way to the purification of thrombin-activated factor V and resulted in a tenfold increase in activity. It was concluded that thrombin, as a proteolytic enzyme, split the molecule of factor V with the production of a new factor V with a smaller molecular weight.

The classical theory of blood coagulation was formulated at the beginning of the century (Morawitz, 1905) and described the interaction of four factors (thromboplastin, calcium, prothrombin, fibrinogen) with the resultant formation of insoluble fibrin. The first addition to the above four factors was factor V,[1] which was described under different names by three independent groups.

Quick (1943) recognized that there existed another factor necessary for the rapid activation of prothrombin by tissue extract; it was named "labile factor" because of its disappearance in stored oxalated plasma. Later, Owren (1947) published his work based on the discovery of a patient with a hemorrhagic state, but lacking none of the then-recognized clotting factors. He consequently postulated the existence of an additional factor necessary for the activation of prothrombin by tissue extracts, which he initially termed "factor V" and later "proaccelerin" (1950). At approximately the same time, Ware *et al.* (1947a) reported on a substance in bovine plasma which could accelerate the activation of prothrombin in the presence of thromboplastin and calcium, and which they termed

* This work was supported by grants from Life Insurance Medical Research Fund, American Cancer Society, and National Heart Institute, U. S. Public Health Service. This communication is part of a dissertation submitted by Demetrios Papahadjopoulos to the Graduate School of the University of Washington in partial fulfillment of the requirements of the Ph.D. degree.

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[1] This name has been adopted by the International Committee on Nomenclature of Blood Clotting Factors (*J. Am. Med. Assoc. 170*, 325, 1959).

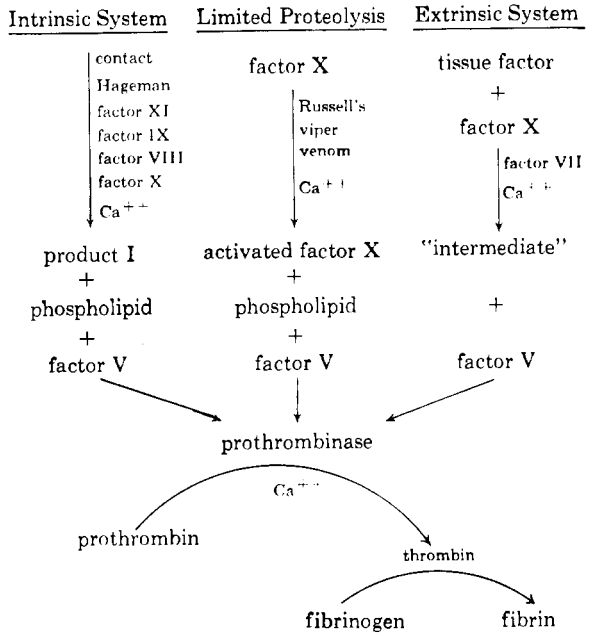

FIG. 1.—Diagrammatic representation of three reaction sequences leading to the activation of prothrombin and eventual formation of fibrin (clot).

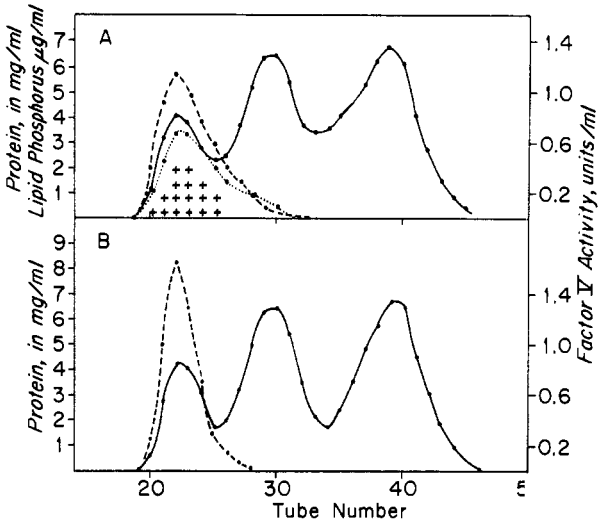

FIG. 2.—Chromatography of plasma fractions on Sephadex G-200. (A) $BaSO_4$-treated bovine plasma, eluted with buffer containing 0.14 M NaCl and 0.01 M Tris-HCl, *p*H 7.35. (B) $BaSO_4$-treated bovine plasma, eluted with buffer containing 0.72 M NaCl and 0.05 M Tris-HCl, *p*H 7.35. Effluent volume in each tube, 3.0 ml.; column volume, 160 ml. Total protein (———), factor V activity (– – –), lipid phosphorus (·····), fibrinogen (+++).

"accelerator globulin" (Ac-globulin). Subsequently a report from the same laboratory (Ware *et al.*, 1947b) noted that a comparable substance isolated from bovine serum was a better accelerator. These investigators were of the opinion that this substance was produced by the action of thrombin on bovine plasma Ac-globulin. Later Ware and Seegers published a more detailed account of the nature and properties of bovine plasma Ac-globulin (Ware and Seegers, 1948a) and showed that the addition of thrombin to bovine plasma produced serum Ac-globulin (Ware and Seegers, 1948b).

Although human serum contains only trace amounts of factor V,[2] Lewis and Ware (1954) showed that human plasma factor V can be activated by thrombin. The activated factor V was found to be much more stable when purified preparations of human plasma were used. The absence of factor V activity in human serum was attributed to the action of an inhibitor.

The first clear evidence that thrombin-activated factor V was a protein with physicochemical characteristics different than those of factor V as it exists in plasma was presented by Cox *et al.* (1956). They demonstrated that the activated factor V from human, but not from bovine, blood could be preferentially retained on ion-exchange columns.

Stormorken (1957), who investigated the levels of factor V activity in the plasma of several different species, and Hjort (1957), who worked with bovine plasma, both confirmed and extended the above evidence concerning the activation of plasma factor V by thrombin. Despite the aforementioned evidence, the activation of factor V by thrombin has not as yet been accepted generally as a significant step in the coagulation process. Nevertheless, it is universally believed that factor V plays a central role in the reactions leading to the formation of prothrombin activator (Biggs and Macfarlane, 1962a; Straub and Duckert, 1961; Macfarlane, 1961). Three possible pathways by which factor V could exert its influence are summarized in Figure 1.

During our own attempts to purify factor V from bovine blood we found that the "molecular sieve" methods of separation revealed a remarkable difference between factor V from plasma and factor V from serum. This present communication presents experimental evidence on the significant difference in molecular size between plasma factor V and serum factor V, and the relationship of this difference in molecular size with the activation of plasma factor V by thrombin preparations.

[2] Throughout the remainder of this article, the terms "factor V" and "thrombin-activated factor V" will be used, irrespective of the name under which the same substances have been described (plasma and serum Ac-globulin, or proaccelerin and accelerin).

MATERIALS AND METHODS

Sephadex G-200 was obtained from Pharmacia Company, and the dry material was passed through standard sieve to obtain the 200–400 mesh fraction. *Barium Sulfate*, U.S.P., suitable for X-ray diagnosis, was obtained from Merck and Co. *Celite* (Filter-cel) was a Johns Mansville product.

Thrombin.—A commercial preparation of thrombin derived from bovine sources (Upjohn, Lot PH 496) was used. The entire contents of one ampoule containing 1000 units was dissolved in 10 ml Tris buffer, *p*H 7.3, and stored at −20° as a stock solution. Immediately before use this solution was diluted 10-fold with the same buffer.

Bovine Plasma.—Bovine blood was obtained immediately after slaughter and mixed in plastic containers with 0.1 volume of 0.1 M sodium oxalate solution. The plasma, obtained by centrifugation at 3000 × *g* for 40 minutes at 4°, was treated at 0° with 100 mg solid $BaSO_4$ per ml for 1 hour under mechanical stirring. The mixture was centrifuged at 1500 × *g* for 30 minutes at 4°. The clear supernatant, subsequently referred to as $BaSO_4$-treated plasma, was removed and stored at −20° until needed.

Bovine Serum.—Bovine blood was collected in a glass container, mixed with a few grams of Celite per liter for maximal contact activation, and stirred with a glass stirrer until a firm clot was obtained. The coagulated blood was either used the same day or left

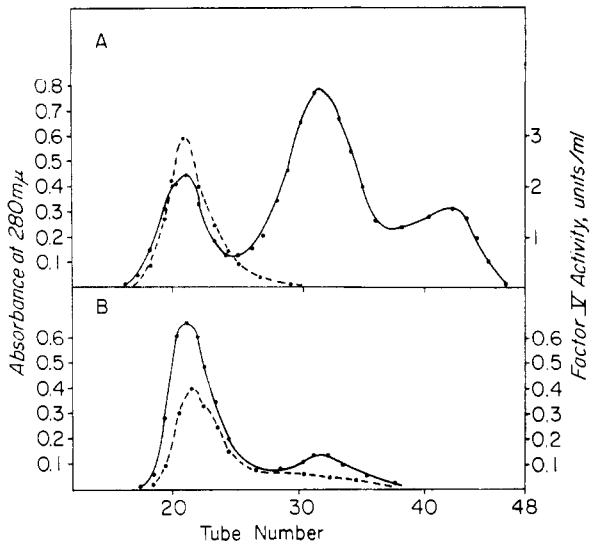

FIG. 3.—Chromatography of plasma fractions on Sephadex G-200. (A) Ammonium sulfate (33–50%) fraction from bovine plasma, eluted with buffer containing 0.72 M NaCl and 0.05 M Tris-HCl, *p*H 7.35. (B) First peak from (A) on another Sephadex column, eluted with buffer containing 0.14 M NaCl, and 0.01 M Tris-HCl, *p*H 7.35. Effluent volume in each tube, 3.0 ml; column volume, 160 ml. Total protein (———), factor V activity (– – – –).

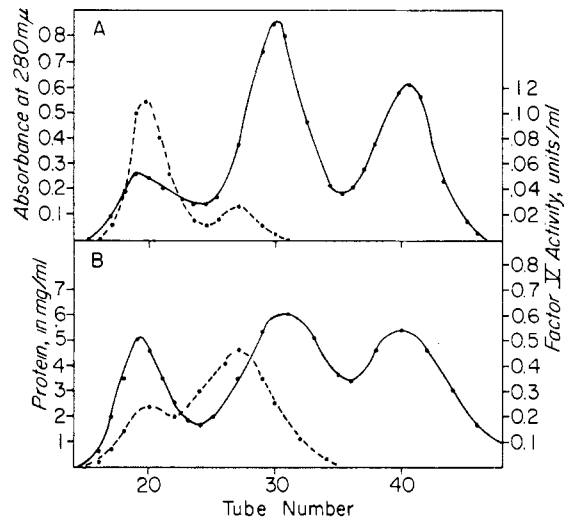

FIG. 4.—Chromatography of bovine serum on Sephadex G-200. (A) Fresh serum eluted with buffer containing 0.72 M NaCl and 0.05 M Tris-HCl, *p*H 7.35. (B) Two-day-old serum eluted with buffer containing 0.14 M NaCl and 0.01 M Tris-HCl, *p*H 7.35. Effluent volume in each tube, 3.0 ml; column volume 160 ml. Total protein (———), factor V activity (– – – –).

at room temperature for 2 days; in both cases, serum was obtained by centrifugation at 4°. The fresh serum was mixed at room temperature with Celite (50 mg/ml) in order to remove most of the remaining thrombin (Soulier and Prou-Wartelle, 1960). After centrifugation at 1500 × *g* for 30 minutes at 4°, the clear supernatant was used directly for column chromatography. The 2-day-old serum was mixed with 0.1 volume of 0.1 M sodium oxalate solution, and then treated with $BaSO_4$ as described for plasma. This $BaSO_4$-treated 2-day-old serum was kept frozen until needed.

Ammonium Sulfate Fractionation.—The precipitation was performed in the cold by addition of the solid salt to the $BaSO_4$-treated plasma or to the $BaSO_4$-treated 2-day-old serum. The fraction which precipitated between 33 and 50% saturation contained 50–70% of the initial factor V activity and was collected and dialyzed for 24 hours against 0.01 M Tris-HCl, *p*H 7.3, in 0.14 M NaCl, at 4°. The dialyzed material was stored at −20° until required. This material is described later in the text as the 33–50% ammonium sulfate fraction.

Fractionation on Sephadex G-200.—Columns of Sephadex G-200, 200–400 mesh, were prepared essentially as described by Flodin and Killander (1962). These columns measured 2.2 × 51 cm and, under gravity conditions, flow rates of 6–8 ml/hour at 4° were obtained. Hydrostatic pressure was avoided since it tends to slow the flow rate over a period of time. The buffer used for the elution was Tris-HCl, *p*H 7.3, with added NaCl. Two different molarities were used: 0.01 M Tris-HCl, 0.14 M NaCl; and 0.05 M Tris-HCl, 0.72 M NaCl. The sample for chromatography, which usually was 3–5 ml in volume, was layered between the surface of the column and the buffer zone. The effluent was collected in 3-ml portions on a Packard fraction collector. The distribution coefficients were calculated as suggested by Flodin (1961). These coefficients are a measure of the part of the stationary phase (the interior of the Sephadex particles) with which a solute equilibrates during the passage through the column.

Sucrose Density Gradient.—The gradient was prepared with an apparatus similar to that described by Britten and Roberts (1960). This unit consisted of a mixing vessel equipped with a small electrically driven stirrer and a reservoir. A narrow polyethylene tube led from the mixing chamber to the plastic centrifuge tube where the gradient was to be established. The reservoir was filled with 2.6 ml of 5 or 7% sucrose solution in 0.02 M Tris-HCl, *p*H 7.35, while the mixing chamber was filled with 2.6 ml of 25 or 14% sucrose in the same buffer. The valve between the two chambers was opened, the stirrer was started, and the solution from the mixing chamber was allowed to run slowly down the side of the centrifuge tube. When all the solution was transferred to the tube, 0.3 ml of the sample for analysis was carefully layered on top of the gradient. This mixing operation was performed at 4° in 5–10 minutes. The tubes were then placed in a precooled rotor SW39 (Spinco) and centrifuged in the Spinco Model L ultracentrifuge at 39,000 rpm for 12 hours at 3–5°. At the end of the run the tubes were pierced at the bottom with a 20-gauge needle, and the effluent was collected in 10 fractions of 7 drops each.

Flotation of Lipoproteins.—The density of plasma was increased by mixing with a solution of NaCl and KBr of density 1.346 g/ml as described by Havel *et al.* (1955). The final mixture had a density of 1.21 g/ml. It was centrifuged at 100,000 × *g* for 24 hours at 4° in a Spinco Model L ultracentrifuge. At the completion of the run the contents of the tubes were found to be separated into a densely colored, yellow-orange top layer; a middle, clear, colorless zone; and a lower zone, which became increasingly yellow toward the bottom. The tubes were sliced in the middle of the clear zone, and the contents of the top and bottom parts were analyzed for lipid phosphorus, protein, and factor V activity.

Factor V Activity.—This assay was performed using human oxalated plasma, which was made deficient in factor V through storage (Biggs *et al.*, 1962b). In the usual assay, 0.1 ml of this reagent was mixed at 37° with 0.1 ml of saline extract of human brain acetone powder and with 0.1 ml of the fraction to be assayed. Then 0.1 ml of 0.025 M $CaCl_2$ was added and the time

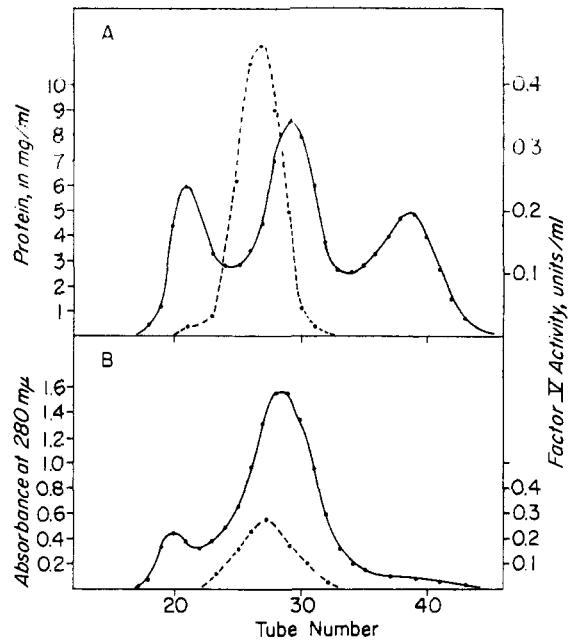

FIG. 5.—Chromatography of serum fractions on Sephadex G-200. (A) Ammonium sulfate (33–50%) fraction from 2-day-old serum, eluted with buffer containing 0.72 M NaCl and 0.05 M Tris-HCl, *p*H 7.35. (B) The first half of the second peak from above (fractions 23–30) on another Sephadex column, eluted with buffer containing 0.14 M NaCl and 0.01 M Tris-HCl, *p*H 7.35. Effluent volume in each tube, 3.0 ml.; column volume, 160 ml. Total protein (———), factor V activity (– – – –).

in seconds between the addition of $CaCl_2$ and the formation of fibrin clot was recorded. These observed times were converted to units per ml by comparison with a standard curve obtained using dilutions of fresh normal human plasma. When the time in seconds was plotted on a double logarithmic scale against the dilutions of normal plasma, a straight line was obtained. A unit of factor V is defined as that amount present in 1 ml of normal plasma.

Fibrinogen.—The presence of fibrinogen was established semiquantitatively by the clotting time of the fractions after the addition of thrombin.

Prothrombin.—Prothrombin was determined by the method of Koller *et al.* (1951).

Lipid Phosphorus.—One volume of the fraction under examination was extracted twice with five volumes of chloroform-methanol 2:1 mixture. The combined chloroform extracts (lower phase) were then analyzed for total phosphorus by the method of King (1932).

Protein Concentration.—Protein concentration, when referred to as mg per ml, was measured by the biuret technique as modified by Weichselbaum (1946); in all other instances protein concentration was determined by the adsorbance at 280 mμ.

RESULTS

"Molecular Sieve" Resolution of Bovine Plasma and Bovine Plasma Fractions

When $BaSO_4$-treated plasma was applied to a column of Sephadex G-200 and eluted with a buffer of 0.15 ionic strength, separation of the proteins into three groups according to molecular size was observed as reported previously by Flodin and Killander (1962). Factor V activity was quantitatively recovered with the first peak, with a distribution coefficient of approximately 0.05 (Fig. 2A). Fibrinogen, as well as a significant portion of the lipoproteins as suggested by the presence of lipid phosphorus, was also found in the first peak. In order to rule out possibility of protein-protein interaction leading to aggregation of factor V with large molecular size proteins, a similar experiment was performed with a buffer of higher (0.77) ionic strength (Fig. 2B). As expected, the separation of the protein peaks was much sharper in the higher ionic strength medium, yet again factor V activity was found exclusively in the first peak. There was 100% recovery of the activity applied and a 5-fold increase in specific activity.

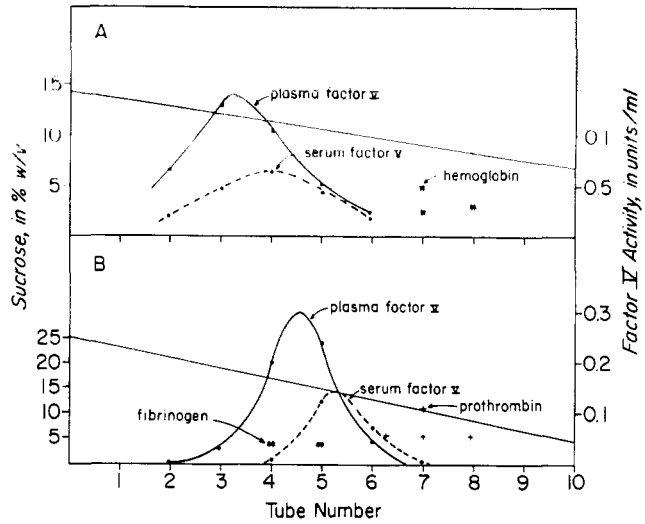

FIG. 6.—Sucrose gradient centrifugation of factor V obtained from plasma and factor V obtained from serum. A portion (0.3 ml) of the protein solution was layered on top of the gradient consisting of 5.2 ml total volume and centrifuged at 100,000 × *g* for 12 hours at 4°. The centrifuge tubes were finally pierced at the bottom with a 20-gauge needle, and the contents were separated manually into fractions containing 7 drops each. (A) Results obtained with a gradient of 7–14% sucrose w/v. (B) Results obtained with a gradient of 5–25% sucrose w/v.

Figure 3A shows the elution pattern obtained when a 33–50% ammonium sulfate fraction from $BaSO_4$-treated plasma was passed through a Sephadex column in a 0.77 ionic strength buffer. Although the amount of proteins in the first and third peaks had been reduced relative to the second peak, factor V activity again appeared with the first peak (distribution coefficient, 0.05). The recovery of the activity varied between 50 and 100%, but the purification achieved was again 5-fold. The material obtained with the first peak upon chromatography of the 33–50% ammonium sulfate fraction showed a specific activity up to fifty times that of the original plasma, since the preliminary ammonium sulfate precipitation involved a 10-fold purification. Figure 3B shows the rechromatography of the material eluted with the first peak (see Fig. 3A). It can be seen that most of the protein material was eluted with a distribution coefficient of 0.05 with only minor contamination from the second peak. Factor V activity was located again exclusively in the first peak (distribution coefficient 0.05).

"Molecular Sieve" Resolution of Bovine Serum and Bovine Serum Fractions

When fresh serum, after treatment with Celite for removal of thrombin, was passed through a Sephadex column, the elution pattern illustrated in Figure 4A was obtained. The protein pattern was similar to that of the plasma, but different in that there were two areas showing factor V activity. One area was in

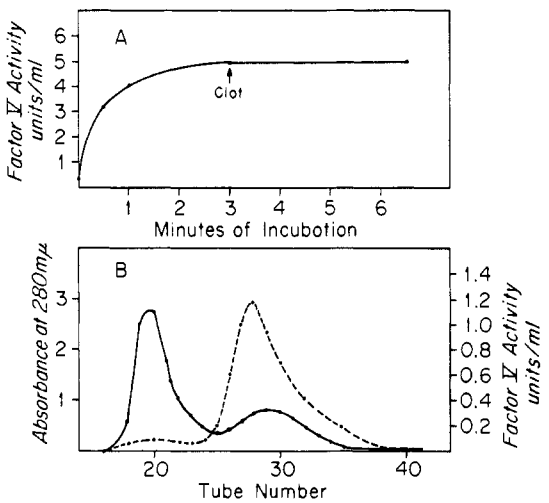

FIG. 7.—Influence of thrombin on factor V. (A) Increase in activity of factor V on incubation with bovine thrombin. (B) Chromatographic behavior of first peak from a previous Sephadex separation (Fig. 3A), after treatment of fraction with thrombin.

the first protein peak, as with plasma, while the other was in the ascending part of the second protein peak and had a distribution coefficient of 0.18. When 2-day-old $BaSO_4$-treated serum was fractionated as stated, it was found that a significantly greater amount of factor V activity was eluted with the second peak, as shown in Figure 4B. A similar elution pattern, but with the bulk of factor V activity in the second peak, was obtained when a 33–50% ammonium sulfate fraction from the same serum was chromatographed, as shown in Figure 5A. The purification achieved when material from serum was used for chromatography was approximately 5-fold. The recovery of total activity was lower than that obtained with plasma fractions, and it usually amounted to 30% of the activity applied on the column.

Rechromatography of the material eluted with the second peak in the latter experiment gave the elution pattern shown in Figure 5B. It can be seen that most of the protein material, as well as factor V activity, had a distribution coefficient of 0.18 which corresponds to the second protein peak. There was some contamination with protein from the first peak, but no detectable factor V activity with a distribution coefficient of 0.05 was present.

When equal amounts of the 33–50% ammonium sulfate fractions from plasma and from 2-day-old serum were mixed together and fractionated on Sephadex as given, a double peak of factor V activity was obtained with a pattern similar to that shown in Figure 4B.

Behavior of Bovine Plasma and Serum Fractions on Ultracentrifugation

The behavior of plasma factor V on Sephadex columns indicated that factor V must be either a high molecular weight protein or a lipoprotein. The following experiment was devised in order to differentiate between these two possibilities. Bovine plasma was mixed with a solution containing NaCl and KBr, of density 1.346 g/ml, in a proportion giving a final density of 1.21 g/ml. The mixture was centrifuged at 100,000 $\times g$ for 24 hours at 4°. The plastic tube was sliced in the middle of the clear zone, and the contents were separated and analyzed. It was found that the lower part of the tube contained all the factor V activity, but less than 10% of the lipid phosphorus. On the basis of these observations it is unlikely that factor V is a lipoprotein.

The possibility that plasma factor V was a high molecular weight protein was strengthened by its behavior on centrifugation on sucrose density gradients. Bovine plasma was layered on top of a sucrose gradient (5–25%) in plastic tubes. After centrifugation at 100,000 $\times g$ for 12 hours at 4°, the tubes were pierced at the bottom and the contents were manually separated into 10 fractions. The results of analyses for factor V activity, fibrinogen, and prothrombin are illustrated in Figure 6B. It can be seen that factor V was found in approximately the same density zone as fibrinogen, whereas prothrombin was found at a higher level. When fractions from 2-day-old serum were used in a similar experiment, factor V activity was found at a higher level than factor V from the plasma fraction, but still substantially lower than prothrombin. The same experiment was repeated with the 33–50% ammonium sulfate fractions and a gradient varying from 14 to 7% (Fig. 6A). In this case factor V activities moved further toward the bottom of the tube but, again, the factor V activity from serum was found at a level higher than that from plasma. The zone at which hemoglobin (by visual examination) was found in this experiment is also illustrated for a comparison with factor V.

Influence of Thrombin on Factor V

The finding that there is a difference in molecular weight between plasma and serum factor V prompted us to investigate the possibility that this change is the reflection of the activation of factor V by thrombin during coagulation.

Activity.—When a small amount of thrombin was added to $BaSO_4$-treated bovine plasma, there was an immediate increase of factor V activity. This increase was more pronounced when a purified fraction containing factor V was used instead of whole plasma. A typical experiment was conducted as follows: One volume of a 33–50% ammonium sulfate fraction from bovine $BaSO_4$-treated plasma containing approximately 0.5 unit of factor V per ml was mixed with 0.1 volume of thrombin solution containing 10 units/ml. Aliquots of this mixture were assayed for factor V activity at various intervals. The results of these assays, which are illustrated in Figure 7A, show that factor V activity rapidly increased over 10-fold after addition of the thrombin preparation.

Molecular Size.—It seemed possible from the foregoing experiment that the profound influence of thrombin on factor V activity could be related to the difference in molecular size between plasma and serum factor V. As already shown in Figure 3B, rechromatography of the material from the first peak showed that most of the proteins, as well as factor V activity, had a distribution coefficient of 0.05. The material from the first peak was then incubated with thrombin as described above and rechromatographed on Sephadex. The results are shown in Figure 7B. Although most of the protein material still possessed a distribution coefficient of 0.05, factor V activity was eluted later in a fraction with a distribution coefficient of 0.18.

In order to substantiate the above observations as to the influence of thrombin on the molecular size of factor V, the following experiment was performed. The material showing factor V activity from the first peak (in Fig. 3B) and the material from the second peak (in Fig. 7B) were layered on top of sucrose density gradients (5–25%) and centrifuged as described. An analysis of the various fractions gave a pattern similar to that shown in Figure 6.

The difference in molecular size before and after activation of factor V by thrombin provided an excellent route to further purification of the activated material. In experiments similar to those described in Figure 7B, factor V, eluted with the first peak from a Sephadex column, was subsequently activated with thrombin and rechromatographed on Sephadex. A purification of 50-fold or more was achieved during the rechromatography of the activated material. The extent of purification depended on the amount of contamination of the first peak with protein material from the second peak.

Discussion

The purification of factor V from bovine plasma was achieved in a reproducible high-yield procedure through application of the molecular sieve (exclusion chromatography) method with Sephadex G-200 columns. When whole bovine plasma was passed through a Sephadex G-200 column, an immediate 5-fold purification was obtained. When a 33–50% ammonium sulfate fraction was used the same purification was obtained, although the recovery of total activity was somewhat lower. Thus, considering the initial purification during the ammonium sulfate fractionation, an over-all purification of 50-fold relative to plasma was obtained for the nonactivated plasma factor V. In addition, most of the lipoproteins could be removed from either the plasma or a fraction thereof by flotation during high speed centrifugation in a 1.21 density medium. By means of this latter procedure factor V activity could be recovered from the lower zone free of most lipid phosphorus and could be chromatographed directly on Sephadex with the same results as described.

In their investigations on the use of Sephadex G-200 for the fractionation of plasma and serum proteins, Flodin and Killander (1962) reported the separation of three groups of proteins according to molecular size. Subsequent examination of the sedimentation and electrophoretic patterns of the different groups showed the following results. The first peak contained lipoproteins, α_2 and γ macroglobulins of 19 S sedimentation coefficient, and minor components of 10 S to 11 S. The second peak contained globulins of 7 S and some α_2, β_1, β_2 globulins of 4 S sedimentation coefficient. The third peak contained mainly albumins with a sedimentation coefficient of 4 S. The experiments described in the present report showed that factor V activity from bovine plasma, or plasma fractions, was eluted exclusively with the highest molecular weight proteins (first peak as described by Flodin and Killander). Inasmuch as it did not float on ultracentrifugation in a density 1.21 medium, factor V did not resemble any of the conventional types of lipoproteins, and hence it can be stated that it belongs in the category of higher molecular weight proteins with a sedimentation coefficient of 19 S to 10 S. It is worth noting in this respect that fibrinogen, with a molecular weight of approximately 400,000, is eluted from Sephadex G-200 with the same distribution coefficient as plasma factor V and that both these proteins sediment to nearly the same zone on density-gradient high speed centrifugation. On a comparative basis, factor V activity isolated from 2-day-old serum behaved, on Sephadex G-200 chromatography and density-gradient centrifugation, as a protein of appreciably smaller molecular weight. Similar physical behavior was observed with purified preparations of plasma factor V after incubation with thrombin (see below).

Since the discovery that plasma factor V could be activated by thrombin (Ware *et al.*, 1947b), only a few attempts have been made to investigate the possible differences in molecular structure between native and activated, or plasma and serum, factor V. Of specific interest here, Cox *et al.* (1956) have reported that thrombin-activated human factor V could be retained on IRC-400 resin with subsequent elution by high ionic strength media, while nonactivated human plasma factor V was not retained on IRC-400 resin even at low ionic strength. A preliminary note from the same laboratory (Lanchantin and Ware, 1955) described the release of trichloroacetic acid–soluble material during the activation of human plasma factor V by thrombin. Although the evidence was only indirect, they interpreted their data as indicating that activated factor V was a small molecular weight protein or, possibly, a peptide. These investigators found no difference between the two forms of factor V from bovine blood. In our present study the data obtained concerning a different molecular size between bovine plasma and serum factors V substantiates the supposition of Cox *et al.* (1956) and provides direct evidence in support of a change of the molecular characteristics of factor V under the influence of thrombin preparation.

On a gross approximation basis, the molecular weight of serum factor V and thrombin-activated factor V is in the range between 80,000 and 400,000, possibly near 200,000. This would allow a 2-fold, or more, molecular weight difference between the two types of factor V. This proposal assumes a molecular weight for plasma factor V of 400,000 or more and does not take into consideration any drastic changes in molecular shape. Thrombin is generally considered to be a specific proteolytic enzyme (Lorand, 1951; Scheraga, 1958), and the activation of factor V by thrombin is well documented. On the basis of this fact, and though our preparations of thrombin contained impurities (Seegers, 1962), we tend to consider the described molecular transformation of factor V as the result of an enzymatic attack by thrombin. It is possible that thrombin splits a bond between two subunits which are more active once separated, or that it splits off part(s) of the factor V molecule thus decreasing its molecular weight while increasing its affinity toward lipid, or factor X, in the assay system (see Fig. 1).

Finally, a high degree of purification of the thrombin-activated factor V can be achieved by taking advantage of the change of molecular size before and after the activation. When factor V from a clean fraction of the first peak on Sephadex G-200 chromatography is activated by thrombin and rechromatographed (see Fig. 7B), it is possible to obtain the activated factor (distribution coefficient 0.18) free from most other contaminating proteins. The degree of purity of this fraction and its physicochemical characteristics need further attention, and studies along these lines are anticipated.

Acknowledgments

The authors acknowledge the expert technical assistance rendered by Miss Margery Carlstrom.

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Naturally Occurring Triglycerides Possessing Optical Activity in the Glycerol Moiety*

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Received September 9, 1963

Seed oils of the Chinese tallow tree, *Sapium sebiferum*, and of *Sebastiana lingustrina* were found to be optically active. By column chromatography, the light-absorbing and optically active triglyceride components of *Sapium sebiferum* were isolated and were found to have $[\alpha]_D^{20°} = -21.5°$ in 20% solution in chloroform. The comparable fraction from *Sebastiana lingustrina* exhibited $[\alpha]_D^{20°} = -17.5°$. The $E_{1cm}^{1\%}$ of the optically active fraction was 900 at 260 mμ. The optically active fraction was separated into four subfractions by countercurrent distribution and these had rotations similar to the optically active fraction, suggesting that all the triglycerides in the optically active fraction are optically active. The unsaponifiable portion of the optically active fraction was demonstrated by thin-layer and gas-liquid chromatography to be glycerol. Fatty acid composition of the four subfractions, measured by gas-liquid chromatography, indicated the ratio of 1 mole unsaturated carboxyl-conjugated fatty acid per mole triglyceride. The unsaturated carboxyl-conjugated acids are considered to be on the α position of the glycerol.

Naturally occurring compounds which possess potentially asymmetric centers usually are found in only one of the optically active enantiomorphic forms in a single source. Racemic mixtures of enantiomorphs are rarely found in nature. The principal components of proteins and carbohydrates exhibit definite optical rotation, whereas the most abundant components of fats, the triglycerides, rarely exhibit measurable optical activity despite the fact that the β-carbon of the glycerol moiety of a triacid triglyceride is potentially asymmetric. The measurable optical activity found in a few natural oils has been traceable to unsaponifiable matter, such as sterols, or to optically active fatty acids within the triglyceride.

Considerable effort has been expended to synthesize asymmetric triglycerides of fatty acids with possible optical rotatory power, but without success, whereas with substituents other than fatty acids glycerol derivatives with high optical activity have been obtained (Fischer and Baer, 1941; Baer and Fischer, 1937). Recently W. Schlenk, Jr. (1962) synthesized 1-trimethylacetyl-2,3-di-*n*-valerin and found it to have a specific rotation of $[\alpha]_D = +1.6°$. He also reported that triglycerides having one (or two) short-chain and two (or one) long-chain normal fatty acids exhibit small but distinct optical rotation in the ultraviolet. Triglycerides such as 1-palmito-2-oleo-3-stearin, containing three long-chain acids, showed no rotation in the visible or ultraviolet spectrum. In these reports the authors show that the naturally occurring triglycerides having mostly long-chain acids may occur in asymmetric forms, but that the structural differences in the three fatty acid radicals are not sufficient to exhibit measurable optical rotation in the visible or near-ultraviolet spectra. To our knowledge, an optically active naturally occurring triglyceride whose center of asymmetry resides in the glycerol moiety has not previously been isolated. We wish to report the isolation and characterization of mixtures of such triglycerides from the seed oil of two species of the botanical family *Euphorbiaceae*.

* Supported in part by a grant (HE 03559) from the National Institutes of Health, U. S. Public Health Service.

EXPERIMENTAL

Seeds of the Chinese tallow tree, *Sapium sebiferum* (otherwise known as *Stillingia sebiferum*), were collected in Houston, Texas, in 1954. The seeds were freed of the external tallow by steam treatment and the dried seeds were ground and pressed. The press cake was extracted with light petroleum hydrocarbon (bp 30–45°) to give a second crop of oil. The seeds of *Sebastiana lingustrina* were collected in eastern Texas in 1962. These were ground in an Omni-mixer and extracted with petroleum hydrocarbon–diethyl ether (1:1) and with chloroform.