The pH Dependence of Quantitative Ristocetin-induced Platelet Aggregation: Theoretical and Practical Implications—A New Device for Maintenance of Platelet-Rich Plasma pH

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Quantitative ristocetin-induced platelet aggregation of normal platelet-rich plasma (PRP) decreases with time after PRP preparation. An increase in pH of the PRP with time proved to be responsible for this finding. Diffusion of CO₂ from the plasma is the prime determinant of the change in pH. Since a complex combination of factors influences CO₂ diffusion (surface area-to-volume relationship, capping, mixing, etc.) the change in pH is variable with time. Thus, quantitative ristocetin aggregation should be pH controlled. A simple device for maintaining PRP pH constant by control of the ambient pCO₂ was designed and found effective in keeping both pH and quantitative ristocetin aggregation constant over a prolonged period of time. It can be adapted for use in platelet aggregation studies employing other reagents. The pH dependence of ristocetin-induced platelet aggregation is consistent with other data supporting an electrostatic interaction between the platelet, von Willebrand factor, and ristocetin. We favor a model wherein ristocetin neutralizes some of the platelet's negative charge and permits the von Willebrand factor to bridge sites on separate platelets to induce agglutination.

Howard and Firkin¹ observed in 1971 that the antibiotic ristocetin induced platelet aggregation of normal platelet-rich plasma (PRP) but did not aggregate the platelets of two of three patients with von Willebrand's disease. Subsequent studies confirmed their observations and established ristocetin-induced platelet aggregation as a diagnostic criterion for von Willebrand's disease.² ³ However, in these later studies most patients with von Willebrand's disease had some aggregation in response to ristocetin although much less than normal. It thus became important to develop a reproducible quantitative technique for evaluating ristocetin-induced aggregation. We observed that quantitative ristocetin aggregation of normal PRP decreased dramatically with increasing time after PRP preparation. When a change in pH of the PRP was found to be responsible for this phenomenon, we analyzed the factors affecting the rate of change of pH in PRP. A simple device for maintaining PRP at a constant pH was designed which improved the reproducibility of the test. The device may be useful in aggregation induced by other agents.

Howard⁴ recently proposed that electrostatic forces are important in ristocetin-induced aggregation. Our pH data are consistent with this hypothesis. A model of ristocetin-induced aggregation is presented, based on recent biochemical analysis of the von Willebrand factor and consistent with a primary role for electrostatic forces.

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MATERIALS AND METHODS

Ristocetin (Lot 714-2041; approximately 95% ristocetin A and 5% ristocetin B) was obtained from Abbott Laboratories (North Chicago, Ill.) and dissolved to 50 mg/ml in buffer (0.01 M barbital-0.15 M NaCl, pH 7.4). For aggregation studies 15 μl of this stock solution were added to 0.4 ml of PRP (final concentration 1.8 mg/ml). Analytical calculations were based upon a $E_{1%}^{1cm} = 49.0 \pm 1$ at 280 nm and an approximate molecular weight for the mixture of ristocetins of 2000.7,8

Platelet aggregation was performed in a Chronolog aggregometer as previously described.9 Donors were normal laboratory personnel who had not ingested medication known to interfere with platelet aggregation for at least 1 wk. Cuvettes and stir bars were siliconized (Siliclad, Clay Adams, Parsippany, N.J.). To minimize the time that PRP would be removed from the buffering action of red blood cells, PRP and platelet-poor plasma (PPP) were prepared simultaneously. Whole blood was collected in polypropylene test tubes (Falcon 2059, Oxnard, Calif.) containing 10% volume 40%, trisodium citrate and divided into aliquots containing 35% and 65% of the total volume. The former was centrifuged 15 min at 2000 g for PPP, and the latter was centrifuged 3.5 min at 700 g for PRP. All specimens were centrifuged at 23°C. The supernatant PPP and PRP were removed with siliconized pasteur pipettes and the platelet count (Coulter Model B, Hialeah, Fla.) of the PRP adjusted to 300,000/cu mm with PPP. The entire volume was distributed in 0.5 ml aliquots into 12 x 75-mm polypropylene tubes (internal diameter 10.7 mm; Falcon 2063) and either capped or left uncapped. Samples were uncapped and the pH determined (Beckman pHasar-I, Irvine, Calif.) using a Ag-AgCl electrode (Beckman 39505) immediately before transferring to an aggregometer cuvette with a plastic pipette (Selectapette, Clay Adams). Samples were allowed to warm to 37°C in the aggregometer until a steady baseline was obtained for 1 min. The addition of ristocetin caused an immediate increase in optical density (OD), which was followed by an initial phase of aggregation, and then in some specimens a more rapid second phase. For most studies quantitation was achieved by measuring the initial slope of aggregation and expressing it as the change in OD units/min. Previous studies by Weiss4 showed good correlation between initial slope and the maximum change in OD in quantitating ristocetin aggregation.

In some experiments, 5% CO2-95% O2 was gently layered over the surface of the PRP for 30 sec before the tubes were capped. The pH of the PRP was adjusted in some experiments by adding either 0.2 N or 0.02 N NaOH or HCl. At the lowest pH value the adjustment resulted in approximately 40% dilution, but all other adjustments caused less than 17% dilution. In other experiments, the pH was adjusted by exposing the PRP to a 100% CO2 environment using the apparatus described below.

Studies were performed to evaluate the factors involved in the rate of pH change of PRP with time. PRP was prepared from citrated whole blood and its pH determined immediately. The PRP was then quickly divided into the polypropylene test tubes of 10.7-mm internal diameter. Three test tubes were filled with 4 ml of PRP giving an air-liquid interface surface area-to-volume ratio of 0.22 cm⁻¹, and three were filled with 0.5 ml giving a surface area-to-volume ratio of 1.80 cm⁻¹. In each group tubes were left in one of three ways: uncapped; capped and left undisturbed; or capped and mixed every 10 min by being aspirated into and forced out of a 1-ml plastic pipette ten times. The pH of each sample was recorded every 30 min.

Assay of the von Willebrand factor in plasma was performed by a modification10 of the macroscopic platelet aggregation test.11 Fresh platelet concentrates prepared at 4°C from ACD blood were kept at 23°C for 2–2 ½ hr so that they regained their disc shape.12 The platelet concentrate was then centrifuged at 750 g for 3 min to reduce red cell contamination. Equal volumes of the supernatant platelet concentrate and a solution prepared by bringing 2 ml of 37% formaldehyde (Fisher Scientific Co., Silver Spring, Md.) to 100 ml with 0.01 M Tris-0.15 M NaCl, pH 7.4, were gently mixed and placed at 4°C overnight. The platelet suspension was then washed three times in 0.01 M Tris-0.15 M NaCl, pH 7.4, buffer and resuspended in the same buffer to a concentration of 300,000/cu mm. Tests were initiated by adding 50 μl of the reference plasma (Hyland Q-Pak, Costa Mesa, Calif.) or the test plasma to 0.4 ml of the platelet solution in a 12 x 75-mm glass test tube and starting a stopwatch. At exactly 30 sec, 5 μl of 50 mg/ml ristocetin solution was blown in and the tube tilted back and forth until macroscopic aggregates were observed. A magnifying mirror under the tube facilitated determination of the endpoint. The clumping time was defined as the number of seconds after ristocetin addition until aggregates first formed. Pre-
liminary studies revealed an essentially linear relationship between the clumping time and dilutions of the reference plasma (in the same Tris NaCl buffer) when plotted on log-log paper. The assay remained linear to dilutions of $\frac{1}{2}$ of the reference plasma giving it a sensitivity of approximately $3\%$ of normal. The plasmas from ten normal individuals contained $91.6\% \pm 29\%$ (mean $\pm$ 1 SD) of the von Willebrand factor of the reference plasma. Three patients with severe von Willebrand's disease tested on five occasions had less than $3\%$ of normal.

To assess the effect of pH on this von Willebrand factor assay, the technique was modified by resuspending the thrice-washed platelet in 0.01 M Hepes (Calbiochem, La Jolla, Calif.) 0.15 M NaCl buffer titrated to various pH's between 6.0 and 8.0. Serial dilutions of the reference plasma were then tested against each of the platelet suspensions.

RESULTS

Changes in Quantitative Ristocetin Aggregation With Time and Relation to pH

Figure 1 shows the tracings of ristocetin-induced aggregation of a single normal PRP kept at room temperature as a function of time after PRP preparation. There is a dramatic decrease in both the initial slope of aggregation and the total change in OD over the course of the 180 min. The results of ten experiments performed on the PRP from ten different donors are shown in Fig. 2, confirming the decrease in quantitative aggregation with time. Since it is well known that the pH of plasma rises as the CO$_2$ diffuses out into the ambient atmosphere, we sampled the pH of the PRP's in these ten experiments just before initiating aggregation. All samples (Fig. 3) showed a significant rise in pH although the rate of increase was quite variable. Analysis of the initial slope of aggregation as a function of pH (Fig. 4) revealed a close inverse relationship over the entire range of pH between 7.6 and 8.3.

To confirm a pH dependence irrespective of time after drawing, fresh PRP was rapidly adjusted to different pH's by the addition of exogenous acid or base. The results of ten experiments performed on PRP from eight donors (Fig. 5) again showed the dramatic decrease in aggregation over the range from 7.5 to 8.5. In addition, there appeared to be a plateau region between about 6.9 and 7.3 with a decrease in aggregation at pH's lower than 6.9. The same pattern was observed when the pH was adjusted by exposing the PRP to a 100% CO$_2$ environment and samples were tested as the pH decreased. Rapidly backtitrating PRP samples to their original pH (7.6) from 8.5 restored the initial rate of ag-
Fig. 2. Relationship between initial slope of ristocetin-induced aggregation and time after PRP preparation in ten experiments performed on the PRP from ten different donors. Lines and bars represent mean ± 1 SEM. Correlation coefficient, -0.802; SE of estimate 0.707; p < 0.001.

Fig. 3. Relationship between pH and time after PRP preparation in the ten experiments shown in Fig. 2. Lines and bars represent the means and ranges.
Fig. 4. Relationship between initial slope of aggregation and pH in the ten experiments shown in Fig. 2. Each point represents a single observation. Correlation coefficient, -0.822; SE of estimate, 0.687; p < 0.001.

Fig. 5. Relationship between initial slope of ristocetin-induced aggregation and pH in ten experiments on eight different donors. The pH of each sample was adjusted with HCl or NaOH. Lines and bars represent the mean ± 1 SEM.
gregation. When left open to the air for 3 hr and then backtitrated with HCl to the original pH, the slope was 80% of the initial value. When the pH of PRP was raised to 8.5 from 7.7 by bubbling nitrogen through the sample (to increase the diffusion of CO₂ out of the plasma), the same dramatic decrease in aggregation was observed.

Incubating the PRP under 5% CO₂-95% O₂ was effective in preventing the rise in pH and maintained a pH of 7.6 ± 0.1 over the 2½ hr of the experiment. Under these circumstances the initial slope of ristocetin aggregation decreased only slightly as a function of time (Fig. 6).

To evaluate if the pH effect resulted from an alteration in platelet energy metabolism, suspensions of formalinized platelets (which are incapable of energy metabolism) were prepared in a Hepes buffer adjusted to different pH's. These platelet suspensions were then tested for their ability to clump in response to ristocetin in the presence of normal reference plasma. The results (Fig. 7) paralleled those observed in PRP aggregation with fastest clumping occurring between 6.8 and 7.6 and slower clumping occurring at higher and lower pH's. The relationship held for all dilutions of the reference plasma tested.

**Factors Affecting pH Changes in PRP**

The results of an experiment to assess the complex interaction of factors affecting the rate of rise in pH of PRP over time are depicted in Fig. 8. The relationship between the volume of PRP and the surface area of the air–liquid interface plays a key role in determining the rate of rise. A large surface area-to-volume ratio maximizes the rate of change of pH, while a small one minimizes it. For a given surface area-to-volume relationship, smaller but quite significant
Fig. 7. Relationship between clumping time of formalinized washed platelets (after addition of dilutions of reference plasma and ristocetin) and pH of the buffer in which platelets were suspended. Inhibition of the reaction at high and low pH parallels the pattern with PRP aggregation. The same pattern is evident at each dilution of the plasma.

differences are noted between capped and uncapped specimens. The advantage conferred by capping, however, is lost if the specimen is periodically mixed.

A Device to Maintain PRP pH Constant

Since the rate of diffusion of CO₂ out of plasma is the prime determinant of pH change, we designed a simple device to maintain a pCO₂ of the surrounding air equivalent to the pCO₂ of the PRP samples at the desired pH. As shown in

Fig. 8. The effects of surface area-to-volume, capping, and mixing on the rate of rise of PRP pH over time.
Fig. 9. A simple device for maintaining the pH of PRP constant over time. A mixture of CO\textsubscript{2}–compressed air flows into the chamber and keeps the pCO\textsubscript{2} of the surrounding air equivalent to the pCO\textsubscript{2} of the PRP at the desired pH. The flow regulator allows for fine control and the humidifier prevents evaporation of samples.

Fig. 9, it is a four-sided Plexiglas structure which fits over a cuvette rack. A Plexiglas lid with a small central hole rests on top of the case. The lid is freely movable so that the hole can be centered over any part of the rack to put in or take out cuvettes. The lid’s large size insures that the rack is not exposed to the air even when the central hole is over the corners of the rack. Gas enters via a low port and exits through the hole in the lid and the two small slots at the top of each plexiglass wall. The fine adjustment of gas flow is controlled by a regulator (E-29-4-150MM3 Air Products and Chemicals, Allentown, Pa.). The gas is humidified (DEM Medical, Inc., Edison, N.J.) just before entering the chamber to prevent evaporation of the PRP samples. A 4\% CO\textsubscript{2}–96\% compressed air mixture (Air Products and Chemicals) can be easily regulated to maintain a PRP pH of 7.7 ± 0.05 for several hours, but a wide range of pH values can be achieved and maintained using different gas mixtures and flow adjustments. In six experiments with the device arranged to maintain a pH of 7.7, the initial slope of ristocetin-induced aggregation remained essentially constant for up to

Fig. 10. The initial slope of ristocetin-induced aggregation (expressed as percentage of the original value) over time when the pH of the PRP was held constant (at 7.7 ± 0.05) by the device shown in Fig. 8. Points represent the means of six experiments. The average coefficient of variation for the six experiments was ±13.5\% of the mean (range 11%–16%).
6 hr (Fig. 10). The average coefficient of variation for the six experiments was \(\pm 13.5\%\) of the mean (range 11\%–16\%). This variation compared very favorably with the coefficient of variation (\(\pm 13\%\) of the mean) obtained when a single PRP sample was tested six times in rapid succession. Moreover, with the pH of the PRP maintained at 7.7, we observed definite, although markedly reduced, aggregation (initial slope 4.0 OD units/min) in response to 1.8 mg/ml ristocetin in a patient with von Willebrand’s disease with 6% plasma von Willebrand factor. Thus, at this pH, quantitative platelet aggregation was sensitive to low levels of von Willebrand factor.

**DISCUSSION**

The pH dependence of ristocetin aggregation of normal PRP has several practical and theoretical implications. In previous studies, aggregation was performed within several hours after blood drawing, but the pH of the samples and the details of the storage conditions were not specified. Since it is possible to have a significant decrease in quantitative ristocetin aggregation in as short a period of time as 1 hr, it is vital that all studies be performed with pH control. It is common practice to test a normal control PRP along with the patient’s sample as a check on the reagents and equipment being used on a given day. If the patient’s sample is drawn first and then the normal control drawn, as it often is, from laboratory personnel just before performing the aggregation, a falsely low comparative value might be obtained for the patient’s sample. While all diagnoses of von Willebrand’s disease should be confirmed by specific assay of von Willebrand factor, the simpler, direct ristocetin aggregation of PRP is likely to become a commonly employed screening test. Failure to control PRP pH may lead to erratic and erroneous results.

The change in pH of PRP over time is variable. The major factors we identified were the air–liquid interface surface area-to-volume ratio, capping, and periodic mixing. All of these factors operate in a manner consistent with the hypothesis that the diffusion of CO\(_2\) out of the PRP is the controlling factor. In addition to these major factors, however, the acid–base properties of the aggregating agent, the concentration of the aggregating agent, the relative proportions of the PRP and aggregating agent employed, and the properties and concentration of the buffer in which the aggregating agent is dissolved will all influence the final pH of the aggregation mixture. Using trisodium citrate, the pH is usually 7.5 by the time the blood is centrifuged and the supernatant PRP removed. Centrifugation for longer periods of time for PPP (which is used to adjust the PRP to a constant platelet count and for establishing the lower OD limit) usually results in a pH of approximately 7.6. This pH is at the beginning of the steep decline in ristocetin aggregation with increasing pH; thus, relatively small differences in pH lead to significant changes in aggregation.

To overcome this problem, we devised a simple apparatus for controlling and maintaining PRP pH by adjusting the ambient pCO\(_2\). A Plexiglas case fits over a cuvette rack, and the flow of humidified CO\(_2\)-compressed air mixture is finely regulated to achieve the desired pH. Recognition of the importance of pH control in coagulation studies and ADP-, epinephrine-, and thrombin-induced platelet aggregation studies has led other investigators to recommend control of
pH by a variety of techniques: these include incubation in tightly capped tubes under a fixed ratio of CO₂ and compressed air,¹³,¹⁴ incubation under mineral oil,¹⁵ collection into a citrate buffer,¹⁶ and the addition of concentrated base immediately before testing.¹⁷ The first three techniques suffer from a lack of fine control and do not allow for varying the pH of a single sample. The use of concentrated base (0.2 N) is technically cumbersome and introduces another potential source of artifact, especially since the microenvironment around the drops of base will be transiently exposed to very high pH values. Our system is simple and easy to control. Since the optimum pH for aggregation studies with different reagents is still uncertain,¹⁴ the flexibility possible with our apparatus is a major advantage. By the appropriate choice of gas ratios, fine adjustment, and surface area-to-volume relationships, virtually any pH in the range of 6.0–8.5 can be quickly attained and then maintained constant for long periods of time. Potentially a different pH could be readily achieved for testing different reagents on a single PRP sample. Several reports in the literature¹³–¹⁵,¹⁷,¹⁸ in addition to our own preliminary studies show epinephrine and ADP aggregation to be maximum in the range 7.7–8.0. As a result, we have chosen to perform our ristocetin studies at pH 7.7. This procedure simplifies sample preparation since a single pH can be used for all studies. We found that a 4% CO₂–96% compressed air mixture can be easily regulated to maintain this pH for long periods of time. Under these circumstances quantitative ristocetin aggregation remains essentially constant. Zucker¹⁹ has observed that epinephrine-induced aggregation and the release reaction are less stable at pH 7.7 than at 7.2–7.4. It is advisable, therefore, to perform epinephrine and ADP aggregation quickly. An alternative approach using our device might be to store the PRP at 7.2–7.4 by adjusting the pCO₂ and then allowing the sample’s pH to rise to 7.7 just before performing the aggregation. Our study also points up a fundamental difference between ristocetin- and epinephrine-induced aggregation. Ristocetin aggregation remains constant over prolonged periods of time as long as the pH is maintained constant, while a decrease occurs in epinephrine aggregation despite a constant pH.¹⁹,²⁰ This difference can be attributed to the dependence of epinephrine aggregation on platelet metabolism and the release reaction, processes which are not important in the initial phase of ristocetin aggregation at a final concentration of 1.8 mg/ml.²¹

The mechanism by which pH affects ristocetin aggregation of PRP may offer insights into the ristocetin-induced aggregation phenomenon. An effect on platelet energy metabolism is unlikely since we found the same pH dependence with formalin-fixed platelets, which are incapable of energy metabolism, in a ristocetin clumping assay. Our studies are in accord with those of Allain et al.,²² who found a similar pH dependence of their von Willebrand factor assay. The influence of pH on ristocetin-induced aggregation of washed platelets was studied by Meyer et al.²³ and Howard.⁵ The former study showed that when plasma was titrated to below pH 6 or above pH 9 for 30 min the von Willebrand factor activity of the plasma dropped significantly. These studies utilized one part of plasma to four or five parts of a 15.4 mM Tris-buffered (pH 7.4) platelet suspension. The data were interpreted as showing the instability of von Willebrand factor at extremes of pH, but the possibility that the low or high pH
of the plasma interfered directly with the assay seems a likely alternative explanation. The latter study showed that the pH’s below 6.5 or above 8.0 caused rapid disaggregation of washed platelets that had been previously aggregated with ristocetin in the presence of normal plasma. In addition, ristocetin-induced aggregation of washed platelets in the presence of normal plasma was completely inhibited by pH’s below 6.8 and above 7.5. From this and other studies using heparin, urea, and anti-human factor VIII antisera, it was concluded that electrostatic forces were crucial to ristocetin aggregation. In studies on bovine factor VIII-induced aggregation of human platelets (a process analogous to the human von Willebrand factor platelet aggregation phenomenon, except that ristocetin is not needed to initiate the reaction10) Kirby and Mills11 also concluded that an electrostatic mechanism was most likely. In unpublished studies we observed that high salt concentrations inhibit both the von Willebrand factor assay and ristocetin-induced aggregation of PRP. This observation lends further support to an electrostatic mechanism. Han and Ardlie12 recently showed that increased pH altered ADP aggregation by decreasing the available calcium ions because citrate and albumin bind calcium more tightly as the pH increases. A similar mechanism is very unlikely in our studies since ristocetin aggregation can occur even in the presence of the much stronger calcium binder, EDTA.1

Nieto and Perkins24 showed that vancomycin (an antibiotic with peptide-binding properties very similar to ristocetin) demonstrated a marked pH dependence in its formation of stable complexes with peptides. They found a dramatic decrease at pH’s greater than 8.0 and less than 3.0. While their upper limit coincided with our data on ristocetin-induced aggregation, their lower limit was considerably lower than ours. They also found an absolute requirement for a free ionized carboxyl group on the peptide, suggesting that these antibiotics actually bind at the carboxyl group. This observation may be very important since there are data to support a pivotal role for the negative charge conferred by free carboxyl groups in maintaining platelets in an unagglutinated state. Neuraminidase treatment of washed platelets (with consequent removal of the negatively charged carboxyl groups on the sialic acid residues) results in spontaneous agglutination,10 and similar treatment of formalinized platelets enhances bovine factor VIII-induced agglutination.10 Similarly, Mester et al. reported a decrease in ADP-induced aggregation when platelets were enriched with sialic acid.25 We have recently observed26 spontaneous agglutination of formalin-fixed platelets when acidified to near the isionic point of platelets of 3.3.27 In addition, carboxyl groups are relatively unaffected by formalinization,28 a process which does not lead to spontaneous agglutination and does not alter ristocetin-induced platelet agglutination. Formalinized platelets will agglutinate, however, with polylysine or histone, both of which carry strong positive charges.10 Since ristocetin, with an isionic point of 8.2–8.3,8 is positively charged at pH’s below this value, it may neutralize some of the negative charges of any carboxyl groups to which it may bind. In a manner analogous to the effects of neuraminidase treatment on bovine factor VIII-induced platelet agglutination, this may permit agglutination to be induced by human von Willebrand factor. This interpretation could also explain the upper pH limit,
since, as the ristocetin loses its net positive charge at pH values above its isoionic point, it can no longer neutralize the negative charge of the carboxyl group. Recent studies by Macfarlane showing inhibition of ristocetin-induced agglutination of formalized platelets in the presence of purified factor VIII by peptides which are known to bind ristocetin and studies by Zucker et al. showing binding of ristocetin to formalized platelets, even in the absence of the von Willebrand factor, are consistent with the above mechanism.

Recent work in our own and other laboratories showed the von Willebrand factor to be a macromolecular glycoprotein (MW > 1.2 x 10^6) composed of multiple identical subunits. Such a molecule by virtue of its large size and multiplicity of identical sites would be ideally suited for acting as a bridge between platelets in a manner analogous to the strong red blood cell agglutinating properties of antigen-specific IgM molecules. We therefore propose a model in which the platelet contains sites that can interact with complementary sites on other platelets and result in agglutination. The carboxyl groups on proteins and sialic acid residues create a negative charge of sufficient intensity to prevent this interaction by electrostatic repulsion between platelets. The large von Willebrand factor glycoprotein can enhance agglutination by acting as a bridge between the underlying complementary sites or perhaps a different set of sites. In the unaltered platelets, the negative charges are great enough to prevent the human von Willebrand factor from causing aggregation. Ristocetin can combine with some of the ionized carboxyl groups, and it can reduce the negative charge to a level where the human von Willebrand factor can cause agglutination. Bovine factor VIII is less affected by the negative charge and can cause direct agglutination. This difference in action between human von Willebrand factor and bovine factor VIII may be related to the greater carbohydrate content of the latter since we have studied three von Willebrand patients with normal antigen but undetectable von Willebrand activity who had no PAS-stainable carbohydrate associated with their von Willebrand factor protein.

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