Factor VIII/von Willebrand Factor Protein: Sensitivity of Periodic Acid Schiff Stain to Carbohydrate Deficiency

By Harvey R. Gralnick, Georgia M. Jackson, Sybil W. Williams, and Mary C. Cregger

We have investigated the periodic acid Schiff (PAS) Coomassie staining ratio of the human factor VIII/von Willebrand factor (fVIII/vWF) protein. The PAS-Coomassie staining ratio is consistent over 8 days. The PAS-Coomassie ratio of the fVIII/vWF protein purified from different starting materials does not appear to be significantly different. The PAS stain can detect as little as 300 ng of carbohydrate in the fVIII/vWF protein. Desialation did not affect the PAS-Coomassie ratio, while removal of penultimate galactose resulted in a marked reduction in the PAS-Coomassie ratio. This reduction was further accentuated with the removal of N-acetylgalcosamine. The smaller multimers of the fVIII/vWF protein have a reduced sialic acid and PAS-Coomassie staining ratio. This difference does not appear to be related to the sialic acid deficiency but may be related to the distribution or organization of the carbohydrate moieties on the smaller fVIII/vWF multimers.

CARBOHYDRATE DEFICIENCY of the factor VIII/von Willebrand factor (fVIII/vWF) protein has been identified as one of the molecular defects in von Willebrand’s disease (vWd).1-4 Three methods have been used to determine carbohydrate deficiency of the fVIII/vWF protein: (1) the decreased precipitation of the vWd fVIII/vWF protein with concanavalin-A5 and abnormalities in two-dimensional immunoelectrophoresis in the presence of the concanavalin-A;6 (2) quantitative analysis of the carbohydrate of the fVIII/vWF protein;4 and (3) the use of the PAS stain of the fVIII/vWF protein in polyacrylamide gel electrophoresis (PAGE).7-8 We have previously reported carbohydrate deficiencies of the purified protein from some patients with vWd using this latter technique,1 while studies by Zimmerman and coworkers8 using an immuno precipitate of plasma fVIII/vWF could not detect any carbohydrate abnormalities in their patients. We have undertaken this study to determine the sensitivity of the PAS stain to the carbohydrate content of the normal fVIII/vWF protein.

MATERIALS AND METHODS

The fVIII/vWF protein was purified from three types of starting materials: (1) a high purity fVIII concentrate (Hyland Method IV, Costa Mesa, Calif.), (2) an intermediate purified vWF protein made by American Red Cross, Bethesda, Md., and (3) another high purity fVIII concentrate (Alpha Therapeutics, Los Angeles, Calif.). A two-step polyethylene glycol precipitation preceded chromatography on Sepharose 4B.7 The void volume fraction was collected after chromatography and dialyzed against imidazole saline, pH 7.0, prior to use in the assays.

The homogeneity of the fVIII/vWF protein was determined by electrophoresis in 5% PAGE in the presence of sodium dodecyl sulfate (SDS) with and without the addition of dithioerythritol (DTE).9 The estimation of protein in all pooled fractions was performed by the method of Lowry et al.7

The protein on the gels was stained with Coomassie brilliant blue 0.25% (BioRad Laboratories, Richmond, Calif.). The PAS stain was performed by the method of Mathieu and Quarles.10 The Coomassie and PAS stains were quantitated either by scanning on a Zeiss Gel Scanner at 550 nm or on a Helco scanner at 525 nm. The gels were rotated 90°-120° and scanned 3 times. A simple formula was used to compare the relative staining intensity determined by planimetry of the Coomassie and PAS related to the amount of protein put on each gel. The absolute staining intensity was divided by the amount of protein put on the gel to obtain a standardized reading. Then the percentage of the standardized PAS stains to the standardized Coomassie stain was calculated. In some experiments, the Coomassie and PAS stains were repeatedly determined on the same individual gels for intervals ranging from 2 days to 21 days. During this procedure, the Coomassie and PAS stained gels were scanned and the ratio compared over time. The gels were stored at 4°C and the tubes of the PAS gels were covered with aluminum foil.

In all studies a normal (intact) protein was part of each set of PAGE and was stained for Coomassie and PAS. A series of different protein concentrations (1.0-256 μg/gel) of the fVIII/vWF protein were subjected to PAGE, and the PAS and Coomassie stains were analyzed.

In some experiments the void volume fraction was divided in half, with the first part of the void volume fraction separated from the second part of the void volume fraction, i.e., the earlier eluting versus the later eluting part of the void volume. These fractions were treated identically, run on the same PAGE, and compared by the PAS and Coomassie staining.

Disulfide bonds of the fVIII/vWF protein were reduced by 2-mercaptoethanol (2-ME) at various concentrations as previously described11 to determine the effect of the size of artificially prepared smaller multimers on the PAS-Coomassie staining of the protein on PAGE.

Carbohydrate Studies

The fVIII/vWF protein void volume was studied before and after removal of sialic acid, penultimate galactose, and N-acetylgalcosamine. Clostridium perfringes neuraminidase (protease-free) was used at a final concentration 0.5-1.0 U/mg of fVIII/vWF protein.12 The bound or total sialic acid was measured by the method of Warren after hydrolysis, and after neuraminidase, the free sialic acid was determined by the same method without hydrolysis of the protein.12 In several experiments, the fVIII/vWF protein was sepa-
rated from free sialic acid and neuraminidase by chromatography on a 1 × 6 cm column of Sepharose 4B.

The asialo fVIII/vWF protein was treated with β-galactosidase (protease-free) derived from Streptococcus pneumoniae. The β-galactosidase was incubated at 0.5–1 U/mg fVIII/vWF protein at room temperature for varying periods of time. The release of penultimate galactose was monitored by the galactose dehydrogenase assay as described by Finch et al. In some experiments, the asialo-agalacto fVIII/vWF protein was subjected to PAGE prior to separation of the released sugars and stained with Coomassie and PAS. In other experiments, before PAGE the free galactose and β-galactosidase were separated from the fVIII/vWF protein by chromatography on small (1 × 6 cm) columns of Sepharose 4B. The asialo-agalacto fVIII/vWF protein was further modified by incubation with β-N-acetylglucosaminidase (0.5–1.0 U/mg fVIII/vWF protein) purified from Streptococcus pneumoniae. The release of N-acetylglucosamine was monitored by a colorimetric assay utilizing dimethylaminobenzaldehyde at 585 nm. This asialo-agalacto-

Fig. 1. Sodium dodecyl sulfate PAGE of fVIII/vWF protein (A) fVIII/vWF protein prior to neuraminidase treatment (Pre). From left to right are the stained gels of the nonreduced fVIII/vWF protein and the reduced fVIII/vWF protein (both stained with Coomassie blue) and the far right reduced fVIII/vWF protein stained with PAS. The protein does not enter the gel in the nonreduced state; in addition to the subunit, two faint bands are seen after reduction. (B) P NEU. The same preparation after treatment with neuraminidase, which removed over 90% of the sialic acid. The PAS and Coomassie stains are the same as the intact.
Factor VIII/von Willebrand Factor Protein

The fVIII/vWF protein that was purified from Sepharose 4B column chromatography was tested for homogeneity by PAGE in the presence of SDS both reduced and nonreduced. Only those materials that did not show multiple bands that entered the gel in the unreduced state were used for the subsequent studies (Fig. 1). When 11 different preparations of Hyland Method IV were assayed as to the PAS to Coomassie staining intensity, a mean value of 18.3% ± 2.7% (1 SD) was found with a range of observed 13.0%–26.6%. Alpha Therapeutics fVIII concentrate had a mean value of 14.6%, with a range of 13.2%–17.3%, while the intermediate purity Red Cross material had a value of 19.8%. The differences between the various preparations were not significant.

Examination of the stability of the PAS-Coomassie stain over time revealed no significant changes from 2 days to 8 days after staining had taken place. However, between 8 and 14 days after staining, there was a reduction of almost 50% in the PAS-Coomassie ratio. This was primarily due to alterations in the intensity of the PAS stain, although the Coomassie stain also decreased slightly. Thus, the gels could be observed and scanned for 2–8 days without significant change, but after that time it was unsafe to do any further studies using the PAS Coomassie ratio.

We examined the staining intensity of the PAS-Coomassie stains in relation to the protein concentration. We found a linear relationship between protein concentration and Coomassie staining (range 1–32 µg/gel) and PAS staining protein (range 2–46 µg/gel) (Fig. 2). Greater concentrations of protein result in a nonlinear relationship of the Coomassie-PAS ratio. When 2 µg of the fVIII/vWF protein were placed on the gel and stained with PAS, a distinct band was visible. Since 15% of the fVIII/vWF protein is comprised of carbohydrate, this PAS stain is sensitive to 300 ng of carbohydrate.

In examining the relative sensitivity of the PAS stain of the fVIII/vWF protein after partial deglycosylation, we found that with removal of over 91% of the sialic acid with neuraminidase, the PAS-Coomassie ratio was not significantly altered compared to the intact material (Table 1). Likewise, we did not find significant differences between 2 and 8 days in the PAS-Coomassie ratios of the asialo and intact material, suggesting that this was a consistent finding. The mean ratio of the asialo PAS-Coomassie ratio to the intact (i.e., considered 100%) was 96% with a range of 81%–109%. However, when the asialo fVIII/vWF protein was treated with β-galactosidase, which removed the penultimate galactose, the asialo-agalacto fVIII/vWF protein had a range of 36%–51% of the intact and a mean of 41% (the change in the percent PAS-Coomassie was related to a decrease in the PAS stain, while the Coomassie did not change) (Fig. 3). After the third modification with the β-N-acetylglucosaminidase, this material had a PAS-Coomassie range of 17%–44%, with a mean value of 25% of the intact material.

The sialic acid content (by acid hydrolysis) of the fVIII/vWF protein was 165 nmole/mg or 38 M/subunit, assuming a subunit molecular weight of 230,000. This comprised 32% of the total carbohydrate. Neuraminidase released 152–163 nmole/mg or 36 M/subunit of penultimate galactose or 18% of the total carbohydrate. This
resulted in a significant change in the relative staining intensity of the PAS stain. After treatment with \( \beta \)-N-acetylglucosaminidase, 151 nmole/mg or 35 M/subunit of N-acetylglucosaminidase were removed. The most marked differences between the PAS and Coomassie ratios were seen in the \( \gamma \) VIII/vWF protein treated with all three enzymes. The N-acetylgluco-
samine accounted for 26% of the total carbohydrate; thus, these enzymes reduced the carbohydrate content of the \( \gamma \) VIII/vWF by approximately 75%. No differences were noted in the PAS-Coomassie ratios before or after column chromatography (Sepharose 4B) to remove the enzymes and/or free carbohydrates.

When the void volume was divided into two parts, the early eluting half and the later eluting half, and the PAS-Coomassie ratios examined, we found that fraction 1 had a PAS-Coomassie ratio of 15% ± 2.0% while fraction 2 had a ratio of 10.0% ± 1.3%, values that were significantly different \( (p < 0.005) \) (Table 2, Fig. 4). When the sialic acid content of the two fractions was examined, the sialic acid content of fraction 1 was 138 ± 8.0 nmole/mg and of fraction 2 was 115 ± 10.0

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**Table 1. Modification of the Carbohydrate of the \( \gamma \) VIII/vWF Protein**

<table>
<thead>
<tr>
<th>( \gamma ) VIII/vWF Protein</th>
<th>Intact</th>
<th>AS(^*)</th>
<th>ASAG(^†)</th>
<th>ASAGAGluN(^‡)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS-Coomassie</td>
<td>100</td>
<td>96</td>
<td>41</td>
<td>25</td>
</tr>
<tr>
<td>Range</td>
<td>—</td>
<td>18–109</td>
<td>36–51</td>
<td>17–44</td>
</tr>
<tr>
<td>Sialic Acid</td>
<td>165 nmole/mg</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>&lt;8</td>
</tr>
<tr>
<td>Galactose§</td>
<td>152 nmole/mg</td>
<td>100</td>
<td>&lt;4</td>
<td>&lt;4</td>
</tr>
<tr>
<td>GlucNAc</td>
<td>151 nmole/mg</td>
<td>100</td>
<td>100</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Decrease of total carbohydrate</td>
<td>—</td>
<td>33</td>
<td>51</td>
<td>75</td>
</tr>
</tbody>
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*AS, asialo, after treatment with neuraminidase.
†ASAG, asialoagalacto, AS treated with \( \beta \)-galactosidase.
‡ASAGAGluN, asialoagalacto \( \alpha \)-N-acetylgalactosamine, ASAG treated with N-acetylglucosaminidase.
§Galactose, penultimate.
||Calculated using data from references 4 and 22.

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**Fig. 3.** The Coomassie and PAS densimetric tracings on SDS-PAGE. (A) The top tracing is the reduced intact \( \gamma \) VIII/vWF protein stained with Coomassie blue. The middle, AS, is the asialo form and the bottom, ASAG, is the asialo-agalacto form stained with Coomassie blue. (B) Identical amounts of protein were run simultaneously as in 3A but stained with PAS. The normal and asialo \( \gamma \) VIII/vWF proteins are similar, while the asialo-agalacto \( \gamma \) VIII/vWF protein has marked reduction in PAS reactivity. The anode of the polyacrylamide gel is to the right. The top (at the far left of each tracing) refers to the top of the polyacrylamide gel, which causes a small change in the densimetric scans.
Table 2. Comparison of Void Volume Fraction 1 and Fraction 2

<table>
<thead>
<tr>
<th></th>
<th>Percent PAS-Coomassie</th>
<th>Sialic Acid (nmole/mg)</th>
</tr>
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<tbody>
<tr>
<td><strong>Fraction 1</strong></td>
<td>15.1 ± 2.0*</td>
<td>138 ± 8</td>
</tr>
<tr>
<td><em>(n = 6)</em></td>
<td><em>(n = 6)</em></td>
<td></td>
</tr>
<tr>
<td><strong>Fraction 2</strong></td>
<td>10.0 ± 1.2</td>
<td>115 ± 10</td>
</tr>
<tr>
<td><em>(n = 6)</em></td>
<td><em>(n = 8)</em></td>
<td></td>
</tr>
<tr>
<td><strong>Fraction 2/fraction 1 (%)</strong></td>
<td>66</td>
<td>83</td>
</tr>
</tbody>
</table>

*Mean ± 1 SD.

\( n \), Number of determinations.

nmole/mg; these values were significantly different \( p < 0.01 \). The 17% reduction in the sialic acid did not explain the marked differences (34% reduction) in the PAS-Coomassie ratios when compared to the result of the asialo fVIII/vWF protein. From previous work\(^{11}\) we knew that there were smaller multimers in the second half of the void volume. We examined the PAS-Coomassie ratio of intact and artificially prepared small multimers. After treatment with 2-ME and alkylation with iodoacetamide, as previously described,\(^{11}\) these materials were then electrophoresed on PAGE and stained with PAS and Coomassie and compared to the intact fVIII/vWF protein. These studies showed that three different concentrations of 2-ME, 0.005%, 0.01%, and 0.1% 2-ME, caused marked alterations of multimeric composition, but did not change the PAS-Coomassie ratio of the subunit.

**DISCUSSION**

Several reports have indicated that the carbohydrate in the normal fVIII/vWF protein is important in its biologic activities. In early studies, Austin and Bidwell\(^{14}\) treated fVIII with an extract of *Trichomonas foetus*, which contained a variety of enzymes. They found that loss of fVIII coagulant activity could be inhibited by addition of glycoproteins or certain sugars. Vermyleen et al. reported that removal of the sialic acid from human cryoprecipitate proteins or semipurified fVIII/vWF protein resulted in a material that caused direct aggregation of human platelets,\(^{15}\) while modification of the penultimate galactose by galactose oxidase abolished this activity. Later, Vermyleen and coworkers reported that the asialo fVIII did not aggregate platelets without ristocetin nor did the loss of sialic acid change vWF activity.\(^{16}\) Levy-Toledano et al. reported that neuraminidase treatment of the bovine fVIII/vWF protein destroyed the platelet aggregating activity without affecting procoagulant activity,\(^{17}\) while Kirby and Mills had reported that treatment of either bovine or human material with neuraminidase did not affect the fVIII/vWF protein interaction with platelets.\(^{18}\) Sodetz et al. reported that removal of the sialic acid, but in addition, found that purified human fVIII/vWF protein reduced the vWF by approximately 50% but did not affect coagulant activity.\(^{19}\) We partially confirmed these findings; however, we did not find a change in vWF activity with removal of the sialic acid, but in addition, found that modification of the penultimate galactose by galactose oxidase resulted in marked reduction of vWF activity without affecting coagulant activity.\(^{7}\) We also could regenerate the vWF activity by reduction of the semialdehyde with borohydride. Further studies by Sodetz and coworkers have agreed with our observations regarding the role of galactose;\(^{20}\) however, the question of the role of sialic acid in vWF activity is still not clear. Rosenfeld and Kirby have subsequently reported that removal of the sialic acid from human or bovine fVIII/vWF protein results in decreased vWF activity.\(^{21}\) The data suggest that the vWF activity of the normal fVIII/vWF protein is at least partly related to the carbohydrate content and that galactose has a major fundamental role; the importance of sialic acid is not yet clear.

Fig. 4. The reduced fVIII/vWF protein from the early and later eluting portion of the void volume; 1 represents the early eluting fVIII/vWF protein and 2 the later eluting fVIII/vWF protein. C represents Coomassie and P represents PAS. The Coomassie stain of the early and later eluting material appears almost identical, while the PAS stain of the later eluting material shows a markedly reduced intensity compared to the PAS stain of the early eluting material.
We previously reported on a group of patients with vWd who had either decreased PAS stain of their fVIII/vWF protein and/or reduced sialic acid content of the fVIII/vWF protein. Bloom and Peake reported a group of vWd patients with decreased binding of their fVIII-related antigen to concanavalin-A, and these studies have in part been confirmed by Howard et al. Zimmerman and coworkers reported a group of patients with vWd in which they could not find any difference between the PAS and Coomassie stain.

Since the PAS stain of the fVIII/vWF protein is one of the simplest methods to partially quantitate carbohydrate abnormalities of this protein, we have undertaken a study to determine the sensitivity of the PAS stain to changes in the carbohydrate content and multimeric composition of the fVIII/vWF. We have found that, unlike other proteins and previous reports in the literature, the PAS is relatively insensitive to the change of the sialic acid content in the fVIII/vWF protein, thus, a reduction of over 30% of the total carbohydrate content does not change the PAS/Coomassie stain of normal fVIII/vWF protein. In fact, it requires almost a 50% decrease in the total carbohydrate content to see a modification of PAS-Coomassie stain ratio.

Our studies also indicate that the differences in PAS staining reactivity may simply not be related to the carbohydrate content but may be related in part to the multimer size of the fVIII/vWF protein, a conclusion not inconsistent with the results of Howard et al. We demonstrated a slight decrease in sialic acid content of the small multimers of fVIII/vWF protein; however, this 17% reduction of sialic acid would not explain the greater reduction of the PAS-Coomassie ratio. In further studies with artificially produced small multimers after treatment with 2-ME, we found no significance between the PAS-Coomassie stain, indicating that the naturally occurring smaller multimers in human plasma may have significant differences from the 2-ME-produced monomers in organization and/or distribution of the carbohydrate side chains detected by the PAS stain.

This study has examined the quantitation of the PAS-Coomassie stain of the normal fVIII/vWF protein. It allows for a simple method for quantitation of this ratio in the normal as well as abnormal fVIII/vWF protein. Similar types of studies may be helpful in examining the interaction of the fVIII/vWF protein with concanavalin-A in both normals and vWd patients and in detecting carbohydrate abnormalities of the fVIII/vWF protein.

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