Role of Galactose in Bovine Factor V*

SESHAIYER SARASWATHI AND ROBERT W. COLMAN
From the Coagulation Unit of the Hematology-Oncology Section, Department of Medicine, University of Pennsylvania 19104

Using galactose oxidase as well as β-galactosidase to produce modifications of the galactose units, the functional significance of these carbohydrate residues on the coagulant activity of bovine Factor V glycoprotein was evaluated. Incubation of native Factor V with galactose oxidase or hydrolysis of asialo-Factor V with β-galactosidase results in a loss of Factor V activity. The inactivation of Factor V by oxidation of galactose moieties is partially reversible upon reduction of the newly formed aldehyde groups with sodium borohydride. The extent of reversal depends upon the degree of inactivation achieved. Thus, Factor V which retained 30% of the original activity following galactose oxidation returns to 75% of the original coagulant activity upon borohydride reduction; but, after destruction of 85% of the original activity treatment with borohydride returns to about 30%.

In the initial stages of the inactivation of Factor V by treatment with galactose oxidase, the loss of Factor V coagulant activity is directly proportional to the moles of galactose oxidized. However, as the reaction progresses, the rate of galactose oxidation exceeds the rate of loss of Factor V activity. Moreover, galactose oxidation continues even after complete inactivation of Factor V. These results suggest that the galactose residues most susceptible to attack by galactose oxidase are those necessary for the activity of this coagulant protein.

Only 15 galactose residues/mol of Factor V are susceptible to galactose oxidase prior to removal of sialic acid. In contrast, 37 galactose residues/mol of Factor V are found after acid hydrolysis. These results suggest that Factor V glycoprotein contains more than one type of sialyl-galactose linkages, the C2,3 or C2,4 linkages susceptible to oxidation in the native protein and the C2,6 linkage which is resistant.

Native Factor V binds with diarachidonyl lecithin forming an active complex of lower buoyant density, while the Factor V oxidized with galactose oxidase does not. The Factor V-phospholipid complex is protected from inactivation by galactose oxidase. Moreover, lipid binding diminishes the extent of oxidation of galactose residues. Certain galactose groups are essential for coagulant activity probably because they are required for binding to phospholipid, a prerequisite to Factor V action.

Many of the proteins involved in blood coagulation are glycoproteins, but the functional role of the covalently linked carbohydrates (1-4) has not been defined. Recent observations that aggregation of platelets by collagen (5-7) or bovine von Willebrand factor (8) is inhibited by modification of galactose in the carbohydrate side chain suggest that sugar residues may play a role in certain hemostatic interactions.

Factor V, the protein which accelerates the conversion of prothrombin to thrombin by Factor Xa in the presence of Ca2+ and phospholipid has been shown to be a glycoprotein containing sialic acid, galactose, mannose, and N-acetylglucosamine (9). Gumprecht and Colman (10) have shown that the removal of sialic acid from Factor V by neuraminidase treatment enhances the specific activity of this coagulation protein and also produces changes in such properties of Factor V as lipid binding and thrombin activation.

In the present report, the role of galactose in Factor V has been probed by modifying the glycoprotein with galactose oxidase and with β-galactosidase. The results indicate that galactose modification destroys Factor V activity and this change is concurrent with interference of the binding of this coagulant protein to phospholipid.

MATERIALS AND METHODS

Galactose oxidase (type II) from Polyergus cinctus was purchased from Sigma Chemical Co. and purified as described below. Neuraminidase from Vibrio cholera was obtained from Behring Diagnostics. β-Galactosidase from Escherichia coli and α-galactosidase from coffee beans were obtained from Boehringer Mannheim Corp. Galactostat kit for galactose measurements was purchased from Worthington Laboratory. Canalco reagents were used for acrylamide electrophoresis. Synthetic diarachidonyl phosphatidylcholine, supplied in benzene (10 mg/ml), was obtained from the Applied Science Laboratories. TEAE-(triethylaminoethyl)cellulose, cellulose phosphate, sodium borohydride, and D(+)-galactose were purchased from Sigma Chemical Co.

Preparation of Factor V—Factor V was purified from bovine plasma by the method of Colman (11) with certain modifications. Following adsorption of bovine plasma with BaSO4 and the adsorp-
tion of supernatant plasma onto TEAE-cellulose, elution of Factor V from TEAE-cellulose was done with 0.25 M potassium phosphate buffer (pH 7.0) rather than with 0.4 M buffer as previously described. Factor V was precipitated from the TEAE-cellulose eluate with a single step 70% ammonium sulfate saturation instead of two steps as previously reported. The precipitate was dissolved in 0.04 M phosphate buffer, pH 7.0, dialyzed overnight against the same buffer, and the inactive precipitate removed by centrifugation. In the cellulose phosphate purification step, the elution of Factor V was done with 0.25 M phosphate buffer, pH 7.0. The pooled Factor V eluate from the cellulose phosphate column was concentrated by using an Amicon ultrafiltration device with an XM-50 membrane. The final step in purification consisted of gel filtration on a Sepharose 4B column. About 30 mg of concentrated cellulose phosphate fraction in 1 ml of 0.02 M Tris-glycine buffer, pH 7.4, was applied through the Sepharose 4B column, which had been equilibrated previously with 0.04 M phosphate buffer, pH 7.4. Elution of Factor V was done with the same buffer at a flow rate of 10 ml/hour. Factor V emerged as a single major peak. The appropriate fractions were pooled and concentrated by ultrafiltration.

**Assay of Factor V—**Factor V-deficient human plasma prepared by the method of Lewis and Ware (12) was used as the substrate for the Factor V assay. Diluted thromboplastin was added to 1 mg of Factor V and incubated at 37°C in human brain acetone powder with 0.15 m saline (0.9% NaCl solution) (20 ml/g) at 50° for 15 min. Solutions to be tested were diluted with 0.02 M Verso buffer containing 0.18 M sodium chloride and 0.1 M sodium oxalate, pH 7.4 (Buffer A). Assays were performed by adding 0.1 ml of the test solution to 0.1 ml of brain thromboplastin and 0.1 ml of 0.02 M CaCl₂. The time required for clot formation was measured with an automatic timer (Fibrometer, Baltimore Laboratories).

A calibration curve was constructed by assaying several dilutions of each of 10 normal human plasmas and plotting the logarithm of the mean clotting times at each dilution. One milliliter of normal human plasma (mean of 10 separate plasmas) was used as containing 1 unit of Factor V.

Protein concentrations were determined using an extinction coefficient of ε₂₈₀ = 11.9 (13).

**Purification of Galactose Oxidase—**Galactose oxidase type I from Sigma Chemical Co. was purified further by chromatography on DEAE-cellulose as described by Morell et al. (14).

**Assay of galactose oxidase** was done according to the method of Amaral et al. (15). One unit of galactose oxidase was defined as the amount which produces an increase in optical density of 1.000 at 425 nm in 10 min using standard galactose as substrate.

**Incubation of Factor V with Galactose Oxidase**—In the inactivation studies, 1 to 2 units/ml of Factor V were incubated with varying amounts of purified galactose oxidase in the presence of 100 mM phosphate buffer, pH 7.4, at 37°C. Aliquots were withdrawn at required intervals, diluted in Buffer A, and assayed for Factor V activity. The protein concentration in each fraction was measured by the micro-Folin method (23).

**RESULTS**

**Acid Hydrolysis and Total Galactose Estimations**—Total galactose content of Factor V was estimated by acid hydrolysis in 2 N H₂SO₄ at 100°C for 4 hours. The neutral sugars were purified from the hydrolysate by chromatography on Dowex 50 H⁺ and Dowex 1 X8 (HCOO⁻) column (16). Galactose was estimated using galactostat.

**Reduction of Factor V with Borohydride—**After incubation with galactose oxidase, as above, the pH of the solution was raised to 8.5 by adding 0.2 ml of Tris HCl buffer, pH 8.5. Sodium borohydride (0.3 to 0.5 mM) dissolved in the same buffer was added so as to maintain a Factor V to borohydride ratio of 1:2 (w/w). The incubation mixture was kept in ice. Aliquots were withdrawn at different times, diluted in Buffer A, and assayed for Factor V activity. In control experiments, borohydride was shown to have no effect on the activity of native Factor V at the concentrations used in these experiments.

**Preparation of Asialo-Factor V—**Removal of sialic acid from Factor V was accomplished by incubation with neuraminidase at 37°C and at pH 7.4 (10). The release of sialic acid was followed by the thiorbituric acid method (17). The incubation mixture was dialyzed overnight against 0.01 M phosphate buffer, pH 7.4. The completion of the removal of sialic acid was assessed by acid hydrolysis of the asialo-Factor V (18) following extensive dialysis.

**Hydrolysis of Asialo-Factor V by β-galactosidase—**Incubation with asialo Factor V was performed at 37°C using 1 to 2 units/ml of Factor V, 1 unit of β-galactosidase, and 100 mM Tris buffer, pH 7.5.

**Polyacrylamide Electrophoresis—**Disc electrophoresis on polyacrylamide gels was performed as described by Ornstein (19) and Davis (20) using 3.5% gels. The electrophoresis was run in (0.025 M) Tris-glycine buffer, pH 8.5, at 3 mA/tube for 2 to 3 hours. Coomassie brilliant blue (21) was used as a protein stain and carbohydrates were visualized by the periodic acid-Schiff method (22).

Factor V activity was eluted from unfixed gels after they were cut into 2-mm slices. The gel slices were incubated in appropriate volumes of 0.05 M Tris-acetate buffer, pH 7.4, for 24 hours at 4°C.

**lipid-binding Studies—**Prior to the lipid-binding studies Factor V and galactose oxidase-treated Factor V were separated from trace residual quantities of contaminating lipoproteins as follows. Solutions of Factor V or galactose oxidase-treated Factor V were diluted with sucrose solution to a final sucrose concentration of 30%. This solution was layered over 50% sucrose solution in a discontinuous sucrose density gradient containing 0.8 M layers of 10, 20, 25, 30, and 50% sucrose solution, respectively, in 0.05 M Tris-acetate, pH 7.4. The tubes were centrifuged in a SW 50.1 swinging bucket of a model L ultracentrifuge for 18 hours at 90,000 × g. After the sedimentation fronts were formed, the tubes were harvested and assayed for Factor V activity. The protein concentration in each fraction was measured by the micro-Folin method (23). The fractions containing Factor V activity (or protein in the case of Factor V treated with galactose oxidase) were pooled and dialyzed against 30% sucrose solution.

**Disaccharidyl phosphatidylcholine (2 mg in 0.1 ml of benzene) was evaporated to dryness in a metal tube using a stream of N₂. One milliliter of cold Buffer A was added and sonication was performed twice in a Branson sonifier at 30 watts for 30 s. The lipid dispersions were left standing in ice for 30 min to 1 hour before use. Four hundred micrograms of the lipid dispersion was mixed with 200 µg of Factor V. The Factor V-phospholipid mixture was centrifuged in the sucrose density gradient as described above. Aliquots were collected and Factor V activity as well as protein concentrations were estimated. Binding of Factor V with the phospholipid was accompanied by a change in the buoyant density as indicated by a migration toward the low density region of the sucrose gradient.

**Stoichiometry of Lipid Binding—**Two hundred micrograms of Factor V (agarose pool) were combined with varying concentrations of sonicated disaccharidyl lecithin and subjected to sucrose density gradient centrifugation as above. Factor V activity was plotted against the fraction number. Total Factor V was taken as the area under the peak of Factor V activity in the control tube (where no lipid was added). In tubes where Factor V-lipid mixture was centrifuged, the area in that portion of the curve corresponding to the Factor V in the control tube was taken as the unbound or free Factor V. The amount (micrograms) of Factor V bound to lipid was calculated from the difference between the total and free Factor V concentration. The concentration of lipid-bound Factor V was plotted against the lipid concentration.

**RESULTS**

**Purification of Factor V—**The results of the purification procedure were essentially identical with those reported previously. Starting from the oxalated bovine plasma, the purification procedure involved five steps: adsorption on BaSO₄, chromatography on TEAE-cellulose, ammonium sulfate precipitation, chromatography on cellulose phosphate, and finally gel filtration on Sepharose 4B. The increase in specific activity was nearly 2,000-fold from the starting plasma with a specific activity of 60 units/mg and with an over-all yield of 15% and the preparation was essentially free of other coagulation factors (11). Prior to gel filtration, larger aggregates of Factor V were seen on disc gel electrophoresis. Therefore, in the experiments
reported subsequently only gel-filtered Factor V ($K_0 = 0.60, M, \approx 300,000$ (24)) was used (Fig. 1, Fractions 60 to 80). After concentration polyacrylamide gel electrophoresis of gel-filtered Factor V gives two closely migrating protein bands both of which stain for carbohydrate. Factor V activity is associated with each of the protein bands eluted from the disc gel. A small amount of protein representing less than $3\%$ of the Factor V activity does not enter the gel (Fig. 2).

**Effect of Galactose Oxidase on Factor V Activity**—When Factor V was incubated with galactose oxidase there was a rapid loss of Factor V activity which was linear with respect to the galactose oxidase concentration (Fig. 3). Nearly $80\%$ of Factor V activity was inactivated upon incubation with 200 units/ml of galactose oxidase for 10 min at $37^\circ$.

When the reaction was performed at different temperatures, it was found that the rate of inactivation was greater at higher temperatures. The decrease in activity was first order with respect to Factor V activity with half-lives of 6, 9, and 11 min. (Fig. 4A) at 37, 25, and $15^\circ$, respectively. An Arrhenius plot of the first order rate constant versus reciprocal of the absolute temperature is shown in Fig. 4B. The activation energy calculated from this curve was 4.3 kcal/mol.

**Acrylamide Electrophoresis**—Using purified galactose oxidase, there was no change in the disc electrophoresis pattern in Factor V associated with galactose oxidase treatment (Fig. 5). When Factor V ($500 \mu g$) was incubated in the presence and absence of galactose oxidase ($90 \mu g$) at $37^\circ$ for 30 min and subsequently precipitated with trichloroacetic acid; the resulting supernatant contained 2.5 to 2.9 $\mu g$/ml of peptide material (micro-Folin assay) in control as well as in the galactose oxidase-containing incubation mixtures.

**Reduction of Factor V with Sodium Borohydride**—The loss of Factor V activity upon galactose oxidase treatment was partially reversible upon reduction with sodium borohydride (Table I). The extent of reversibility depended largely on the extent of inactivation induced by galactose oxidase. When the residual activity was $30\%$ of the original Factor V, the reduction with sodium borohydride resulted in a return to $75\%$ of the original activity.

However, when the inactivation proceeded up to $85\%$ the recovery upon sodium borohydride reduction was only up to $35\%$; sodium borohydride had no effect on control Factor V under the conditions of these experiments.

**Relation of Factor V Inactivation to Galactose Oxidation**—The correlation of the rate of Factor V inactivation with quantity of galactose oxidized is shown in Fig. 6, A and B. Inactivation of Factor V by exposure to galactose oxidase was related directly to the number of galactose residues oxidized. As the time of incubation was increased, there was progressive decrease in Factor V activity and a gradual increase in the amount of galactose oxidized (Fig. 6A). In the initial stages of the reaction the loss of Factor V activity was directly proportional to the amount of galactose oxidized (Fig. 6B). However,

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**Fig. 1.** Gel filtration of Factor V. Gel filtration of Factor V was done in a Sepharose 4B column (2.5 × 56 cm) which had been equilibrated with 0.04 M phosphate buffer, pH 7.4. Twenty milligrams of Factor V were applied to the column and elution was done with the above buffer, at the rate of 10 ml/hour. The assay for Factor V and the protein estimation was done as described in the text. $\bullet$--$\bullet$, Factor V activity.

**Fig. 2.** Polyacrylamide disc electrophoresis of Factor V. Samples were applied in 10% glycerol. Twenty micrograms of protein were used in gels stained for protein (upper gel) and 50 $\mu g$ for the carbohydrate staining (lower gel). Standard methods for disc electrophoresis (19, 20) and staining for protein (20) and carbohydrate (21) were used and described under "Materials and Methods." Factor V was recovered from the unfixed gels by slicing to approximately 2-mm pieces and eluting with 0.05 M Tris-acetate buffer, pH 7.4. The elution was done for about 48 hours at $4^\circ$.

**Fig. 3.** Inactivation of Factor V as a function of the galactose oxidase concentration. Galactose oxidase was purified and suitable dilutions were done with 0.04 M phosphate buffer, pH 7.4. One to two units per milliliter of Factor V were incubated with varying concentrations of galactose oxidase as shown for 10 min at $37^\circ$. Aliquots of the incubation mixture were diluted in Buffer A and assayed for Factor V.
FIG. 4. Inactivation of Factor V by galactose oxidase as a function of time and temperature. A, incubation of Factor V (1 unit/ml) with 100 units of galactose oxidase was performed at the temperatures indicated in the figure. At various times aliquots were removed and assayed as described under "Materials and Methods." B, Arrhenius plot of the first order rate constant versus the reciprocal of the absolute temperature. The half-life in minutes ($t_1/2$) at each temperature was calculated by plotting the log of Factor V activity versus time. The pseudo-first order rate constant was calculated from the equation $k = \frac{0.693}{t_1/2}$. The activation energy of the reaction was calculated from the curve.

FIG. 5. Disc gel electrophoresis of Factor V and galactose oxidase (GAL. OX)-treated Factor V. The details of the electrophoresis are described under Fig. 2. Galactose-oxidized Factor V was prepared by incubation of 30 μg of Factor V with 10 μg of galactose oxidase for 45 min in a total volume of 0.1 ml. The inactivation of Factor V was 90% at this stage. An equivalent amount of galactose oxidase was added to the control Factor V prior to application to the gel column.

After nearly 60% of Factor V activity was lost, the extent of oxidation exceeded the extent of inactivation of Factor V. When the incubation was continued after complete loss of activity had occurred, galactose oxidation continued to take place.

Quantitation of Available and Total Galactose Residues—Acid hydrolysis released 22 μg of galactose/mg of Factor V. Thus, there are 37 galactose residues/mol of Factor V, assuming the molecular weight of Factor V to be 300,000. Incubation of native Factor V with galactose oxidase resulted in an oxidation of 15 galactose residues/mol (8.5 μg/mg). Thus, 15 out of 37 galactose residues have the CH$_2$OH group available for oxidation in native Factor V. When asialo-Factor V was incubated with galactose oxidase all of the 37 galactose residues were oxidized (Table II).

Binding of Factor V with Phospholipids—When Factor V was centrifuged in the sucrose density gradient, all the Factor V activity could be recovered in the first three to four fractions.
Fig. 6. Inactivation of Factor V as a function of concentration of galactose oxidized. A, incubation was done at 37° with 70 units (1 mg) of Factor V, 100 units of galactose oxidase, and 100 µmol of phosphate buffer, pH 7.0, in a volume of 1 ml. The loss in Factor V activity was followed every 5 min. The amount of galactose oxidized was measured collected from the bottom of the tube. When a mixture of Factor V and phospholipid was subjected to sucrose density gradient centrifugation, two Factor V activity peaks were obtained. The first peak, which corresponds with the control Factor V, represents the concentration of this protein not bound to the lipid. The second peak, which has a lower buoyant density than the control Factor V, represents Factor V bound to the lipid (Fig. 7, top). The two Factor V activity peaks correspond with the two peaks obtained when the fractions were assayed for protein concentrations (Fig. 7, bottom).

When the lipid concentration was increased for a constant amount of Factor V, there was a corresponding increase in the amount of Factor V bound to lipid (i.e. the second peak). This result thus indicates that the Factor V peak with lower buoyant density observed in the presence of lipid is due to the complex formations between the two constituents. The amount of Factor V binding to lipid approaches an optimum when the ratio of Factor V to lipid is 1:2 (µg/µg) (Fig. 8).

Effect of Galactose Oxidation on Binding of Factor V to Phospholipid—When Factor V which had been inactivated by the oxidation of galactose residues was subjected to sucrose density gradient centrifugation in the presence of phospholipid (Fig. 9), only one protein peak was obtained irrespective of whether phosphatidylethanolamine was added or not. This peak was in a position corresponding to that of control Factor V without phospholipid, indicating the absence of any lipid binding by the galactose-oxidized Factor V. The Factor V activity lost upon galactose oxidase treatment was not recovered upon the addition of lipids. Heat inactivation of galactose oxidase prior to incubation with Factor V allowed normal lipid binding of Factor V to occur.

Effect of Galactose Oxidase on Factor V-Phospholipid Complex—Since galactose oxidation prevents the binding of Factor V to phospholipid, it was of interest to study whether in an identical reaction mixture, as indicated in the text. B, per cent inhibition was calculated as: (initial activity − activity after galactose oxidation)/(initial activity) × 100 and plotted against the concentration of galactose oxidized.

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Fig. 8. Formation of Factor V-phospholipid complex as a function of the phospholipid concentration. Two hundred micrograms of aga-
rose pool were combined with different concentrations of diarachidonyl lecithin and subjected to sucrose density gradient centrifugation. The 
amount of Factor V bound to the phospholipid was calculated as described in the text.

Fig. 9. Binding of galactose oxidase (Gal.ox)-treated Factor V with phospholipid. Factor V was incubated with galactose oxidase until the 
inactivation was about 90%. Sucrose density gradient centrifugation in the presence and absence of phosphatidylcholine (PC) was done as in 
the case of Fig. 7. •—•, galactose-oxidized Factor V; x—x, galactose-oxidized Factor V + phospholipid; Δ—Δ, Factor V + phospholipid.

oxidase for 60 min, the loss of activity was only 20% when Factor V-lipid complex was incubated under similar conditions.

Oxidation of galactose residues in the Factor V-lipid complex by galactose oxidase proceeds at an initial rate almost identical with that of Factor V. In both cases about 3.7 μg/mg of Factor V are oxidized in the first 40 min. Thus, these groups 
are equally accessible in the presence and absence of phospholipid. However, the galactose oxidation of the Factor V-phospholipid complex reaches a maximum by 40 min of incubation (Fig. 10, bottom). In contrast, the oxidation of uncomplexed Factor V continues over a much longer period. This further oxidation is best appreciated from the data in Table II where, 
in the absence of lipid, a plateau level after 3 hours of 8.5 μg of

galactose oxidase/mg of Factor V is reached. Thus, binding with phospholipid makes about 4.8 μg of galactose/mg of 
Factor V eventually unavailable for galactose oxidation. Using asialomucin or asialotransferrin as a substrate, phospholipid 
failed to interfere with galactose oxidase activity.

Experiments with β-Galactosidase—The importance of ga-
lactose in Factor V is illustrated further by the effect of 
β-galactosidase upon asialo-Factor V. Although β-galactosi-
dase had no effect on native Factor V and removal of sialic acid from Factor V resulted in an increase of activity (10), incubation of asialo-Factor V with β-galactosidase resulted in rapid inactivation. The inactivation was higher at higher temperature with half-lives of 7, 11.5, and 15 min at 37, 25, and 15°, respectively. The plot of \( K \) versus \( 1/T \) is linear and the first order rate constant calculated from the Arrhenius plot was 4.0 kcal/mol.

α-Galactosidase from coffee beans had no effect on Factor V activity.

**DISCUSSION**

This investigation documents a significant role for the galactose residues of Factor V glycoprotein in the coagulant activity of this plasma protein. This conclusion has been arrived at from the various observations which link Factor V activity to the presence of intact galactose units. Virtually complete loss of Factor V activity occurred upon treatment with galactose oxidase and this loss of activity was proportional to the amount of galactose oxidized in the initial stages of the reaction. The inactivation caused by the oxidation of galactose units was partially reversible by reduction with sodium borohydride, indicating the absence of any gross irreversible conformational or other related changes in the molecule. Finally the removal of galactose by β-galactosidase from asialo-Factor V also resulted in loss of activity.

The role of galactose in hemostasis has been reported by earlier workers. Thus, the ability of asialo-bovine von Willebrand's factor (6) to promote platelet aggregation has been shown to be destroyed by incubation with galactose oxidase. In a similar way, the binding of collagen to platelets, the primary event in hemostasis, has been shown to be destroyed by galactose oxidase treatment and reversed by borohydride reduction (7). For quantitative conclusions about the number and role of galactose groups in Factor V, enzymatic modification of the coagulant protein was used as the primary tool. This approach demands purity of both the substrate and the enzyme. Although the cellulose phosphate eluate contains only Factor V protein as previously shown by activity and immunological studies (25), the preparations show considerable molecular weight heterogeneity including the presence of aggregates as well as active fragments derived from in vivo proteolysis. These were eliminated by using a single peak of gel-filtered Factor V with an estimated molecular weight of 300,000 probably representing Factor V monomer. When the preparation was analyzed by alkaline acrylamide disc gel electrophoresis two protein bands both of which have Factor V activity were evident. They grossly appeared to have similar carbohydrate as measured by periodic acid-Schiff staining, but heterogeneity of carbohydrate composition, particularly sialic acid, might still account for the slightly different migration of the two bands of Factor V. In evaluating the purity of the enzyme, proteolytic activity was found in the galactose oxidase preparation (20). Purification of galactose oxidase eliminated any proteolytic activity toward Factor V and thus the inactivation is not due to peptide bond cleavage.

Only a small fraction of the total galactose units are involved in Factor V activity. Thus, complete loss of activity resulted from the oxidation of about 15% of the total galactose content of the Factor V protein. Assuming the molecular weight of Factor V to be 300,000 (21) the oxidation of only 7 to 8 galactose residues out of 37 residues/mol of Factor V, leads to the complete inactivation. Galactose units which are important for Factor V activity are probably in 2,3- or 2,4-glycosidic linkage with the terminal sialic acid since inactivation by galactose oxidase occurred without prior removal of sialic acid.

Oxidation of galactose by galactose oxidase takes place specifically at the hydroxyl group at carbon 6 (27). The observation that only 15 galactose residues of 37/mol of Factor V are available to the enzyme prior to hydrolysis of sialic acid indicates that these sugar residues are in 2,3 or 2,4 linkages with adjacent sialic acid residues. Since removal of sialic acid by neuraminidase results in the oxidation of all the galactose residues by galactose oxidase, this finding suggests the existence of 2,3- or 2,4- as well as 2,6-sialyl galactose linkages in Factor V, as in the case of α-glycoprotein (28). However, the increased availability of galactose for oxidation in asialo-Factor V might also be due to the minor conformational changes in protein resulting from the removal of negatively charged sialic acid residues (29, 30).

While reduction with sodium borohydride restored the activity of galactose-oxidized Factor V, the recovery of the activity was never complete. Since no loss of activity occurred when Factor V was incubated with borohydride alone in the absence of galactose oxidase, the reason for lack of complete reversibility is unclear. Perhaps this is due to a conformational change such as that documented by Harper et al. (31) after galactose oxidation of collagen.

The binding of Factor V and Factor Xa, the components of the prothrombinase complex, to phospholipids and the importance of such a binding in the optimal activity of prothrombinase complex has been reported (32–34). Our studies imply that Factor V can bind in a stoichiometric fashion with the highly unsaturated synthetic phospholipid-diarachidonyl phosphatidylcholine to alter its buoyant density.

Interaction of Factor V with phospholipids has been demonstrated previously by gel filtration (32) and by precipitation methods (34). We chose density gradient centrifugation since this approach allows not only the separation of free Factor V from phospholipid-bound Factor V but also the determination of whether the complex is heavier than native Factor V or lighter. No protein-phospholipid interaction as demonstrated by sucrose density gradient ultracentrifugation was observed when Factor V previously inactivated by galactose oxidase was incubated with phospholipid. The hypothesis that galactose oxidation prevents the binding of Factor V to phospholipid, thus leading to a loss of coagulant activity, is further substantiated by the observation that phospholipid protects Factor V from galactose oxidase-induced inactivation and also make some of the galactose residues unavailable for oxidation by galactose oxidase. In view of the fact that only a fraction of the total oxidizable galactose residues are involved directly in Factor V activity, it seems probable that the galactose residues which are oxidized in Factor V-phospholipid complex are those which are not involved in Factor V activity. Alternatively, the possibility exists that binding with phospholipid has caused conformational changes in the Factor V molecule (35, 36), which restricts the number of available galactose residues for oxidation.

The role of galactose in facilitating the formation of Factor V-phospholipid may be analogous to the attachment of glycoproteins with plasma membranes. It has been suggested (37–39) that the association of glycoproteins with membranes involves the attachment of lipophilic groups of the protein.
molecule to the lipoidal membrane with the peptide region containing the carbohydrate chain extending outside the membrane barrier. According to the theory of Spiro (40), the membrane glycoproteins in the externally located hydrophilic area serve as a probe for the noncovalent attachment of proteins to the lipoidal membrane. By analogy with the binding of glycoproteins to membranes, it seems logical to postulate that the carbohydrates of Factor V glycoprotein play a role in regulating the binding to platelet membrane with the concomitant binding of the other participants of the prothrombinase complex.

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