THE INFLUENCE OF FIBRINOGEN AND ALBUMIN ON A RISTOCETIN COFACTOR (VWD) ASSAY

By

Jayne A. Zuhlke

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
of the degree of

MASTER OF SCIENCE

Department of Pathology

1981

ABSTRACT

THE INFLUENCE OF FIBRINOGEN AND ALBUMIN

ON A RISTOCETIN COFACTOR (VWD) ASSAY

By

Jayne A. Zuhlke

It has been noted that ristocetin causes the precipitation of fibrinogen. In ristocetin cofactor assays this precipitate can mask the platelet agglutination endpoint. If high concentrations of ristocetin (greater than 1.5 mg/ml) are used the precipitate is heavier. This can lead to falsely normal results obtained in plasmas from von Willebrand's disease (vWD) patients. Bovine albumin when added to the assay will inhibit the formation of the precipitate.

This study was undertaken to identify the proper albumin and ristocetin concentrations needed to minimize this effect. Optimum concentrations for this assay were found to be 20 mg/ml bovine albumin with 1.0-1.2 mg/ml of ristocetin.

DEDICATION

To my parents, Charles and Donna Chrisman for their interest and support in my education. To my husband, Dave, for bearing with me while I worked on this.

ACKNOWLEDGMENTS

I wish to express my appreciation to the following people:

Joan C. Mattson, M.D., my major professor for her guidance throughout my research.

Thomas G. Bell, D.V.M., Ph.D., for his interest in the development of a sensitive assay for von Willebrand factor.

Garson H. Tishkoff, M.D., Ph.D., for serving as a committee member.

Martha T. Thomas, M.S., M.T.(ASCP), my academic advisor, for her continued support and guidance in my graduate study.

Belinda J. Oxender, for her excellent typing.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
LITERATURE REVIEW	2
von Willebrand's Disease and its Relationship to the Factor VIII complex	2
History	2
Biochemistry of the Factor VIII:vWF and its Relationship to the VIII Complex	5
Role of von Willebrand Factor	7
Clinical Aspects	8
Congenital and Variant Forms	8
Acquired Forms	10
Quantitative and Qualitative Defects of VIII:vWF	10
Platelet-von Willebrand Factor-Ristocetin Interaction	12
MATERIALS AND METHODS	15
Preparation of Formalin-Fixed Washed Platelets (FWP).	15
Standard and Sample Collection and Preparation	16
Modified Ristocetin Cofactor (Ristocetin Plate Test).	16
Interpretation of Test Results	19
Canine Ristocetin Cofactor Assay (Plate Test)	19
RESULTS	20
Fibrinogen Precipitation Induced by Ristocetin as Measured by Changes in Light Transmission	20
Effect of Presence or Absence of FWP on Fibrinogen Precipitation Induced by Ristocetin	22
Inhibitory Effect of Bovine Albumin on Ristocetin-Induced Fibrinogen Precipitation	26
Effect of Albumin Concentration on a vWD Plasma Tested with Ristocetin Plate Test	33
Varying Ristocetin Concentrations	34
Effect of Serum and Defibrinated Plasma Samples on von Willebrand Factor Activity, Factor VIII Clotring Activity and Factor VIII Antigen Levels	36
Effect of Ristocetin Concentration on Canine Ristocetin Cofactor Assays	37

Page

Effect of Albumin Concentration on Canine Ristocetin Cofactor Assays..... 38
Effect of Ristocetin on Clottable Fibrinogen Levels of Fibrinogen Solutions, Normal Plasma and vWD Plasma..... 39

DISCUSSION..... 42

CONCLUSION..... 46

LITERATURE CITED..... 47

APPENDIX..... 52

VITA..... 53

LIST OF TABLES

TABLE		Page
1.	Subtypes of vWD	9
2.	Effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation (ristocetin 1.5 mg/ml) in the absence of FWP	24
3.	Effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation (ristocetin 1.5 mg/ml) in the presence of FWP	26
4.	Time for platelet agglutinates to form with ristocetin and varying albumin concentrations. Ristocetin concentration 1.2 mg/ml	28
5.	Effect of various fibrinogen concentrations on the ability of albumin (20 mg/ml) to inhibit precipitation (plate test) with and without FWP present. Ristocetin concentration 1.2 mg/ml	31
6.	Fibrinogen precipitation in the presence and absence of albumin (20 mg/ml) with FWP present (plate test). Ristocetin concentration 1.2 mg/ml	32
7.	Fibrinogen precipitation in the presence and absence of albumin (20 mg/ml) in the absence of FWP (plate test). Ristocetin concentration 1.2 mg/ml	32
8.	Percent activity of a vWD plasma with varying albumin concentrations in the ristocetin plate test. Ristocetin concentration 1.2 mg/ml	33
9.	Effect of various ristocetin concentrations on the time for precipitate formation with serum, fibrinogen, PNP and VIII deficient plasma	35
10.	Effect of ristocetin concentration on quantitating the VIII:vWF activity of a known vWD patient (normal range 50-160%). Albumin present in a concentration of 20 mg/ml	35
11.	Results of VIII:C, VIIIR:Ag and VIII:vWF assays on 2 normal serum samples and defibrinated plasma samples	37

TABLE Page

12. Canine vWD plasma levels of ristocetin cofactor activity with varying ristocetin concentrations. Albumin in a concentration of 20 mg/ml..... 37

13. Effect of varying albumin concentrations in a canine ristocetin cofactor assay (plate test). Ristocetin concentration 1.0 mg/ml..... 39

14. vWD plasma fibrinogen levels before and after the addition of ristocetin (1.0 mg/ml, 1.5 mg/ml, and 3.0 mg/ml) 40

15. PNP fibrinogen levels before and after the addition of ristocetin (1.0 mg/ml, 1.5 mg/ml, and 3.0 mg/ml)..... 40

LIST OF FIGURES

FIGURE		Page
1.	Typical standard curve for the ristocetin plate test....	18
2.	The effect of two concentrations of ristocetin on fibrinogen as measured by a decrease in light transmission.....	21
3.	The effect of ristocetin on various concentrations of fibrinogen (3.25 mg/ml, 6.5 mg/ml and 13.0 mg/ml) as measured by changes in light transmission.....	23
4.	The effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation in the presence and absence of FWP.....	25
5.	The effect of various albumin concentrations (0 to 40 mg/ml) on ristocetin-induced fibrinogen precipitation as measured by changes in light transmission.....	27
6.	The effect of various albumin concentrations (0 to 40 mg/ml) on the slope of standard curves for the ristocetin plate test.....	29
7.	The effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation in the presence of 20 mg/ml albumin (with and without FWP).....	30

Abbreviations and terminology used in this thesis:

vWD	von Willebrand's disease
VIIIR:Ag*	Factor VIII-related antigen
VIII:C*	Factor VIII-procoagulant activity
VIII:vWF	Factor VIII-von Willebrand factor
VIIIR:RCF*	Factor VIII-related ristocetin cofactor
FWP	formalin-fixed washed platelets
PPP	platelet-poor plasma
PNP	pooled-normal plasma

*Nomenclature proposed by the International Committee on Thrombosis and Haemostasis.

INTRODUCTION

von Willebrand factor plays an important role in primary hemostasis. It is necessary for normal platelet adhesion to the exposed collagen and basement membrane of a damaged blood vessel wall. In von Willebrand's disease this factor is deficient and individuals with this disorder characteristically have prolonged bleeding from minor wounds. Besides its role in platelet adhesion, von Willebrand factor also stimulates the release and/or production of Factor VIII clotting activity; the activity that is decreased or absent in hemophilia.

This research project investigates the influence of albumin and fibrinogen on a ristocetin assay for quantitating von Willebrand factor. In these studies, it became apparent that most of the ristocetin assays do not take into account ristocetin-induced precipitation of fibrinogen and other plasma proteins. It is apparent that precipitation can mask the ristocetin-platelet-von Willebrand factor interaction and that a deficiency of von Willebrand factor could therefore be missed. Others have found that, albumin will inhibit the interfering precipitation. Determining the proper concentrations of albumin and ristocetin needed to accurately measure the von Willebrand factor in human and canine plasma, are the subject of this report.

LITERATURE REVIEW

von Willebrand's Disease and its Relationship to the Factor VIII Complex

History

In 1926 Erik von Willebrand published his findings of a bleeding disorder he found in several family members. The family lived on the Aland Islands located off the coast of Finland. He described a hemorrhagic diathesis characterized by bleeding from the nose, gums, and tooth extraction sites and menorrhagia. In his publication he pointed out that hemarthrosis, common in hemophilia, was not seen in these family members. Further, both sexes were affected. He found prolonged bleeding times with normal platelet counts in this family. He called this disease hereditary pseudohemophilia. In 1933, von Willebrand and Jürgens reevaluated the patients in Aland and concluded that the disorder was due to impaired platelet function and a platelet factor 3 deficiency. This bleeding disorder was then renamed von Willebrand-Jürgens Thrombopathy (1).

It was not until the 1950's that more literature was published on similar bleeding problems. It was at this time that Factor VIII clotting activity was studied. Nilsson *et al.* in 1957 found that the Factor VIII clotting activity was decreased when he studied the affected family members on the Islands of Aland (2). Nilsson and coworkers also found that infusion of Factor VIII concentrate corrected the Factor VIII deficiency and also corrected the prolonged bleeding

times in these patients. In 1959, Nilsson and Blömback infused plasma from severe hemophiliacs into their von Willebrand patients and found that the bleeding time was again corrected and also Factor VIII production was stimulated. These significant findings lead to the conclusion that there is a plasma factor present in normal and hemophiliac plasma but missing in the plasma of von Willebrand patients. It is this factor, the von Willebrand factor that is responsible for normal platelet function and for the stimulation of Factor VIII production (3). These findings have been confirmed time and time again.

In 1960 Borchgrevink studied platelet adhesion by comparing venous platelet counts with platelet counts from a capillary lesion. He found that patients with von Willebrand's disease (vWD) had decreased platelet adhesiveness (4). Three years later Salzmann using a modified technique of Hellem, found that platelets from von Willebrand patients show decreased adhesiveness to glass beads (5). When normal plasma, hemophiliac plasma or Factor VIII concentrates were infused into vWD patients platelet adhesiveness returned to normal.

In the 1970's Baumgartner and colleagues demonstrated decreased adhesion of vWD platelets to the subendothelium of rabbit aorta. This decreased adhesion was corrected by addition of normal plasma (6). These were similar findings to those of Jorgensen and Borchgrevink (7). These findings indicate that von Willebrand factor is necessary for normal primary hemostasis - its presence enhances platelet adhesiveness.

In the 1970's much more information about von Willebrand factor has been obtained. In 1971 antisera to Factor VIII were produced in rabbits (8). Laurell's electroimmunoassay (9) and gel chromatography (10) showed that hemophiliac patients have normal amounts of Factor VIII

related antigen (VIIIR:Ag) as identified by rabbit antisera, even-though this molecule has virtually no coagulant activity. Patients with vWD on the other hand had low levels of VIIIR:Ag.

Bouma *et al.* found that human VIIIR:Ag from normal and hemophiliac plasma would correct platelet adhesion and bleeding times of dogs with vWD (11). This antigen thus possesses the von Willebrand factor activity (VIII:vWF). In 1975 Zimmerman and colleagues and Nilsson and Holmberg studied VIIIR:Ag by crossed-immunoelectrophoresis. Because of the broad band that was found by this method it was agreed that there are multiple molecular forms and thus molecular heterogeneity of the normal VIIIR:Ag (12, 13).

Using SDS agarose electrophoresis Ruggeri and Zimmerman found 10 distinct multimers of VIIIR:Ag which can be separated according to size. The smaller forms have little or no VIII:vWF activity while the larger molecular forms appear to have most of the VIII:vWF activity (14). This has been repeated by others (15). In vWD there are variants which are apparently molecular abnormalities of the VIIIR:Ag where some of the larger molecular forms are decreased or absent.

Much work has been done to show that patients with vWD have no intrinsic platelet defects. In 1971 Howard and Firkin discovered that ristocetin, an antibiotic, induced platelet aggregation of normal platelets but did not cause aggregation of most of the vWD platelets that they tested. When normal plasma or hemophiliac plasma was added to the vWD platelets their ability to aggregate in the presence of ristocetin was restored (16). Meyer *et al.* in 1973 observed that antisera to VIIIR:Ag inhibited ristocetin-induced aggregation of normal platelets (17). These observations indicate that VIIIR:Ag

is responsible for the ristocetin cofactor activity (VIIIR:RCF) in plasma. Several methods have been developed to quantitate VIIIR:RCF (18, 19, 20, 21, 22, 23, 24). Most of the methods use washed (20, 23), gel-filtered (24), or formalin-fixed normal platelets (18, 19, 20, 21) although more recently lyophilized normal platelets (25) are being used. In general, all the methods use a normal platelet substrate, test plasma and ristocetin. Ristocetin is generally in a final concentration of 1.0-1.5 mg/ml in these assays. In addition to ristocetin, polybrene (26) and snake venoms (27) have been used to quantitate VIII:vWF activity. These methods have not been widely used.

Biochemistry of the Factor VIII:vWF and its Relationship to the VIII Complex

The Factor VIII complex is characterized by three major activities: Factor VIII-related antigen (VIIIR:Ag), procoagulant activity (VIII:C) and von Willebrand factor activity (VIII:vWF). The exact relationship of VIII:vWF to VIIIR:Ag and VIII:C has been the subject of much debate. There have been three main hypotheses concerning the nature of the Factor VIII complex. It has been suggested that it consists of one molecule that exhibits all of the VIII related activities (23). Another hypothesis is that it may consist of 2 molecules one with the coagulant activity and one with the platelet-related activity (28). Most experimental data suggests that Factor VIII is a complex of a lower molecular weight procoagulant molecule linked by non-covalent bonds to a high molecular weight glycoprotein that exhibits the VIII:vWF activity and the VIIIR:Ag activity.

Hemophiliac plasma has been shown to correct both the abnormal platelet retention (29) and the abnormal ristocetin aggregation (30). This suggests that on the Factor VIII molecule, Factor VIII:vWF may be located at a different site than that which determines the Factor VIII:C activity.

Using gel chromatography to purify Factor VIII from cryoprecipitate Weiss *et al.* showed that antisera from human hemophiliacs with known inhibitor activity inhibits VIII:C activity but does not abolish VIII:vWF activity. Even though VIII:C activity was blocked, ristocetin-induced aggregation of normal washed platelets still occurred (23). This further supports the postulate that VIII:vWF and VIII:C are located on different sites of the VIII molecule.

There is evidence that VIII:vWF activity is closely associated with VIIIR:Ag. Until recently no one has been able to clearly separate VIII:vWF from VIIIR:Ag. In 1980 Barrow *et al.* used two antibodies to Factor VIII to demonstrate that passage of human plasma over a rabbit antibody column completely removed VIIIR:Ag but not the VIII:C or VIII:vWF activities. The VIIIR:Ag-free plasma was then passed over a column containing human VIII:C antibody (hemophiliac plasma with an inhibitor) which removed VIII:C. The amount of VIII:vWF that remained was 60% of the original plasma. Thus Barrow's work appears to demonstrate three separable activities of the Factor VIII complex (31).

According to Olson *et al.*, VIII:vWF is a high molecular weight polymer of disulfide-linked subunits, each having a molecular weight of 230,000 daltons (32). Ruggeri and Zimmerman also agree that VIII:vWF has a multimeric composition. Ten distinct bands are present in normal

plasma on reduced SDS agarose gel electrophoresis (14). Counts *et al.* (33) and Perret *et al.* (34) found that mild reduction of purified VIII:vWF resulted in a band with a molecular weight of about 500,000. They concluded that this was a dimer of the 230,000 MW subunit. In 1978 Fass *et al.* studied porcine VIII:vWF and found it to be a polymeric series of multimers each of which contains 6 to 8 subunits of 230,000 MW (35). There is evidence that the higher molecular weight forms exhibit most of the ristocetin cofactor activity. They bind to platelets in the presence of ristocetin and may be involved in the platelet activities of VIII:vWF (14, 15).

Role of von Willebrand Factor

Individuals with reduced or absent von Willebrand factor activity have a defect in primary hemostasis and so demonstrate prolonged bleeding from minor wounds. VIII:vWF is necessary for normal platelet adhesion to collagen of the subendothelium of an injured blood vessel (6). In the absence of VIII:vWF platelets do not adhere and no platelet plug forms. Bleeding will continue through the injured vessel wall.

The synthesis and/or release of VIII:C into the plasma has been found to be stimulated by VIII:vWF. Blombäck and Nilsson in 1959 observed that Factor VIII:C increases during a 24 hour period in vWD patients following transfusion of normal plasma. The normal plasma presumably supplied VIII:vWF which stimulated the synthesis and/or release of more VIII:C (1).

Another role of VIII:vWF has been suggested by Weiss *et al.* They found that VIII:vWF has a stabilizing affect on VIII:C. VIII:C is more labile when VIII:vWF is not present (36).

Clinical Aspects

Individuals with vWD clinically present with symptoms such as epistaxis, menorrhagia, excessive bleeding from minor wounds and mucous membrane bleeding. The severity of bleeding is variable depending on the level of VIIIR:Ag and VIII:vWF.

Congenital and Variant Forms

Both dominant and recessive forms of vWD have been reported. Classic vWD (Type I) is inherited as an autosomal dominant trait. Individuals who are heterozygous have moderate bleeding problems. This is the most common form of the disease. This form is characterized by prolonged bleeding times, reduced platelet aggregation in response to ristocetin, decreased platelet adhesion (retention), decreased VIIIR:Ag, decreased VIII:C activity, and decreased VIII:vWF as determined by the ristocetin cofactor assay.

Homozygous individuals with autosomal dominant inheritance have not been clearly described. This is the most severe form of the disorder with hemarthroses as a common clinical symptom (37).

Autosomal recessive inheritance has been described in some families (38). Heterozygotes are essentially symptomless while homozygotes have manifestations of vWD.

Bloom *et al.* has described both dominant and recessive patterns within the same kindred (39). In these families it is unlikely that two different abnormal genes coexist. A possible explanation for this occurring may be the biochemical nature of VIII:vWF. The normal VIII:vWF is thought to have homologous oligomers with the high molecular weight forms having the ristocetin cofactor activity. Abnormal genes

inherited from one parent may result in biologically abnormal VIII:vWF oligomer(s). Which oligomers or how many oligomers are affected may influence the severity of the bleeding disorder.

The variant forms of the disease may also be created in this manner. In the variants some of the diagnostic tests will be normal, some abnormal thus making a definitive diagnosis difficult (Table 1). Bleeding times, VIII:C activity, VIIIR:Ag and VIII:vWF levels must all be determined to avoid missing a variant of the disease. Much emphasis must be placed on clinical symptoms since laboratory evaluations may leave many questions unanswered. For example, ristocetin cofactor assays can show markedly increased VIII:vWF activity if the variant is subtype IIb (14) compared to reduced VIII:vWF activity in subtype IIa (Table 1).

Table 1: Subtypes of vWD (1, 14)

	BT*	VIII:C	VIIIR:Ag	VIIIR:RCF
Type I	Prolonged	Reduced	Reduced	Reduced
Type IIa	Prolonged	Normal or Reduced	Normal	Reduced
Type IIb	Prolonged	Normal or Reduced	Normal	Increased
Type III	Prolonged	Normal	Reduced	Reduced
Type IV	Normal to Slightly Prolonged	Reduced	Reduced	Reduced

*Bleeding Time

Acquired Forms

Acquired vWD has recently been reported. In these acquired cases the VIII:RAg, VIII:C and VIII:vWF levels were decreased. All cases had negative family histories for vWD.

Handin *et al.* in 1976 described an antibody-induced vWD in a patient with lymphosarcoma (40). This antibody prevented aggregation of normal platelets by ristocetin, but did not interfere with the measurement of VIIIR:Ag nor did it inhibit VIII:C. The patient was not thrombocytopenic. It appeared that the antibody was directed specifically against VIII:vWF. Rosborough and Swaim in 1978 reported a case of angiodysplasia with acquired vWD (41). No VIIIR:Ag and VIII:vWF were detectable in this patient's plasma. No inhibitor to VIII:vWF was found. This patient also had abnormal platelet release which also contributed to his bleeding problems.

In general acquired vWD has been reported in association with autoimmune (42) or lymphoproliferative disease including systemic lupus erythematosis (43) and monoclonal gammopathy (44). With further study of acquired vWD perhaps a better understanding of the defect in this disease will be gained.

Quantitative and Qualitative Defects of VIII:vWF

In classic vWD (subtype I) it is probable that there is a quantitative defect of the VIII complex and thus all the hemostatic activities associated with this complex are diminished. This type can be inherited or acquired.

In the other subtypes of vWD there are a variety of qualitative molecular defects creating the reduced activities and thus the bleeding

problems. Nilsson and Holmberg feel that the VIII:vWF activity is primarily a function of a highly aggregated Factor VIII. Factor VIII that is less polymerized will not function normally (1). Ruggeri and Zimmerman (1980) in extensive research have suggested that molecular defects may be due to mutations causing the inability to produce the proper multimers. In vWD subtype IIa the larger multimers (needed for normal ristocetin cofactor activity) cannot be produced. In subtype IIb there is evidence that the VIII:vWF multimers produced are functionally defective, even though this subtype can produce the larger multimers (14).

In variants studied by Gralnick and colleagues using PAS reactions, they observed a decreased carbohydrate content of VIII:vWF. In these variants both the VIIIR:Ag and the VIII:C were normal while the bleeding times, ristocetin-induced platelet aggregations, platelet adhesions and the ristocetin cofactor assays were abnormal. This finding suggests that the carbohydrate moiety of the VIII:vWF is important in regulating hemostatic platelet activities. A specific glycolytic enzyme could not be identified in the plasma of these patients to account for the deficiency (45). Gralnick *et al.* concluded that the carbohydrate deficiency was due to a defect which did not allow the carbohydrate to attach in the proper sequence or in the quantity seen in normal VIII:vWF. Since platelet retention was reduced and ristocetin-induced aggregation was abnormal this suggests that carbohydrate deficient VIII:vWF cannot interact normally with normal platelets or the blood vessel wall, or both.

In a 1980 publication, Morisato and Gralnick treated VIII:vWF with neuraminidase, galactose oxidase and β-galactosidase. They found that

asialo VIII:vWF retained its ability to agglutinate platelets in the presence of ristocetin, while asialo agalacto VIII:vWF lost most of this activity. This suggests that galactose controls the attachment of VIII:vWF to platelets so that agglutination can occur (46).

Platelet-von Willebrand Factor-Ristocetin Interaction

In the presence of VIII:vWF, washed, normal or formalin-fixed human platelets agglutinate following the addition of ristocetin. Ristocetin is a cationic glycopeptide antibiotic (47). At present, the mechanism by which VIII:vWF interacts with platelets to cause aggregation is still unclear. It is not known if VIII:vWF and ristocetin form a complex or if VIII:vWF is modified by ristocetin so that it can bind to and agglutinate platelets. There are many hypotheses of the platelet-ristocetin-VIII:vWF interaction.

A list of the hypotheses of the platelet-ristocetin-VIII:vWF interaction would include:

1. Binding sites for VIII:vWF on platelet surfaces, exposed by ristocetin.
2. Ristocetin interacts with VIII:vWF so that VIII:vWF molecules both aggregate and bind to platelet membranes forming bridges.
3. Ristocetin binds to platelets directly and bridges platelets in the presence of VIII:vWF.
4. Mutual binding of ristocetin and VIII:vWF to platelets to cause agglutination.
5. Ristocetin neutralizes the negative charge on platelets so that they have reduced repulsion.

Kao *et al.* in 1979 demonstrated specific binding sites on human platelets by using ^{125}I-VIII:vWF. Kao and colleagues found VIII:vWF binding to platelets was ristocetin-dependent and increased with increasing concentrations of ristocetin (48). This was also observed by Morisato and Gralnick (46) and by Moake and colleagues (47). Immunofluorescent techniques using fluorescein tagged antibody to VIII:vWF showed fluorescent staining of normal platelets while platelets from patients with vWD showed no staining (49).

Kattlove and Gomez feel that ristocetin interacts with VIII:vWF so that VIII:vWF molecules both aggregate and bind to platelet membranes forming bridges between adjacent platelets (50). Several workers have attempted to demonstrate that ristocetin binds to platelets directly. They have not been able to successfully show this (51).

Another theory of the mechanism of interaction is the mutual binding of ristocetin and VIII:vWF to platelets. The binding of one may promote the binding of the other (51). This has not been observed. Conformational change in VIII:vWF caused by interaction with ristocetin may render VIII:vWF more reactive so that it now binds to and agglutinates platelets, or the binding of ristocetin to VIII:vWF may cause polymerization of the VIII:vWF thus forming large aggregates. Platelets could be trapped within or stick to these aggregates (51). Ristocetin precipitates fibrinogen which could form fibers to entrap platelets (16, 52). Possibly fibrinogen is involved in the ristocetin-VIII:vWF-platelet interaction.

Ristocetin has also been shown to neutralize platelet negative charge. At physiologic pH ristocetin has a net positive charge which would decrease the platelet negative charge. Repulsion between

platelets would be reduced and agglutination could occur (46, 51). This theory of interaction seems unlikely since formalin-fixed platelets will agglutinate in the presence of ristocetin and they presumably carry very little charge since they are not living cells. The neutralization of charge may play a part in the interaction but it is probably not the sole reason for the ristocetin-induced platelet aggregation.

Platelet membrane glycoproteins have been implicated in ristocetin-induced aggregation. Removal of glycoproteins by proteolytic enzymes such as chymotrypsin, results in platelets which show reduced aggregation in the presence of ristocetin (53). The mechanism of ristocetin-induced aggregation somehow requires both a platelet receptor (probably membrane glycoproteins) and VIII:vWF. Recently there has been evidence published that glycoprotein I complex on the human platelet membrane is the site for ristocetin-VIII:vWF-platelet agglutination. Nachman and colleagues using double-antibody immunoprecipitation showed that the antibody which blocked ristocetin reactions interacted with a protein of MW 155,000 located on the platelet membrane. When they added glycoprotein I, ristocetin reactions were blocked indicating that either ristocetin or VIII:vWF or both attached to the glycoprotein (54). It seems likely that the platelet membrane has specific binding sites for VIII:vWF and recent studies have clearly demonstrated this (46, 47, 48, 49). Ristocetin is necessary for this binding to occur but the exact mechanism of interaction is still unclear.

MATERIALS AND METHODS

Preparation of Formalin-Fixed Washed Platelets (FWP)

Platelets were prepared by a modified method of Allain *et al.* (18). Platelet concentrates from the American Red Cross were used. Concentrates less than 48 hours out-dated were incubated at 37 C for one hour. After incubation the unit of platelets was poured into large plastic tubes. An equal volume of 2% paraformaldehyde was added to each tube. If more than one unit of platelets was prepared they were not pooled but prepared separately. The platelet-formalin mixture was mixed by gentle inversion and then allowed to incubate at 4 C for at least 18 hours.

After the 4 C incubation the tubes of platelets were centrifuged at 300 g for 10 minutes at 4 C. The supernatant was discarded and the remaining platelet pellet was gently resuspended in 10 ml of Tris-saline, pH 7.4. This suspension was centrifuged at 300 g for 10 minutes at 4 C. After centrifugation the supernatant was discarded and the platelet pellet was again resuspended in 10 ml of Tris-saline buffer. This washing procedure was repeated 4 more times. After the final wash, the platelets were resuspended in about 3 ml of Tris-saline buffer containing 0.05% sodium azide. A platelet count was performed and the platelet suspension was adjusted to a count of 1,000,000/cmm. This suspension when stored at 4 C was stable for at least six weeks.

Just prior to running an assay, the formalin-fixed washed platelets (FWP) were diluted 1:2 in Tris-saline buffer containing 60 mg/ml bovine albumin (final concentration 20 mg/ml). The FWP were brought to room temperature just before use in the assay.

Standard and Sample Collection and Preparation

Whole blood was collected in a siliconized vacutainer tube containing 3.8% sodium citrate. When this method was not possible to perform, a plastic syringe was used for blood collection. In both cases nine parts of whole blood weremixed with one part of 3.8% sodium citrate. Blood and anticoagulant were gently mixed and then centrifuged at 800 g for 10 minutes to obtain platelet-poor plasma (PPP). PPP was placed into plastic tubes.

To prepare a normal standard, PPP from 10-15 normal donors was pooled. The pooled plasma was then aliquoted into plastic 12 x 75 mm tubes, capped and frozen at -25 C.

A lyophilized assayed reference plasma (ARP from Helena Labs, Beaumont, TX) was also used to construct the standard curves. ARP produced curves similar to pooled normal plasma curves.

Samples to be assayed were collected in a similar manner and frozen in plastic tubes at -25 C until tested. Just prior to testing, standard PPP (or ARP) was diluted 1:2, 1:4, 1:8 in Tris-saline, pH 7.4. Test samples were diluted 1:2 and 1:4 in Tris-saline, pH 7.4.

Modified Ristocetin Cofactor (26, 55) (Ristocetin Plate Test)

The major modification of this assay was the use of a black plastic plate to visualize the macroscopic platelet agglutination. A standard curve was prepared from the dilutions described above.

Twenty microliters of undiluted or diluted PPP were mixed with 0.1 ml of FWP that had been previously pipetted on the black plate. A wooden applicator stick was used to mix these together. After a 30 second incubation, 0.03 ml of ristocetin (final concentration of 1.2 mg/ml) was added to the PPP-FWP mixture. Simultaneously a stopwatch was started. The plate was gently rocked while observing it through a magnifying glass lamp. When the first small platelet agglutinates were visible the stopwatch was stopped and the time recorded. Using log-log graph paper a standard curve was plotted. Seconds for platelet agglutination was on the ordinate and percent von Willebrand factor activity (ristocetin cofactor) was on the abscissa (undiluted standard equals 100%, standard diluted 1:2 equals 50%, etc.).

Example:

Standard	Seconds to Agglutination (duplicates)	% vWF Activity
PPP (undiluted)	32-30	100
PPP (1:2)	41-40	50
PPP (1:4)	56-59	25
PPP (1:8)	78-74	12.5
Buffer (no PPP)	>120	0

An example of a standard curve is shown in Figure 1.

Test samples, diluted and undiluted are tested in exactly the same manner. The seconds until platelet agglutination occurred was compared to the standard curve and percent ristocetin cofactor activity was read from the curve.

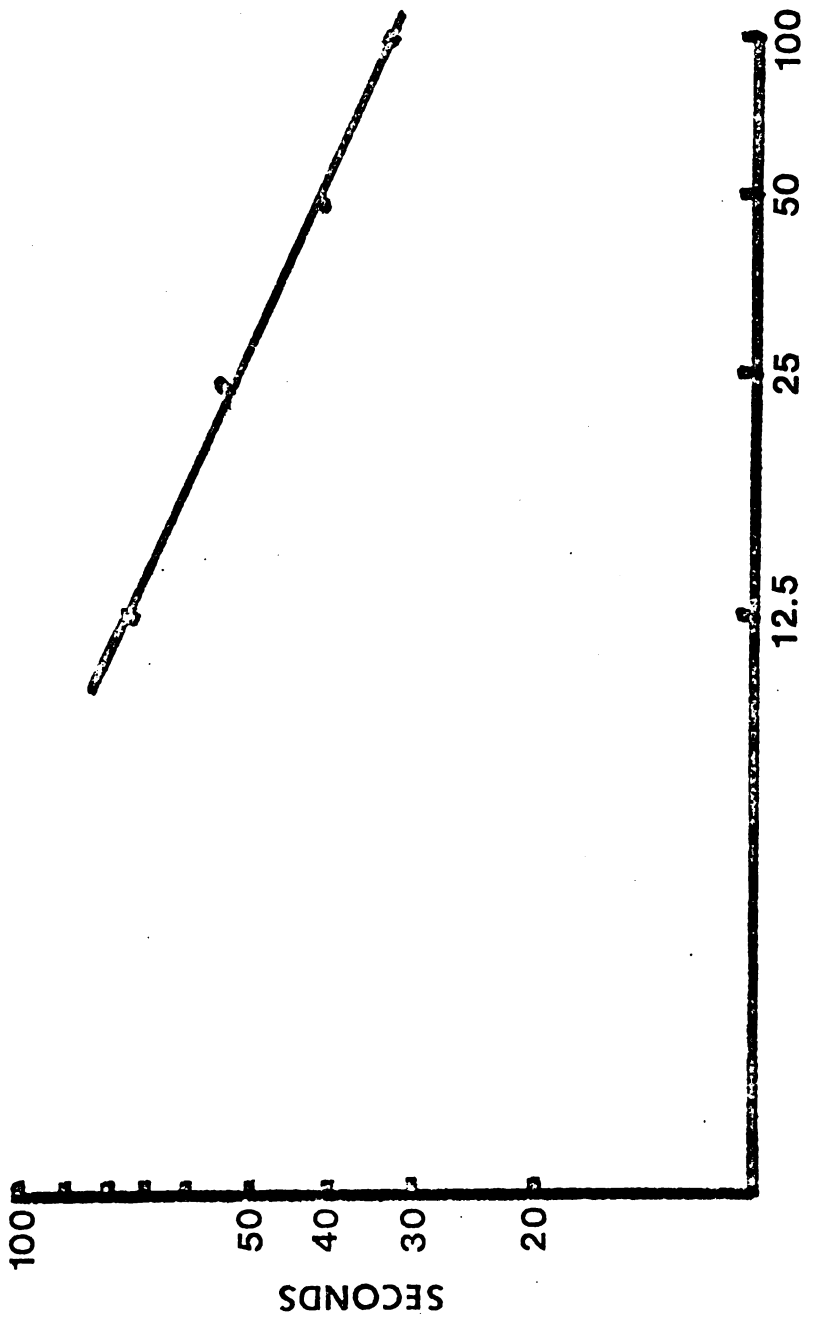

Figure 1: Typical standard curve for the ristocetin plate test.

Interpretation of Test Results

Samples with low von Willebrand factor activity show prolonged times. When buffer was used in place of the plasma or plasma dilution in the assay, no platelet agglutination occurred since there was no von Willebrand factor in the test system.

Canine Ristocetin Cofactor Assay (Plate Test)

The canine ristocetin cofactor assay was identical to the human assay except that pooled normal canine plasma was used to construct the standard curve and the ristocetin concentration was 1.0 mg/ml. Human FWP containing 30 mg/ml bovine albumin (final concentration of 20 mg/ml) were used in the canine assay, as in the human assay.

RESULTS

Fibrinogen Precipitation Induced by Ristocetin as Measured by Changes in Light Transmission

To characterize the effect of ristocetin on fibrinogen, a solution of Kabi (Grade L) fibrinogen was diluted in saline to give a clottable fibrinogen range of 300-400 mg/dl as quantitated by the Dade Data-Fi Fibrinogen Determination Kit. An appropriate solution of fibrinogen was prepared by diluting 12-13 mg of fibrinogen in 1 ml of saline which produced a concentration range that was comparable to normal human fibrinogen levels in plasma.

The initial experiment was performed using a single concentration of fibrinogen and testing the effect of two concentrations of ristocetin commonly used in the clinical laboratory in ristocetin cofactor assays. When ristocetin was added to fibrinogen the solution immediately turned cloudy. The precipitate formed was measured by the change in light transmission as recorded by a standard aggregometer (Chrono-log Corporation). Addition of ristocetin (1.2 mg/ml) resulted in a decrease in light transmission and a cloudy precipitate was observed visually. This was followed immediately by some clearing of the sample as evidenced by a slow but definite increase in light transmission. Visually the clearing could be seen as the conversion of a fine precipitate to larger aggregates (Figure 2). In a separate experiment this conversion from fine precipitate to large aggregates was also demonstrated in the light microscope.

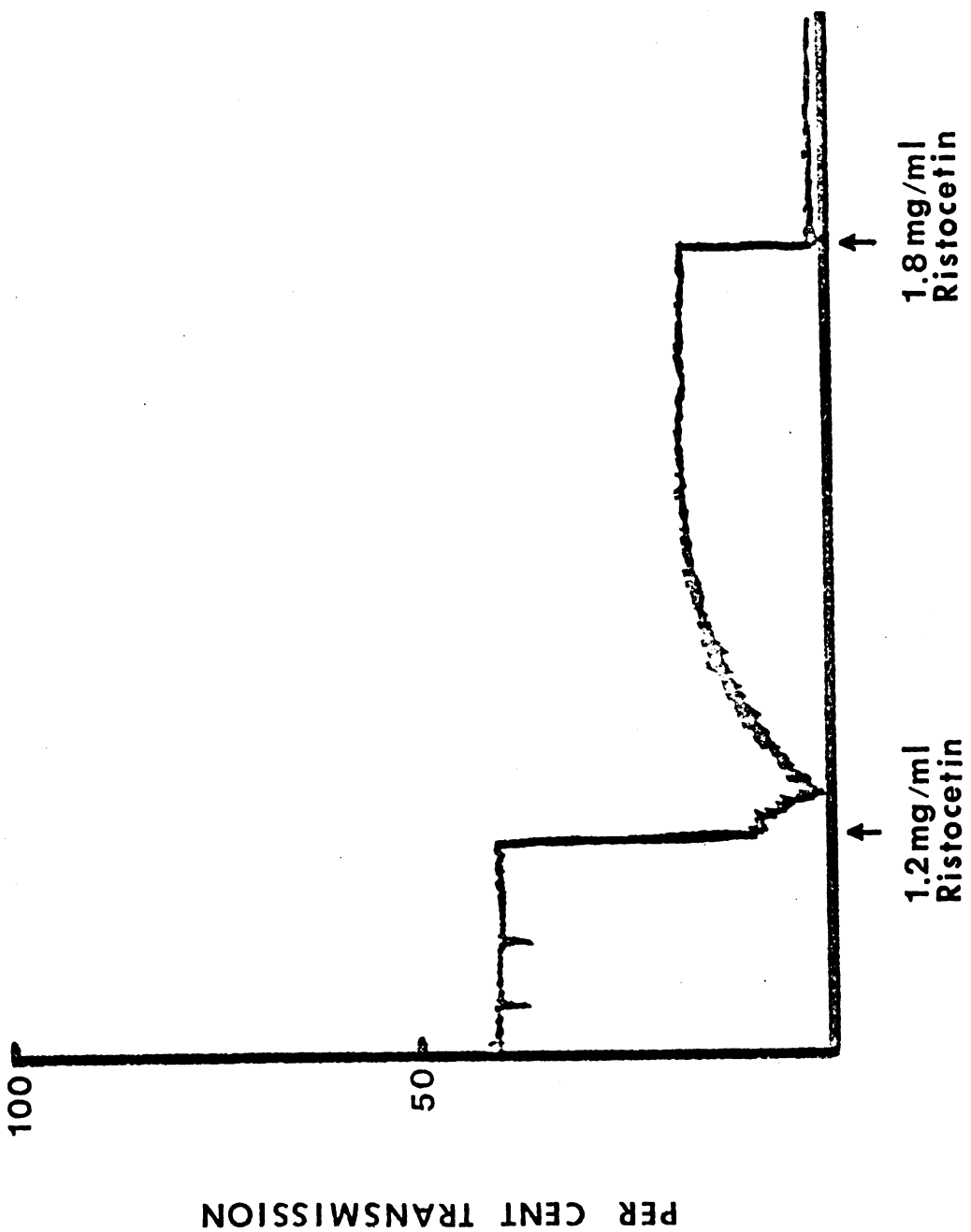

Figure 2: The effect of two concentrations of ristocetin on fibrinogen as measured by a decrease in light transmission.

When additional ristocetin was added (final concentration 1.8 mg/ml) to the sample that had been precipitated by 1.2 mg/ml ristocetin, it was possible to induce further precipitation of fibrinogen (Figure 2).

Ristocetin-induced precipitation of fibrinogen was observed in a fibrinogen range of 3.25-13.0 mg/ml. The amount of precipitate was dependent on the amount of fibrinogen present, i.e., greater precipitation being formed with higher concentrations of fibrinogen. Similarly, the amount of macroscopic aggregates formed was dependent upon the fibrinogen concentration. At a concentration of 3.25 mg/ml of fibrinogen only a fine precipitate formed and no macroscopic aggregates were observed. With 6.5 mg/ml and 13.0 mg/ml of fibrinogen, macroscopic aggregates could be observed, more being present in the solution which had the higher fibrinogen concentration (13.0 mg/ml) (Figure 3).

With all concentrations of fibrinogen tested it was possible to induce further precipitation by the addition of more ristocetin (Figure 3).

Effect of Presence or Absence of FWP on Fibrinogen Precipitation Induced by Ristocetin

The production of fibrinogen precipitate was examined in the ristocetin plate test, where the endpoint was the time of aggregate (precipitate) formation. Ordinarily the test is performed with FWP but in experiments performed to test ristocetin's effect on fibrinogen, the FWP were replaced by Tris-saline buffer. Fibrinogen (13 mg/ml) was diluted 1:2, 1:4, 1:8 and 1:16. Fibrinogen dilution

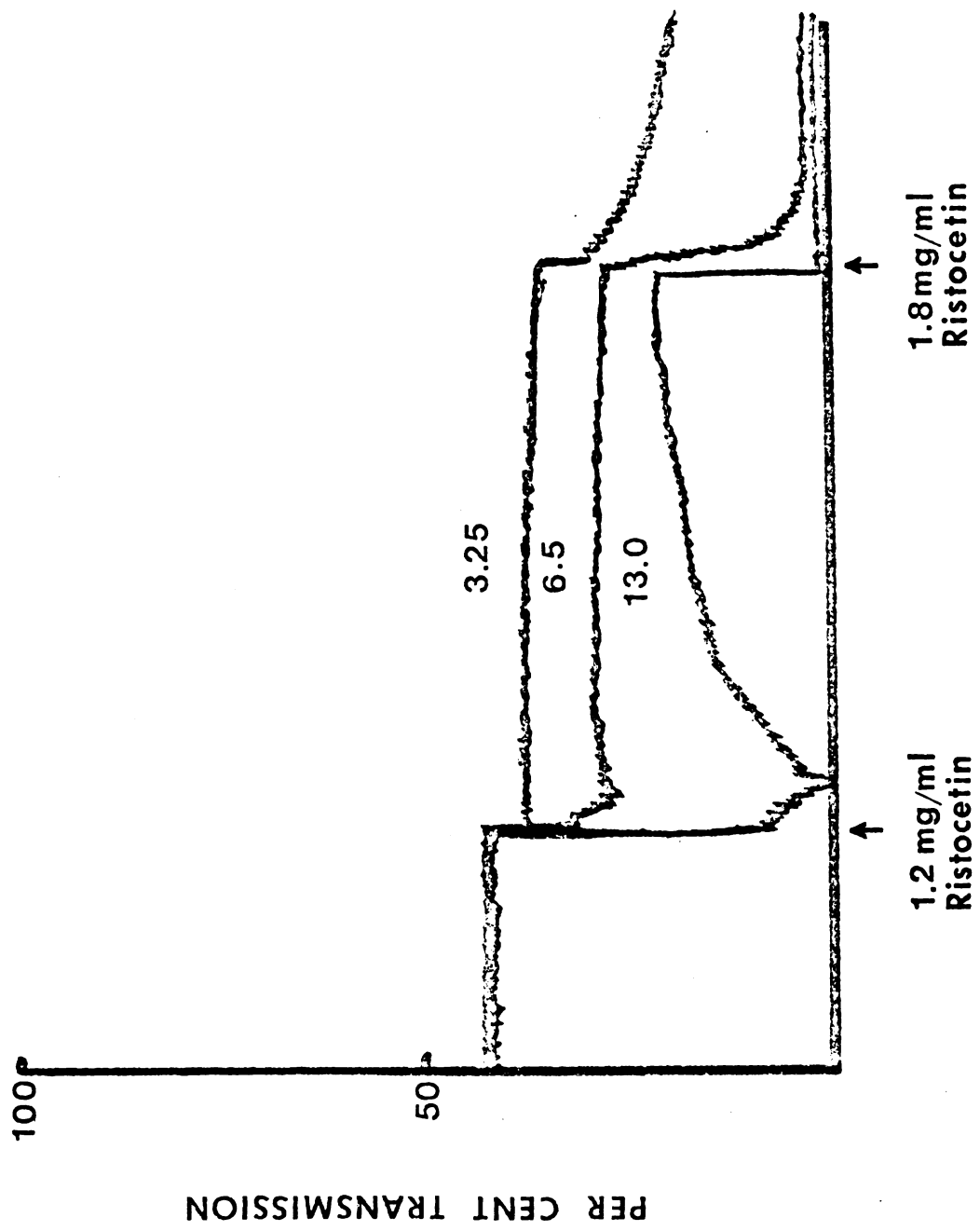

Figure 3: The effect of ristocetin on various concentrations of fibrinogen (3.25 mg/ml, 6.5 mg/ml and 13.0 mg/ml) as measured by changes in light transmission.

resulted in increased time of onset for precipitation to occur (Table 2). This relationship is nearly linear on log-log graph paper (Figure 4).

Table 2: Effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation (ristocetin 1.5 mg/ml) in the absence of FWP.

Fibrinogen Dilution		Seconds
undiluted	(13.0 mg/ml)	10
1:2	(6.5 mg/ml)	18
1:4	(3.25 mg/ml)	22
1:8	(1.63 mg/ml)	47
1:16	(0.81 mg/ml)	75
0		>120

These experiments were repeated in the presence of FWP in Tris-saline buffer. In the presence of FWP the dilutional effect on the onset of fibrinogen precipitation was still present but was much less (Table 3).

No precipitation occurred in the absence of fibrinogen suggesting that the precipitate is due to the interaction of ristocetin and fibrinogen and does not involve agglutinated platelets. It is possible that platelets contribute fibrinogen which would explain the more rapid fibrinogen precipitation observed in the presence of FWP as compared to the absence of FWP. The relationship of onset of fibrinogen precipitation to fibrinogen concentration in the absence of FWP is nearly linear on log-log graph paper (Figure 4). With FWP present this relationship is no longer linear on log-log graph paper (Figure 4).

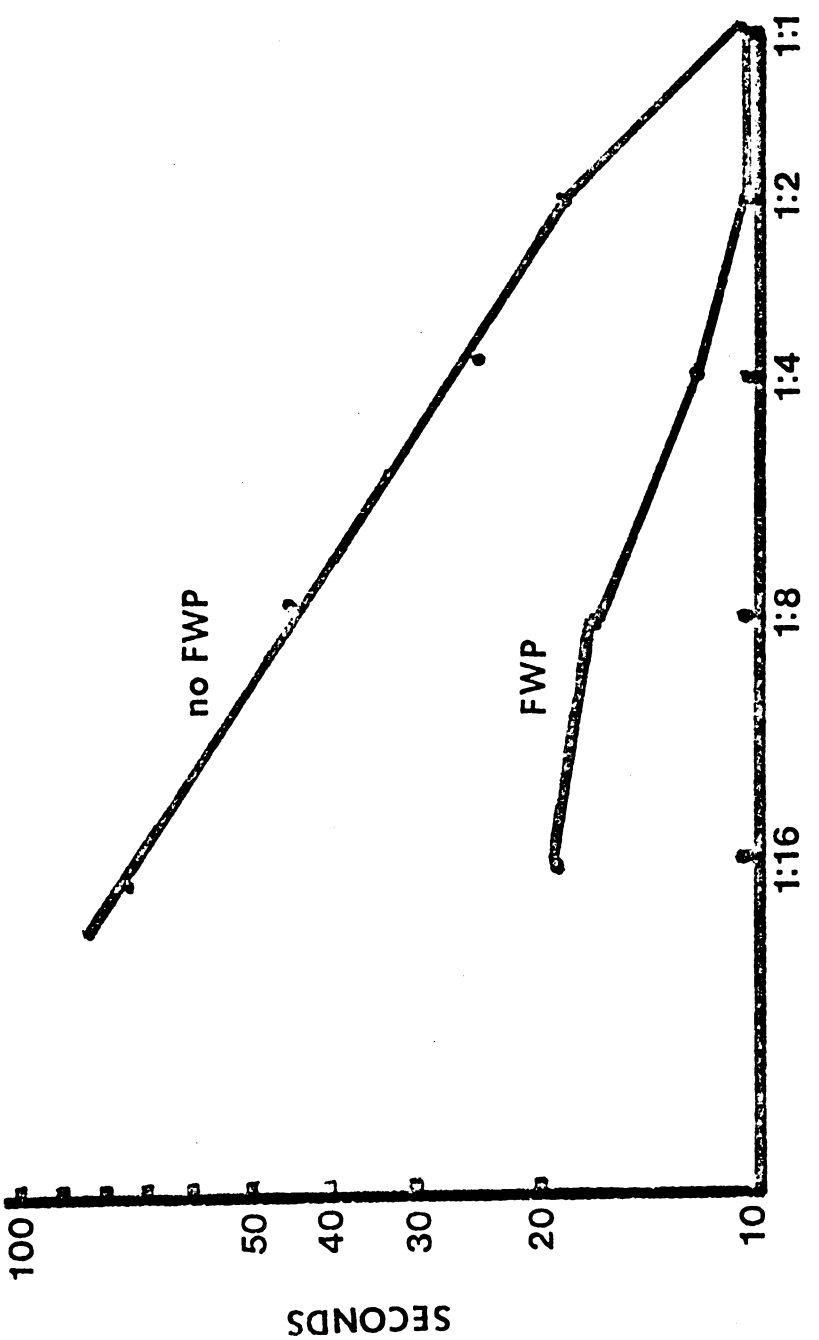

Figure 4: The effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation in the presence and absence of FWP.

Table 3: Effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation (ristocetin 1.5 mg/ml) in the presence of FWP.

Fibrinogen Dilution		Seconds
undiluted	(13.0 mg/ml)	10
1:2	(6.5 mg/ml)	10
1:4	(3.25 mg/ml)	12
1:8	(1.63 mg/ml)	16
1:16	(0.81 mg/ml)	18
0		>120

Inhibitory Effect of Bovine Albumin on Ristocetin-Induced Fibrinogen Precipitation

Previous reports indicate that albumin alters fibrinogen precipitation by ristocetin. A series of experiments were performed to study this phenomenon. Serial dilutions of bovine albumin were tested with a constant fibrinogen concentration (13.0 mg/ml) and two concentrations of ristocetin (1.2 mg/ml and 1.8 mg/ml). The amount of fibrinogen precipitation was quantitated by changes in light transmission.

The inhibitory effect of albumin on ristocetin-induced fibrinogen precipitation was proportional to the albumin concentration. At a final concentration of 40 mg/ml of albumin no change in light transmission was detected. Albumin at this concentration apparently blocked precipitation when both concentrations of ristocetin were used. Albumin at a concentration of 20 mg/ml partially blocked the precipitation (Figure 5). Based on these studies albumin in a range of 20-40 mg/ml appeared to be most useful in reducing fibrinogen precipitation.

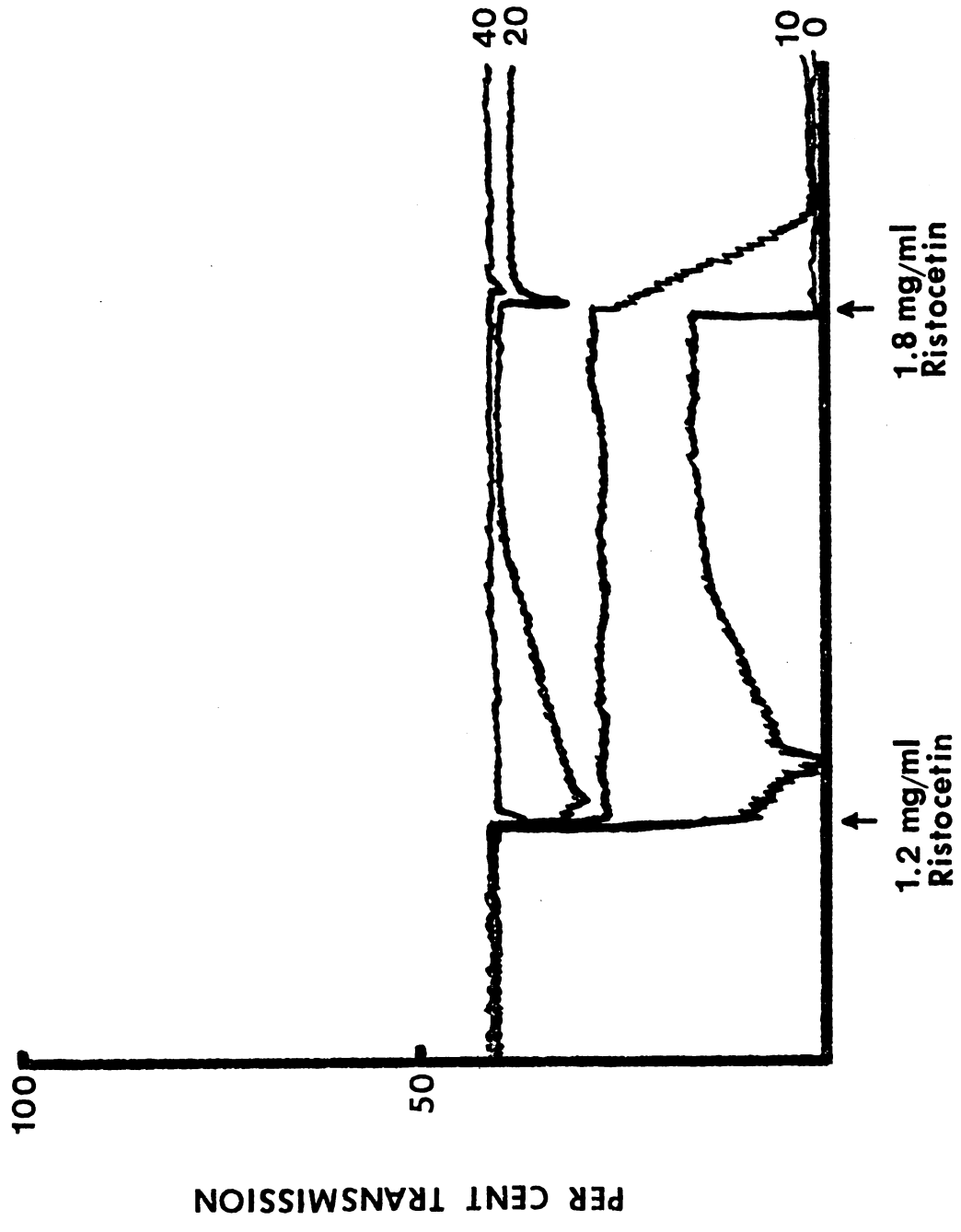

Figure 5: The effect of various albumin concentrations (0 to 40 mg/ml) on ristocetin-induced fibrinogen precipitation as measured by changes in light transmission.

Further experiments to demonstrate the inhibitory effect of albumin on fibrinogen precipitation were performed using ristocetin (1.2 mg/ml). Albumin in concentrations of 0-40 mg/ml was tested in the ristocetin plate test in which plasma provided the fibrinogen and VIII:vWF. FWP were present in this study. In this test system no endpoint could be obtained with albumin in a concentration of 40 mg/ml. An albumin concentration of 30 mg/ml could be used with undiluted plasma but no curve could be constructed because plasma dilutions of 1:4 failed to produce an endpoint (Table 4) (Figure 6). Based on these results 20 mg/ml of albumin was chosen as the optimum concentration.

The effect of fibrinogen concentration on the ability of albumin (20 mg/ml) to inhibit precipitation in the plate test was studied. This experiment was performed with and without FWP. Again it was observed that in the presence of FWP and albumin (20 mg/ml) there was a shortened time of onset of precipitation (Table 4). The curve produced was not linear on a log-log plot (Figure 7).

Table 4: Time for platelet agglutinates to form with ristocetin and varying albumin concentrations. Ristocetin concentration 1.2 mg/ml.

PNP Dilution	Seconds Albumin Concentrations (mg/ml)					
	0	10.1	13.6	22.5	30	40
undiluted	20	20	22	24	50	>120*
1:2	20	21	29	32	89	>120*
1:4	20	26	35	40	>120*	>120*

*Endpoints of approximately 100 seconds were hard to visualize. The data in Table 4 represented in log-log graph form can be seen in Figure 6. Undiluted PNP represented 100% activity, 1:2 was 50%, etc.

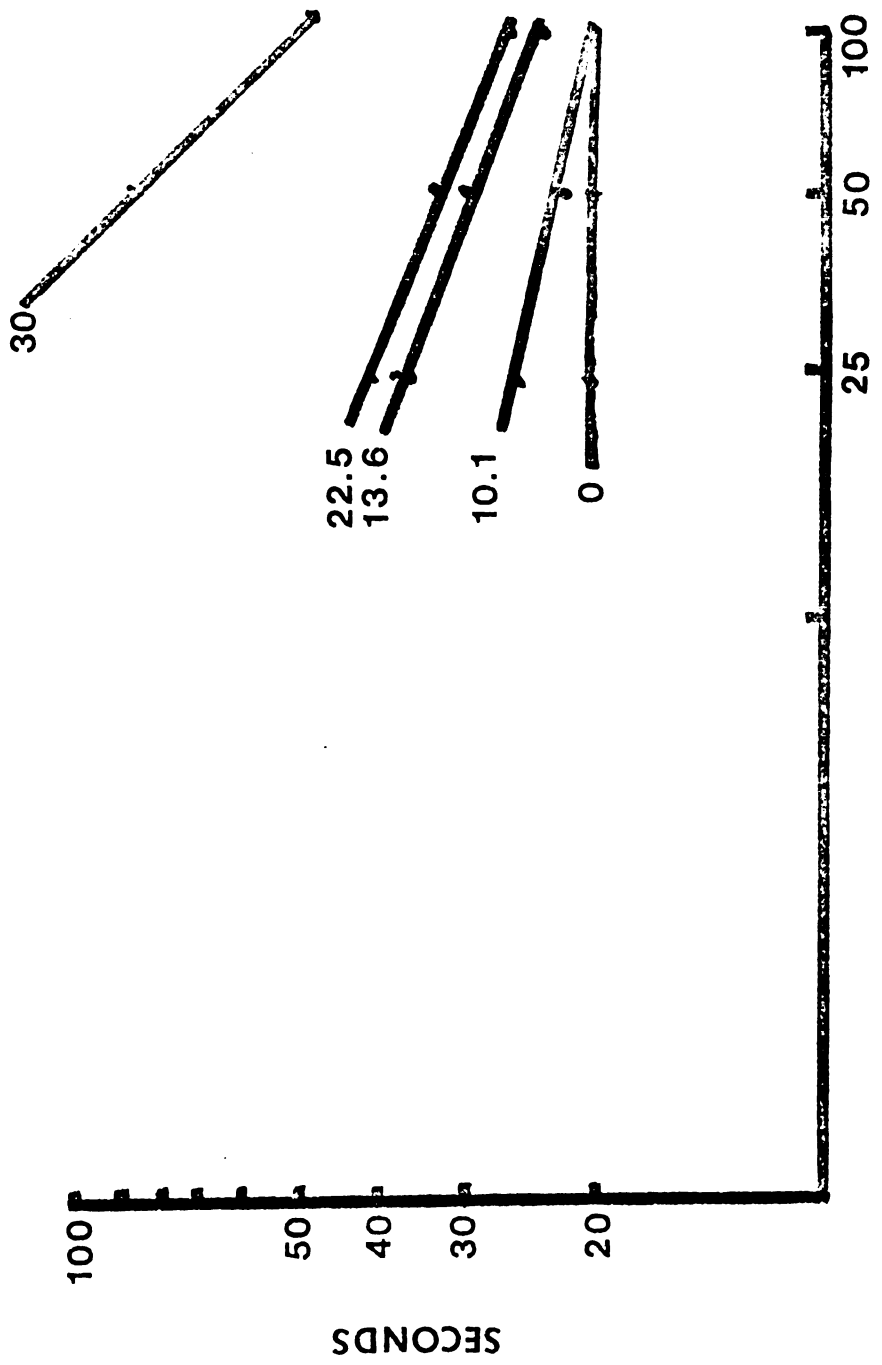

Figure 6: The effect of various albumin concentrations (0 to 40 mg/ml) on the slope of standard curves for the ristocetin plate test.

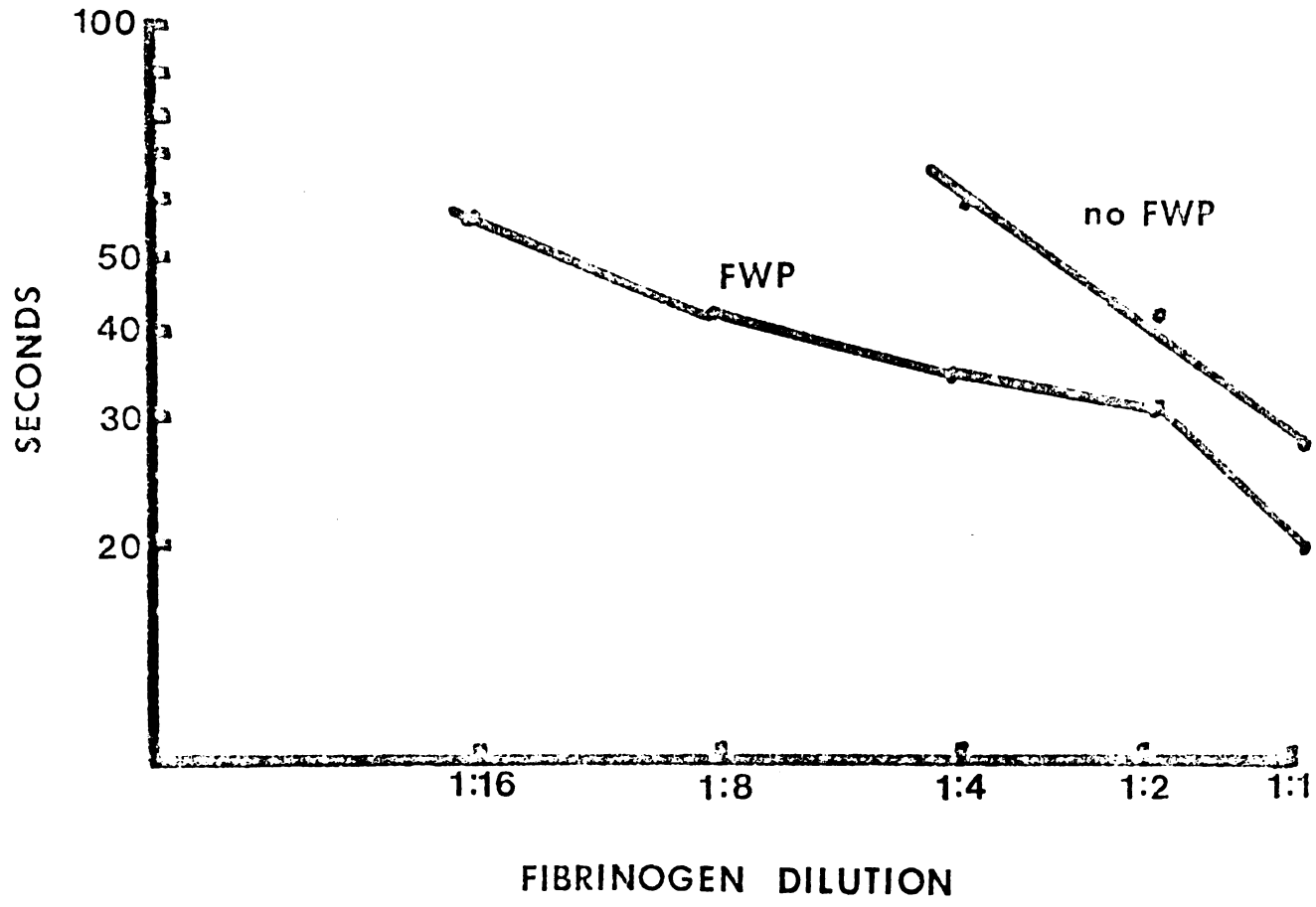

Figure 7: The effect of fibrinogen concentration on ristocetin-induced fibrinogen precipitation in the presence of 20 mg/ml albumin (with and without FWP).

Table 5: Effect of various fibrinogen concentrations on the ability of albumin (20 mg/ml) to inhibit precipitation (plate test) with and without FWP present. Ristocetin concentration 1.2 mg/ml.

Fibrinogen Dilutions		Seconds with F.W.P.	Seconds without F.W.P.
undiluted	(13.0 mg/ml)	20	29
1:2	(6.5 mg/ml)	31	43
1:4	(3.25 mg.ml)	32	60
1:8	(1.63 mg/ml)	42	>120
1:16	(0.81 mg/ml)	58	>120
0		>120	>120

In contrast, when FWP were absent but fibrinogen dilutions were made in the presence of albumin (20 mg/ml) there was a prolongation of the onset of precipitation (Table 5) and a linear curve was produced on log-log graph paper (Figure 7). These results are comparable to those found in similar studies previously described where albumin was omitted. This data is contrasted with that previously obtained in the absence of albumin (20 mg/ml) in Table 6 and 7.

Table 6: Fibrinogen precipitation in the presence and absence of albumin (20 mg/ml) with FWP present (plate test). Ristocetin concentration 1.2 mg/ml.

Fibrinogen Dilution		Seconds	
		No albumin	Albumin (20 mg/ml)
undiluted	(13.0 mg/ml)	10	20
1:2	(6.5 mg/ml)	10	31
1:4	(3.25 mg/ml)	12	32
1:8	(1.63 mg/ml)	16	42
1:16	(0.81 mg/ml)	18	58
0		>120	>120

Table 7: Fibrinogen precipitation in the presence and absence of albumin (20 mg/ml) in the absence of FWP (plate test). Ristocetin concentration 1.2 mg/ml.

Fibrinogen Dilution		Seconds	
		No albumin	Albumin (20 mg/ml)
undiluted	(13.0 mg/ml)	10	29
1:2	(6.5 mg/ml)	18	43
1:4	(3.25 mg/ml)	22	60
1:8	(1.63 mg/ml)	47	>120
1:16	(0.81 mg/ml)	75	>120
0		>120	>120

Effect of Albumin Concentration on a vWD Plasma Tested with Ristocetin Plate Test

Citrated plasma from a patient with confirmed von Willebrand's disease was tested for VIII:vWF activity using the ristocetin plate test and various albumin concentrations. von Willebrand's disease (vWD) had previously been diagnosed by Laurell immunoelectrophoresis which revealed zero factor VIIIR:Ag and low ristocetin cofactor activity as determined by a method of Penner (55). When this vWD plasma was tested in the ristocetin plate test with albumin concentrations of 10.1 mg/ml, 13.6 mg/ml, 20 mg/ml and 30 mg/ml the following results were obtained (Table 8).

Table 8: Percent activity of a vWD plasma with varying albumin concentrations in the ristocetin plate test. Ristocetin concentration 1.2 mg/ml.

	Albumin Concentration				
	0	10.1	13.6	20	30
% Activity of vWD plasma	100%	100%	100%	39%*	53%*

*Average normals for ristocetin assays = 50-150%.

In the absence of albumin and at low concentrations of albumin (10.1 mg/ml and 13.6 mg/ml) fibrinogen precipitation masked the absence of significant platelet agglutination when vWD plasma was tested. When albumin was in a concentration of 20 mg/ml, fibrinogen precipitation was inhibited and it was possible to quantitate platelet agglutination by ristocetin and obtain a true value for VIII:vWF activity of the vWD plasma. A higher percent

VIII:vWF activity was obtained when albumin was at a concentration of 30 mg/ml. This was most likely due to a problem in endpoint visualization at high concentrations of albumin. Endpoints greater than 100 seconds were weak and hard to detect. This particular vWD plasma showed an endpoint of 125 seconds with albumin at a concentration of 30 mg/ml in the test system.

Varying Ristocetin Concentrations

Ristocetin in various concentrations was mixed with either serum, Kabi fibrinogen (12.0 mg/ml), pooled normal plasma or Factor VIII deficient plasma. Final concentrations of ristocetin used were 0.5, 0.75, 1.0, 1.2, 1.5 and 2.0 mg/ml. Platelets were not used but were replaced by Tris-saline buffer (containing no albumin).

Kabi fibrinogen was the most sensitive to lower ristocetin concentrations, e.g., at 0.5 mg/ml of ristocetin a precipitate was seen at 55 seconds. Possibly the albumin and other proteins present in PNP and VIII deficient plasma have an inhibitory effect on precipitation when low concentrations of ristocetin are used. As expected no precipitate formed when serum was used since serum lacks fibrinogen (Table 9).

The ristocetin plate test (containing FWP, plasma, and ristocetin) was performed with varying concentrations of ristocetin to see if there is an effect of ristocetin concentration on quantitating von Willebrand factor activity of a known vWD patient. Ristocetin was used in final concentrations of 0.5, 1.0, 1.2, 1.5 and 2.0 mg/ml. Albumin (20 mg/ml) was present in the assay. Results of this study are displayed in Table 10.

Table 9: Effect of various ristocetin concentrations on the time for precipitate formation with serum, fibrinogen, PNP and VIII deficient plasma

Ristocetin Concentration	Seconds			
	Serum	Fibrinogen	PNP	VIII Deficient Plasma
2 mg/ml	*	10	14	18
1.5	*	10	33	20
1.2	*	14	80	25
1.0	*	16	>120	70
0.75	*	33	>120	>120
0.5	*	55	>120	>120
0	*	>120	>120	>120

*No precipitate seen in any concentration at >120 seconds. Fibrinogen in a concentration of 12.0 mg/ml.

Table 10: Effect of ristocetin concentration on quantitating the VIII:vWF activity of a known vWD patient (normal range 50-160%).
Albumin present in a concentration of 20 mg/ml.

	Ristocetin Concentration (mg/ml)				
	0.5	1.0	1.2	1.5	2.0
% Activity of vWD plasma	*	18%	21%	54%	57%

*No endpoint detected.

From this data it is apparent that higher concentrations of ristocetin (greater than 1.5 mg/ml) have a normalizing effect on plasma from vWD patients. The endpoints were very short when high concentrations of ristocetin were used, which was probably due to fibrinogen precipitation obscuring the true endpoint. From these results ristocetin in concentrations of 1.0-1.2 mg/ml were determined to be optimum.

Effect of Serum and Defibrinated Plasma Samples on von Willebrand Factor Activity, Factor VIII Clotting Activity and Factor VIII Antigen Levels

Because of the problems encountered with fibrinogen precipitation it seemed important to determine if it would be possible to use serum or defibrinated plasma in the ristocetin plate test.

Two citrated plasmas were defibrinated by heating for 5 minutes at 56 C followed by centrifugation to remove denatured fibrinogen. Two additional citrated plasmas were defibrinated with snake venom (Bothrops atrox) by a method of Cutter Laboratories, ATIII package insert. Two serum samples were produced by allowing unanticoagulated whole blood to clot at room temperature for 1 hour, followed by centrifugation. Factor VIII clotting assays, Factor VIII antigen and von Willebrand factor activity were then measured (Table 11).

The results demonstrate that VIIIR:Ag is quite stable. Both VIII:C and VIII:vWF activity are sensitive to treatment with heat and snake venoms and are also greatly reduced in serum samples. It appears to be extremely difficult to separate fibrinogen from Factor VIII:C and VIII:vWF without also removing these activities.

Table 11: Results of VIII:C, VIIIR:Ag and VIII:vWF assays on 2 normal serum samples and defibrinated plasma samples.

Sample	VIII:C	VIIIR:Ag	VIII:vWF
#1 Untreated Plasma	88%	110%	90%
Serum	51%	110%	20%
Heated Plasma	<1%	110%	0%
Venom Plasma	*	110%	18.5%
#2 Untreated Plasma	83%	95%	100%
Serum	10%	95%	28%
Heated Plasma	22%	82%	22%
Venom Plasma	*	82%	20%
NORMAL RANGE FOR PLASMA	55-145%	50-150%	62-126%

*Immediate clot in assay, could not quantitate.

Effect of Ristocetin Concentration on Canine Ristocetin Cofactor Assays

A vWD canine plasma was tested with varying ristocetin concentrations in the presence of 20 mg/ml bovine albumin. The following results were obtained (Table 12).

Table 12: Canine vWD plasma levels of ristocetin cofactor activity with varying ristocetin concentrations. Albumin in a concentration of 20 mg/ml.

	Ristocetin Concentration			
	0.5 mg/ml	1.0 mg/ml	1.25 mg/ml	1.5 mg/ml
% vWF activity	*	0%	80%	70%

*No agglutination of platelets at >120 seconds.

Ristocetin concentrations of 1.25 mg/ml and greater result in false normal results for a known canine vWD plasma. Fibrinogen precipitation was most likely masking the platelet agglutination. The endpoint (precipitation) was shortened when higher concentrations of ristocetin were used. This observation was similar to that seen in human ristocetin cofactor assays.

In the human assay ristocetin concentrations of 1.2 mg/ml produced the expected results of low ristocetin cofactor activity for vWD plasma (Table 12). This was not observed in the canine assay at ristocetin concentrations of 1.2 mg/ml.

Effect of Albumin Concentration on Canine Ristocetin Cofactor Assays

Bovine albumin must be added to canine ristocetin cofactor assays to inhibit fibrinogen precipitation. The ristocetin cofactor assay was performed with 1.0 mg/ml ristocetin and the following final concentrations of albumin: 0, 6.6 mg/ml, 13.6 ml/ml, 19.9 mg/ml, 26.6 mg/ml and 41.6 mg/ml. A canine vWD plasma sample was also assayed along with dilutions of pooled normal dog plasma.

The canine vWD plasma (diagnosed by W. J. Dodds' Laboratory, Albany, N. Y.) showed decreased VIII:C activity and zero VIIIR:Ag. The expected low ristocetin cofactor results were obtained only when albumin concentrations were greater than 13.6 mg/ml. An albumin concentration of 26.6 mg/ml appeared to be optimum in canine ristocetin assays (Table 13).

Table 13: Effect of varying albumin concentrations in a canine ristocetin cofactor assay (plate test). Ristocetin concentration 1.0 mg/ml.

Sample	Seconds Albumin Concentration (mg/ml)					
	0	6.6	13.6	19.9	26.6	41.6
PPP undiluted	28	29	30	40	49	56
1:2	30	32	37	48	58	62
1:4	34	36	42	60	60	70
vWD canine plasma (undiluted)	25	33	42	55	81	80
% vWF activity	>100%	42%	25%	30%	4%	12%

Effect of Ristocetin on Clottable Fibrinogen Levels of Fibrinogen Solutions, Normal Plasma and vWD Plasma

To demonstrate that ristocetin has an affect on fibrinogen, clottable fibrinogen levels were determined before and after an incubation with ristocetin. Fibrinogen was quantitated by the Dade fibrinogen determination kit; a modified thrombin clotting time. Since this method lacks sensitivity, high concentrations of fibrinogen and ristocetin were used. When a fibrinogen (Kabi, Grade L) solution (13.0 mg/ml) was incubated for 10 minutes with ristocetin in a final concentration of 3.0 mg/ml the clottable fibrinogen level decreased from 400 mg/dl to 98 mg/dl. The precipitate formed would not dissolve in saline.

In a similar experiment, fibrinogen levels of vWD plasma were determined before and after exposure to ristocetin. Ristocetin was in

final concentrations of 1.0 mg/ml, 1.5 mg/ml and 3.0 mg/ml in the vWD plasma. Plasmas turned immediately cloudy when 1.5 mg/ml and 3.0 mg/ml of ristocetin were added. After a 10 minute incubation with ristocetin the plasmas appeared clear and the fibrinogen levels remained essentially unchanged (Table 14).

Table 14: vWD plasma fibrinogen levels before and after the addition of ristocetin (1.0 mg/ml, 1.5 mg/ml, and 3.0 mg/ml).

Ristocetin Concentration	Fibrinogen (mg/dl)	
	Before	After
1.0 mg/ml	220	215
1.5 mg/ml	205	207
3.0 mg/ml	198	207

Similar results were obtained when pooled normal plasma was incubated for 10 minutes with three concentrations of ristocetin (1.0 mg/ml, 1.5 mg/ml and 3.0 mg/ml) Table 15).

Table 15: PNP fibrinogen levels before and after the addition of ristocetin (1.0 mg/ml, 1.5 mg/ml, and 3.0 mg/ml).

Ristocetin Concentration	Fibrinogen (mg/dl)	
	Before	After
1.0 mg/ml	225	213
1.5 mg/ml	207	215
3.0 mg/ml	199	220

Perhaps the albumin of the plasma has a protective effect against fibrinogen precipitation and after 10 minutes the fibrinogen that precipitated

(as indicated by the cloudiness) redissolved in the plasma. Kabi fibrinogen solutions did not show this since no albumin was present.

DISCUSSION

This study has provided further evidence that ristocetin causes the precipitation of fibrinogen as originally reported by Howard and Firkin (16). In addition the data presented here shows the importance of albumin in minimizing precipitation and the importance of standardized ristocetin concentrations in ristocetin cofactor assays.

The mechanism by which ristocetin induces fibrinogen precipitation is still unclear. It is also unclear how albumin interacts to inhibit fibrinogen precipitation. Stibbe and Kirby have suggested that albumin may bind ristocetin thereby lowering the concentration of free ristocetin below that necessary for agglutination (56). This may explain the observations seen here that in the presence of albumin and high ristocetin concentration precipitation can still occur. Perhaps the albumin concentration was not high enough to completely bind ristocetin, so free ristocetin was in optimal concentations to precipitate fibrinogen.

Ts'ao and colleagues have studied ristocetin-induced fibrinogen precipitation by TEM observations of platelets exposed to ristocetin. They observed fibrinogen clumps in the surface cannalicular system of the platelets (52). These observations may help to explain the data reported here, that endpoints were shorter in the presence of FWP than in the absence of FWP. Possibly the FWP contributed fibrinogen to the precipitate thus creating the faster endpoints. When no fibrinogen was added to FWP, ristocetin-induced precipitation did not occur. This data

further supports the observation that fibrinogen is precipitated by ristocetin and that the interaction of ristocetin with fibrinogen does not require the presence of platelets.

Others have shown that there is an initial decrease in light transmission when ristocetin is added to formalin-fixed washed platelets (18,52). Decreased light transmission also occurs in the absence of FWP as reported in the present study. When the initial decrease in percent transmission was occurring the sample was very cloudy. This was followed by clearing as aggregates of precipitated material formed. When FWP were present and ristocetin was added, both precipitated material and loose platelet aggregates could be seen microscopically.

It is apparent that the precipitated material can mask the platelet agglutination endpoint if suboptimal concentrations of albumin and ristocetin are used. In this study the masking of the true endpoint was demonstrated. vWD plasma (both canine and human) was tested with various albumin and ristocetin concentrations. It was found that 20 mg/ml of albumin and 1.0-1.2 mg/ml of ristocetin were optimum for the ristocetin plate test.* Lower albumin concentrations along with higher ristocetin concentrations had a false normalizing effect on VIII:vWF activity of vWD plasma.

Clottable fibrinogen levels of pure fibrinogen solutions were measured before and after the addition of ristocetin. Clottable fibrinogen was lower after the addition of ristocetin. However, when human plasma samples, both normal and vWD plasma, were exposed to ristocetin

---

*The stoichiometry of the system results in a final albumin concentration in a 4-fold excess as compared with native plasma.

the clottable fibrinogen levels remained essentially unchanged. The presence of albumin in the plasma samples may explain these findings, since albumin has an inhibitory affect on fibrinogen precipitation. The plasma samples did show immediate cloudiness following the addition of ristocetin but after a 10 minute incubation the cloudiness had disappeared indicating that fibrinogen was no longer precipitated. It may be that, the ristocetin-fibrinogen reaction is reversible in the presence of albumin. Nevertheless, this cloudiness interferes with seeing endpoints in ristocetin cofactor assays.

While other plasma proteins can be precipitated by ristocetin (26, 56), Stibbe and Kirby agree that ristocetin concentrations less than 2.5 mg/ml affect only fibrinogen in plasma samples (56). The results of the study undertaken here support their findings since serum (containing no fibrinogen) showed no precipitate formation with ristocetin concentrations of 2.0 mg/ml and less.

Stibbe and Kirby feel that vWD patients who have been transfused with fibrinogen-rich concentrates will have high fibrinogen levels that will interfere with ristocetin cofactor assays. Furthermore, they say pregnant hemophilia A carriers often have increased fibrinogen and lowered albumin levels and that caution must be exercised when interpreting ristocetin cofactor results from these individuals (56). This study again supports Stibbe's and Kirby's findings in that ristocetin-induced fibrinogen precipitation was found to be dependent on fibrinogen concentration. The higher the fibrinogen concentration, more precipitate formed.

Perhaps fibrinogen and VIII:vWD together, are necessary for ristocetin aggregation of platelets. It is well-known that fibrinogen is essential for the aggregation of platelets with adenosine diphosphate (ADP). Fibrinogen has been shown to bind specifically to platelets in the presence of ADP and this binding is essential for aggregation (57). In the case of ristocetin, perhaps fibrinogen binding to platelets is necessary for aggregation to occur with this agent. VIII:vWF is also necessary, because vWD patients have normal fibrinogen levels but their platelets do not aggregate with ristocetin. Ristocetin has been shown to enhance the binding of VIII:vWF to the platelet membrane (46,47,48). Possibly ristocetin stimulates the binding of fibrinogen to platelets along with VIII:vWF to cause aggregation of those platelets. When serum samples (no fibrinogen) were tested in the ristocetin plate test no platelet agglutination occurred. VIIIR:Ag levels of serum were comparable to plasma levels but possibly because no fibrinogen is present in serum, platelet agglutination did not occur. Further studies on the ristocetin-platelet interactions are needed. From this study is it established that fibrinogen has a direct effect on ristocetin agglutination of platelets but the nature of this relationship remains unclear.

CONCLUSION

This report suggests that caution is necessary when interpreting patient results from ristocetin cofactor assays. When concentrations of albumin and ristocetin are not optimal, false negative results can occur due to fibrinogen precipitation masking the true endpoint. Possibly the diagnosis of von Willebrand's disease would be missed.

Ristocetin in concentrations of 1.0-1.2 mg/ml and bovine albumin in a concentration of 20 mg/ml provided optimal conditions for the ristocetin plate test described here.

Literature Cited

LITERATURE CITED

1. Nilsson, I. M., and Holmberg, L. von Willebrand's Disease Today. Clinics Haematol. 8: 147, 1979.

2. Nilsson, I. M., Blömback, M., and von Franckin, I. On an Inherited Autosomal Hemorrhagic Diathesis with Antihemophilic Globulin (AHG) Deficiency and Prolonged Bleeding Time. Acta Medica Scand. 159: 35, 1957.

3. Nilsson, I. M. Blömback, M., and Blömback, B. von Willebrand's Disease in Sweden. Its Pathogenesis and Treatment. Acta Medica Scand. 164: 263, 1959.

4. Borchgrevink, C. F. A Method for Measuring Platelet Adhesiveness In Vivo. Acta Medica Scand. 168: 157, 1960.

5. Salzman, E. W. Measurement of Platelet Adhesiveness: A Simple In Vitro Technique Demonstrating an Abnormality in von Willebrand's Disease. J. Lab. Clin. Med. 62: 724, 1963.

6. Weiss, H. J., Baumgartner, H. R., Tschopp, T. B., Turitto, V. T. and Cohen, D. Correction by Factor VIII of the Impaired Platelet Adhesion to Subendothelium in von Willebrand Disease. Blood 51: 267, 1978.

7. Jörgensen, L. and Borchgrevink, C. F. The haemostatic mechanism in patients with haemorrhagic diseases. Acta Path. Micro. Scand. 60: 55, 1964.

8. Zimmerman, T. S., Ratnoff, O. D., and Powell, A. E. Immunologic differentiation of classic hemophilia (factor VIII deficiency) and von Willebrand's disease. With observations on combined deficiencies of antihemophilic factor and proaccelerin (factor V) and on an acquired circulating anticoagulant against antihemophilic factor. J. Clin. Invest. 50: 244, 1971.

9. Zimmerman, T. S., Hoyer, L. W., Dickson, L. and Edington, T. S. Determination of the von Willebrand's Disease Antigen (factor VIII-related antigen) in plasma by quantitative immunoelectrophoresis. J. Lab. Clin. Med. 86: 152, 1975.

10. Van Mourik, J. A., Hellings, J. A., and Hoorweg, E. M. Micro-heterogeneity of human factor VIII. Abstract of the VI Int. Cong. on Thrombosis and Haemostasis, XII Cong. of World Fed. of Hemophilia. Thrombosis and Haemostasis 38: 10, 1977.

11. Bouma, B. N., Dodds, W. J., Van Mourik, J. A., Sixma, J. J., Webster, W. P. Infusion of human and canine factor VIII in dogs with von Willebrand's disease: studies of the von Willebrand factor VIII synthesis stimulating factors. Scand. J. of Haem. 17: 263, 1976.

12. Zimmerman, T. S., Roberts, J. and Edington T. S. Factor VIII related antigen: multiple forms in human plasma. Proc. Natl. Acad. Sci.USA 72: 5121, 1975.

13. Nilsson, I. M., and Holmberg, L. (1975). Subtypes of factor VIII deficiencies. In Transfusion and Immunology (Ed.) Ikkala, E. and Nykänen, A. Pp. 235-248.

14. Ruggeri, Z. M., and Zimmerman, T. S. Variant von Willebrand's Disease: Characterization of two subtypes by analysis of multimeric composition of factor VIII/von Willebrand factor in plasma and platelets. J. Clin. Invest. 65: 1318, 1980.

15. Martin, S. E., Marder, V. J., Francis, C. W., Loftus, L. S., and Barlow, G. H. Enzymatic Degradation of the Factor-VIII-von Willebrand Protein: A Unique Tryptic Fragment with Ristocetin Cofactor Activity. Blood 55(5): 848, 1980.

16. Howard, M. A. and Firkin, B. G. Ristocetin--a new tool in the investigation of platelet aggregation. Thrombosis et Diathesis Haemorrhagica 26: 362, 1971.

17. Meyer, D., Jenkins, C. S. P., Dreyfus, M. and Larrieu, M. J. Experimental Model for von Willebrand's Disease. Nature 243: 293, 1973.

18. Allain, J. P., Cooper, H. A., Wagner, R. H. and Brinkhous, K. M. Platelets fixed with paraformaldehyde: a new reagent for assay of von Willebrand factor and platelet aggregating factor. J. Lab. Clin. Med. 85: 318, 1975.

19. Brinkhous, K. M., Graham, J. E., Cooper, H. A., Allain, J. P., and Wagner, R. H. Assay of von Willebrand factor in von Willebrand's disease and hemophilia: use of an macroscopic platelet aggregation test. Thrombosis Research 6: 267, 1975.

20. Kelton, J. G., Bishop, J., Carter, C. J. and Hirsch, J. A comparison of the quantitative ristocetin von Willebrand factor assay by using fresh and fixed platelets. Thrombosis Research 18: 477, 1980.

21. Ramsey, R. and Evatt, B. L. Rapid assay for von Willebrand factor activity using formalin-fixed platelets and microtitration technic. A. J. C. P. 72: 996, 1979.

22. Sarji, K. E., Stratton, R. D., Wagner R. H. and Brinkhous, K. M. Nature of von Willebrand factor: A new assay and a specific inhibitor. Proc. Nat. Acad. Sci. USA 71: 2937, 1974.

23. Weiss, H. J., Hoyer, L. W., Rickles, F. R., Varma, A., and Rogers, J. Quantitative Assay of a Plasma Factor Deficient in von Willebrand's Disease that is Necessary for Platelet Aggregation. J. Clin. Invest. 52: 2708, 1973.

24. Olson, J. D., Fass, D. N., Bowie, E. J. W. and Mann, K. G. Ristocetin-induced Aggregation of Gel-filtered Platelets: A Study of von Willebrand's Disease and the Effect of Aspirin. Thrombosis Research 3: 501, 1973.

25. Brinkhous, K. M., and Read, M. S. Preservation of Platelet Receptors for Platelet Aggregating Factor/von Willebrand Factor by Air Drying, Freezing or Lyophilization: New Stable Platelet Preparation for von Willebrand Factor Assays. Thrombosis Research 13: 591, 1978.

26. Rosborough, T. K., Johnson, G. S., Benson, R. E., Swaim, W. R., and Dodds, W. J. Measurement of Canine von Willebrand Factor Using Ristocetin and Polybrene. J. Lab. Clin. Med. 96: 47, 1980.

27. Read, M. S., Shermer, R. W., and Brinkhous, K. M. Venom Coagglutinin: An Activator of Platelet Aggregation Dependent on von Willebrand Factor. Proc. Natl. Acad. Sci. 75: 4514, 1978.

28. Blatt, P. M., Brinkhous, K. M., Culp, H. R., Krauss, J. S. and Roberts H. R. Antihemophilic Factor Concentrate Therapy in von Willebrand Disease. Dissociation of Bleeding-time Factor and Ristocetin-cofactor Activities. J. A. M. A. 236: 2770, 1976.

29. Weiss, H. J., Rogers, J. and Brand, H. Properties of the platelet retention (von Willebrand) factor and its similarity to the anti-hemophilic factor (AHF). Blood 41: 809, 1973.

30. Weiss, H. J., Rogers, J. and Brand, H. Defective ristocetin-induced platelet aggregation in von Willebrand's disease and its correction by factor VIII. J. Clin. Invest. 52: 2697, 1973.

31. Barrow, E. S., Reisner, H. M. and Graham, J. B. Separation of von Willebrand Factor from Factor VIII Related Antigen. VI Int. Cong. Throm. Haem.-XII Cong. World Fed. Hemoph. 1977. Abstract 38(1): 38, 1977.

32. Olson, J. D., Brockway, W. J., Fass, D. N., Bowie, E.J.W. and Mann, K. G. Purification of Porcine and human ristocetin-Willebrand factor. J. Lab. Clin. Med. 89: 1278, 1977.

33. Counts, R. B., Paskell, S. L., and Elgee, S. K. Disulfide bonds and the quarternary structure of factor VIII/von Willebrand factor. J. Clin. Invest. 62: 702, 1978.

34. Perret, B. A., Furlan, M., and Beck, E. A. Studies on Factor VIII-related protein. II Estimation of molecular size differences between factor VIII oligomers. Biochim. Biophys. Acta. 578: 164, 1979.

35. Fass, D. N., Knutson, G. J. and Bowie, E. J. Porcine Willebrand factor: A population of multimers. J. Lab. Clin. Med. 91: 307, 1978.

36. Weiss, H. J., Sussman, I. I. and Hoyer, L. W. Stabilization of Factor VIII in Plasma by the von Willebrand factor. J. of Clin. Invest. 60: 390, 1977.

37. Bloom, A. L. The von Willebrand Syndrome. Semin. Hematol. 17: 215, 1980.

38. Bloom, A. L. and Peake, I. R. Molecular Genetics of Factor VIII and its Disorders. Semin. Hematol. 14: 319, 1977.

39. Bloom, A. L. and Peake, I. R. Apparent "dominant" and "recessive" inheritance of von Willebrand's disease within the same kindreds. Possible biochemical mechanism. Thrombosis Research 15: 505, 1979.

40. Handin, R. I., Martin, V. and Maloney, W. C. Antibody-induced von Willebrand's Disease: A Newly Defined Inhibitor Syndrome. Blood 48: 393, 1976.

41. Rosborough, T. K. and Swaim, W. R. Acquired von Willebrand's Disease, Platelet-release Defect and Angiodysplasia. A. J. M. 65: 96, 1978.

42. Pizzuto, J., Ambriz, R., De La Paz Reyna, M., Monrroy, L. M., Morales, M. R., Aviles, A., Conte, G. and Enriquez, R. Acquired von Willebrand's Syndrome during Autoimmune disorder. Thrombos. Haem. 12: 1523, 1979.

43. Simone, J. V., Cornet, J. A. and Abildgaard, C. F. Acquired von Willebrand's Syndrome in Systemic lupus erythematosis. Blood 31: 806, 1968.

44. Mant, M. S., Hirsch, J., Gauldie, J., Bienenstock, J., Pineo, G. F. and Luke, K. H. von Willebrand's Syndrome presenting as an acquired bleeding disorder in association with a monoclonal gammopathy. Blood 42: 429, 1973.

45. Gralnick, H. R., Coller, B. R. and Sultan, Y. Carbohydrate deficiency of the factor VIII von Willebrand protein in von Willebrand's disease variants. Science 192: 56, 1976.

46. Morisato, D. K. and Gralnick, H. R. Selective Binding of the VIII/von Willebrand Factor Protein to Human Platelets. Blood 55: 9, 1980.

47. Moake, J. L., Olson, J. D., Troll, J. H., Weinger, R. S., Peterson, D. M. and Cimo, P. L. Interaction of platelets, von Willebrand factor and ristocetin during platelet agglutination. J. Lab. Clin. Med. 96: 168, 1980.

48. Kao, K-J., Pizzo, S. V., and McKee, P. A. Demonstration and characterization of specific binding sites for factor VIII/von Willebrand factor on human platelets. J. Clin. Invest. 63: 656, 1979.

49. Green, D., and Potter, E. V. Platelet-bound ristocetin aggregation factor in normal subjects and patients with von Willebrand's disease. J. Lab. Clin. Med. 87: 976, 1976.

50. Kattlove, H. E. and Gomez, M. H. Studies on the mechanism of ristocetin-induced platelet aggregation. Blood 45: 91, 1975.

51. Kirby, E. P. Factor VIII-Associated Platelet Aggregation. Thromb. and Haem. 38: 1054, 1977.

52. Ts'ao, C., Green, D. and Rossi, E. C. Some factors affecting fibrinogen precipitation by ristocetin: ultrastructure of precipitates. Blood 45: 621, 1975.

53. Jenkins, C.S.P., Phillips, D. R., Clemetson, K. J., Meyer, D., Larrieu, M-J. and Lüscher, E. F. Platelet Membrane Glycoproteins Implicated in Ristocetin-induced Aggregation. J. Clin. Invest. 57: 112, 1976.

54. Nachman, R. L., Jaffe, E. A. and Weksler, B. B. Studies on Ristocetin-induced Platelet Aggregation. Invited symposium abstract VI Int. Cong. Throm. Haem. - XII Cong. World Fed. Hemoph. Thromb. and Haem. 38(1): 239, 1980.

55. Penner, J. A. Factor VIII-related von Willebrand's factor. Blood Coagulation Laboratory Manual. September, 1980, pp.

56. Stibbe, J. and Kirby, E. P. The Influence of Haemaccel, Fibrinogen and Albumin on Ristocetin-induced Platelet Aggregation. Relevance to the quantitative measurement of the ristocetin cofactor. Thromb. Res. 8: 151, 1976.

57. Peerschke, E. I., Zucker, M. B., Grant, R. A., Egan, J. J., and Johnson, M. M. Correlation Between Fibrinogen Binding to Human Platelets and Platelet Aggregability. 55(5): 841, 1980.

Appendix

APPENDIX: Reagent Preparation

1. Tris-saline, pH 7.4 (0.14 M NaCl and 0.03 M Tris). 8.2 grams of NaCl + 3.6 grams Trizma (base) were dissolved in approximately 800 ml of distilled water. This solution was brought to a pH of 7.4 with 1 N HCl and then diluted to 1000 ml with distilled water.

2. 60 mg of Bovine Albumin in Tris-saline, pH 7.4. 0.6 grams of bovine albumin (Sigma, St. Louis, MO) was dissolved in 10 ml of the Tris-saline, pH 7.4.

3. Ristocetin (6.25 mg/ml) - Helena Laboratories, Beaumont, TX. To one vial of ristocetin 1.2 ml of distilled water was added. When used in the assay this was final concentration of 1.2 mg/ml.

4. 2% Paraformaldehyde. 2 ml of formaldehyde solution, HCHO 37% (Mallinckrodt, Paris, KY) was diluted to 100 ml with Tris-saline, pH 7.4.

VITA

The author was born in Kalamazoo, Michigan on June 17, 1950. In June 1972 she graduated from Michigan State University with a Bachelor of Science in Medical Technology. Immediately following graduation she started an internship in Medical Technology at E.W. Sparrow Hospital, Lansing, Michigan which she completed in June of 1973. In September 1974 the author started graduate school (part-time) in the Clinical Laboratory Science program at Michigan State University while working full-time in local hospitals. The author is married to David J. Zuhlke.