the man-mouse hybrid clones may indicate that the GAPDH, TPI, and LDH-B loci are close together.

The PEP-B locus has also been assigned to chromosome 12 (*13*) and may be located at 12q21 (*14*). The expression of PEP-B was independent of the expression of GAPDH, TPI, and LDH-B in 6 of 23 man-hamster hybrid clones (Table 1) and was independent of the expression of GAPDH and TPI in 3 of 28 man-mouse hybrid clones. The degree of discordancy in the expression of the PEP-B : LDH-B : TPI syntenic group in the man-hamster hybrid clones is three times greater than that observed in this series of hybrid clones for the PGD : PPH : AK-2 : PGM-1 syntenic group, the IDH-1 : MDH-1 pair, the ME-1 : SOD-2 pair, and the LDH-A : ACP-2 pair (*15*). If this is not due to chance, it may reflect the presence of a fragile site on a chromosome 12 from this particular white blood cell donor. The degree of discordancy in the expression of PEP-B and TPI in the man-mouse hybrids is comparable to that observed for the other known syntenic groups in these clones (*15*).

Nine loci specifying enzymes of the Embden-Meyerhof pathway of glycolysis have been assigned to particular human chromosomes (Table 2) and only three of the nine loci are syntenic (GAPDH, TPI, and LDH-B). If not due to chance, the synteny of these three loci may reflect their evolutionary relationship, which should also be evidenced by the structure of the enzymes.

Both GAPDH and LDH are tetramers whose molecular weight is 144,000 (*8, 16*). The four subunits of GAPDH are identical, as are those of the LDH-A and LDH-B homopolymers, and each subunit binds one molecule of the coenzyme NAD or NADH (*8, 16, 17*). The binding of NAD to GAPDH shows cooperativity, whereas in LDH the coenzyme binding sites do not interact (*17*). The three-dimensional structure of the coenzyme binding site of GAPDH is strikingly similar to that of LDH (*3*) and a lesser degree of structural homology is observed in the remainder of the two molecules (*3*). The structural and catalytic similarities of GAPDH and LDH may indicate that both dehydrogenases have evolved from a common ancestral gene (*18*).

TPI and GAPDH are sequential enzymes in the metabolism of glucose and 3-carbon intermediates. TPI is a dimer with a molecular weight of 52,000, is composed of identical subunits, and catalyzes the reversible isomerization of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (*19*). The TPI subunit is composed of alternating segments of α-helix and β-sheet structure (*20*), a structure which has also been observed in the dehydrogenases, in phosphoglycerate kinase, and in phosphoglycerate mutase (*3, 21*). These observations may indicate a distant evolutionary relationship of the glycolytic enzymes (*22*). The syntenic association of the genes specifying GAPDH and TPI may also be of significance for the regulation of these two glycolytic enzymes.

Further understanding of the significance of the syntenic association of the human genes specifying GAPDH, TPI, and LDH-B will require a determination of the distance between these loci and knowledge of the degree of conservation of this syntenic group in diverse species.

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Carbohydrate Deficiency of the Factor VIII/von Willebrand Factor Protein in von Willebrand's Disease Variants

Abstract. *Study of the normal human factor VIII/von Willebrand factor reveals a macromolecular glycoprotein composed of apparently identical subunits. This purified glycoprotein has procoagulant, antigen, and von Willebrand factor activities. In three patients with a variant of the von Willebrand's disease syndrome, their factor VIII/von Willebrand factor protein was present in normal amounts and had normal procoagulant and antigen activities; however, this protein was deficient in both carbohydrate and von Willebrand factor activity. The carbohydrate portion of the factor VIII/von Willebrand factor glycoprotein is of major importance in its interactions with platelets or the blood vessel wall, or both.*

Hemophilia A and von Willebrand's disease (vWd), two congenital hemorrhagic diseases, are similar in that they are both associated with reduced levels of procoagulant factor VIII (fVIII). Von Willebrand's disease can be distinguished from hemophilia by the findings of a long bleeding time, reduced ability of platelets to adhere to a column of glass beads (platelet retention), and autosomal dominant inheritance pattern (rather than X-linked recessive of hemophilia)

Table 1. Properties of chromatographed cryoprecipitate. α-Chymotrypsin–digested cryoprecipitate was chromatographed on Sepharose 4B as previously described (*2*). The void volume of the column was 48 ml. Protein elution was monitored at 280 nm. Factor VIII activity was measured in a one-stage assay utilizing the partial thromboplastin time; factor VIII assays were performed on all column fractions. For the measurement of fVIII/vWf antigen and vWf activity column fractions were pooled to form five separate pools (I to V). (The Roman numerals indicate in which pool the peak of antigen and of vWf were found.) Each pool was made up of 13 to 14 ml of column eluate. When necessary these pools were concentrated against powdered Ficoll and subsequently tested in polyacrylamide gel electrophoresis, counterimmunoelectrophoresis against goat antiserum to human fVIII, and in a gel-filtered washed, normal platelet system for vWf (*2, 6*). Antigen units are expressed as the highest reactive dilution per 1.0 $O.D._{280}$, and the vWf units are expressed as percentage of vWf activity (against a standard plasma curve per 0.1 $O.D._{280}$ of each column fraction). V_e, elution volume in milliliters; O.D., optical density.

	Peak procoagulant activity		Peak protein in the void volume		Peak antigen		Peak vWf	
	Units per milliliter	V_e (ml)	$O.D._{280}$	V_e (ml)	Units	Pool	Units	Pool
Normal (N = 15)	0.22 0.10–0.41	54 ± 1.5*	0.160 ± 0.38*	53 ± 1.5*	31.8 ± 7.6*	I	53.2 ± 15.6*	I
vW I	0.22	54	0.142	53	26.8	I	5.3	I
vW II	0.14	64	0.122	64	18.4	II	< 3.0	
vW III	0.30	55	0.167	54	21.9	I	12.1	I

*Mean ± 1 standard error.

(*1, 2*). Recently, immunologic studies with heterologous antibodies to fVIII have revealed that in most patients with vWd the fVIII-related antigen is reduced or undetectable. In hemophilia A despite the severe procoagulant deficiency the antigen is present in normal or increased amounts (*3*). Howard and Firkin (*4*) first noticed that the platelet-rich plasma (PRP) from patients with vWd did not aggregate in response to the antibiotic ristocetin, while the PRP from normal subjects and patients with hemophilia was reactive. Meyer *et al.* and Weiss *et al.* (*5*) devised tests to measure a plasma ristocetin cofactor by utilizing von Willebrand factor (vWf) platelets or normal washed platelets (unresponsive to ristocetin without the presence of the cofactor). Ristocetin-induced platelet aggregation is defective in PRP of patients with vWd, and vWd plasma does not support the aggregation of normal washed platelets or vWd platelets with ristocetin. Thus, an immunologic deficiency of the fVIII-related antigen and absence or reduction of ristocetin aggregation and the ristocetin cofactor have been added to the diagnostic abnormalities in vWd.

The human factor VIII/von Willebrand factor (fVIII/vWf) (*6*) has been characterized as a macromolecular glycoprotein. The normal purified glycoprotein possesses both procoagulant fVIII activity and vWf activity (as judged by the ristocetin aggregation assay) (*7, 8*). This glycoprotein has an estimated molecular weight of 1.2 to 1.6 million (*9–11*). It elutes in the void volume in all forms of gel chromatography and fails to enter 3 percent polyacrylamide gels when electrophoresed in the presence of sodium dodecyl sulfate (SDS). After reduction with dithioerythritol or 2-mercaptoethanol, a single subunit with a molecular weight of approximately 230,000 is present (*10, 11*). Both the intact glycoprotein and the subunit contain carbohydrate as judged by positive periodic acid–Schiff (PAS) stains (*10*). In addition, analysis of the purified human fVIII/vWf glycoprotein indicates that 5 to 7 percent of the molecule is made up of carbohydrate (*11, 12*). In hemophilia, a disease characterized by deficient procoagulant activity but intact vWf activity, a protein similar to normal is found which also contains carbohydrate (*10*).

We have studied three patients from three separate families with variants of the vWd syndrome who have a qualitative defect of their fVIII/vWf glycoprotein. In these unusual patients fVIII/vWf antigen and procoagulant activity were normal while the bleeding time, ristocetin-induced platelet aggregation (of their own PRP), plasma vWf activity (plasma added to vWd or nonreactive normal platelets), and platelet retention were all abnormal. Clinical data and studies on the purification of fVIII/vWf glycoprotein in two of these patients (vW I and vW II) have been described previously (*13*). In patients vW I and vW III (Table 1) the chromatogram of α-chymotrypsin–digested cryoprecipitate or intact plasma on a Sepharose 4B column (2.5 by 40 cm) were indistinguishable from normal. The procoagulant activity, protein, and antigen all had peak activity in the void volume fractions. In the other patient, vW II, no protein peak and minimal procoagulant activity were found in the void volume fractions. The peak of protein and fVIII procoagulant activity and antigen were delayed in elution compared to normal (Table 1).

Polyacrylamide gel electrophoresis analysis of the purified proteins from vW I and vW III were indistinguishable from normal. In the nonreduced state the protein did not enter the gel, and after reduction the protein of vW I and vW III had a subunit identical in mobility to the normal fVIII/vWf protein. In vW II the void volume fractions did not contain the fVIII/vWf protein, while the second fraction contained a protein that on polyacrylamide gel electrophoresis was indistinguishable from the normal.

Studies of the functional capacity of these proteins revealed normal procoagulant activity and normal antigenic reactivity; however, they were all defective in vWf activity (impaired support of ristocetin aggregation of vWd platelets or washed, gel-filtered, or formalinized normal platelets) (Table 1). The purified fVIII/vWf protein or plasma from these three patients did not inhibit or augment the ristocetin-induced platelet aggregation of either normal plasma or the normal purified fVIII/vWf protein.

Despite the normal polyacrylamide gel appearance of the intact protein and the subunit after reduction in these three patients, both the intact fVIII/vWf protein and the subunit were deficient in carbohydrate content as judged by repeatedly negative PAS stains (Fig. 1). These findings were confirmed on three polyacrylamide gel electrophoresis analyses performed on two separate preparations of the fVIII/vWf protein from each of these patients. We have described three other vWd patients with severe deficiencies of procoagulant vWf and antigen activities (*8*). The fVIII/vWf protein, its subunit, and PAS reactivity were not detectable in polyacrylamide gel electrophoresis. In contradistinction all normal (N = 18) and hemophilic (N = 10) preparations have been PAS positive.

In patients vW I and vW III and in

normal subjects the void volume fractions from chromatographed undigested plasma were tested in polyacrylamide gel electrophoresis. In the normal subjects the protein and subunit in the void volume were stained with PAS; in vW I and vW III the void volume protein and its subunit had a normal appearance, but they did not stain with PAS. This suggests that the negative PAS reactions with the cryoprecipitate were not related to cleavage of the carbohydrate by α-chymotrypsin.

In order to assess whether a specific glycosidase was present in the plasma of these three patients, we incubated samples of these vWd plasmas with the purified normal fVIII/vWf protein in the ratio of 1:9 for 30 minutes at 37°C. The mixtures were then subjected to electrophoresis in the presence of SDS on polyacrylamide gels. Identical gels were stained with either Coomassie blue or PAS. There was no alteration in the intensity or the position of the PAS-stained band after incubation with either the normal or the vWd plasmas.

The PAS reaction is thought to be specific for glycoproteins and glycolipids that contain vicinal hydroxyl groups. A positive PAS reaction requires an insoluble polyaldehyde of sufficient concentration density to be visible. This, in turn, depends upon a sufficient quantity of vicinal hydroxyl groups or of hydroxy-amino substitutions; a positive reaction indicates polyvicinal glycols or the equivalent. No unsaturated fats or glycolipids have been demonstrated in our preparation of fVIII (*11*); therefore, the positive PAS reaction of the normal and hemophilic fVIII is related to its carbohydrate content.

Some investigators have attributed a positive PAS reaction solely to the presence of sialic acid (*14*), while others (*15*) have demonstrated positive PAS reactions in glycoproteins in which sialic acid is absent. Recently, it was shown that the PAS-positive globules in the livers of patients with α_1-antitrypsin deficiency do not contain sialic acid and yet stain intensely positive with the PAS reagent (*16*). Although there may be an increased sensitivity of the PAS reaction for sialic acid residues, it is not specific for sialic acid.

It is well known that the presence of sialic acid residues plays an important role in the intravascular half-life of glycoproteins and cells. Prothrombin (*17*), ceruloplasmin (*18*), a variety of hormones and hormone-binding proteins (*18*), and erythrocytes (*19*) all have shortened intravascular half-lives after removal of sialic acid, while asialo fibrinogen (*20*) and transferrin (*18*) appear to have normal half-lives. The role of sialic acid in glycoprotein function, however, is not quite as clear since asialo gonadatropic hormones, follicle-stimulating hormones (*21*), and erythropoietin all lose their activity while prothrombin (*19*), fibrinogen (*20*), and transferrin (*22*) all maintain their biological activities. From our studies of these patients with vWd, it appears that sialic acid and other carbohydrates do not have primary importance in either procoagulant or antigenic activity, but are of major importance in maintaining vWf activity.

A specific glycolytic enzyme could not be identified in the plasma of these patients to account for the carbohydrate deficiency. It is, therefore, likely that a defect in which the carbohydrate cannot be attached in the proper sequence or in the proper total quantity compared to normal is responsible for the observed carbohydrate deficiency. This could be due to a specific amino acid substitution that results in the inability to attach carbohydrate to the base protein or a deficiency of an enzyme needed for attachment of carbohydrate to the protein, which is in contrast to the three previously described patients with vWd (*8*) in whom the defect appears to be in the synthesis of the fVIII/vWf protein.

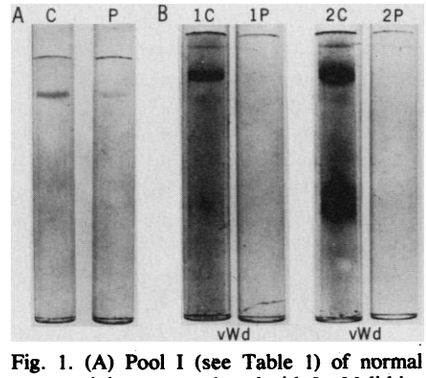

Fig. 1. (A) Pool I (see Table 1) of normal cryoprecipitate was reduced with 5 m*M* dithioerythritol (final concentration) and electrophoresed in the presence of SDS in 5 percent polyacrylamide gels. The protein stain was Coomassie blue (*C*), and the carbohydrate stain (*P*) was PAS. With normal and hemophilia A (not shown) column pools 20 to 30 μg of protein were electrophoresed for the protein stain and 40 to 60 μg for the carbohydrate stain. In all instances, with both protein and carbohydrate stains a band corresponding to an apparent molecular weight of 230,000 was observed. (B) Sodium dodecyl sulfate polyacrylamide gels of the reduced proteins of vW I (column "Pool," I, in Table 1) and vW II (column "Pool," II). On the left is the Coomassie stain (*C*) and PAS (*P* stains of patient vW I, and on the right are the gels of vW II. In patients vW I and vW III (not shown) 20 to 100 μg of the first column fraction protein were electrophoresed for the protein stain, while as much as 200 μg were electrophoresed for carbohydrate content. In patients vW I and vW III no carbohydrate (PAS-positive material) was seen in the first or second column fractions in the region of the 230,000-molecular-weight protein band. In vW II no protein bands were seen in fraction I. When 20 to 40 μg of protein fraction II were electrophoresed a distinct protein band was seen. However, electrophoresis of up to 150 μg of this fraction did not reveal any staining with the PAS reagents.

Despite this altered carbohydrate content of the vWd fVIII/vWf protein in these patients, the antigenicity of the protein appears to be normal by the Laurell and counterimmunoelectrophoresis techniques. Similarly, the antigenic determinants are not significantly altered, since lines of complete identity with normal were observed in immunodiffusion against goat antiserum to human fVIII/vWf. These findings are in contrast to blood group substances A, B, and H in which specific terminal carbohydrate results in a completely separate antigenic determinant (*23*).

The long bleeding time, reduced platelet retention, and abnormal ristocetin-induced aggregation suggests that these carbohydrate-deficient vWd proteins cannot interact appropriately with normal platelets or the blood vessel wall, or both. Neuraminidase treatment of normal cryoprecipitate results in direct aggregation of platelets. This activity can be abolished by further incubation of the cryoprecipitate with galactose oxidase (*24*). There is further evidence of an impaired ability of platelets from patients with vWd to adhere to denuded endothelial surfaces (*25*). Since recent studies (*26*) show that endothelial cells are capable of synthesizing the vWf, close association between the platelet, vWf, and endothelium or subendothelium may be responsible for the primary arrest of bleeding. Perhaps more intriguing is the suggestion that an exaggeration of this process may be responsible for the development of atherosclerosis. Harker *et al.* (*27*) postulate that endothelial smooth muscle proliferation stimulated by a factor released from platelets upon adhesion to the vessel wall may be the first step in the development of atheroma. The vWf is probably needed for platelet adhesion to noncollagenous subendothelial structures, and therefore a deficiency of vWf might be expected to result in protection from atherosclerosis. While no reliable human statistics are available, just such an effect was observed in pigs with vWd when compared to normal controls (*28*). This may be an interesting model for investigating the role of carbohydrates in coagulation glycoproteins in relation to atherosclerosis.

It is interesting to speculate on the mechanism of action of the vWf that would be consistent with a primary role for the carbohydrate moiety. One possibility is that the platelet membrane glycosyl transferases that are capable of transferring single units from one glycoprotein acceptor onto other glycoprotein acceptors actually result in a complex between the enzyme (that is, the platelet enzyme) and the acceptor (that is, the glycosyl residue of the fVIII/vWf protein) (*29*). Perhaps a similar enzymatic activity situated in the subendothelium would permit the vWf to act as a bridge between the subendothelium and the platelet. This type of mechanism may be of more general interest since it may underlie a variety of cell-cell adhesion phenomena (*30*).

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Insect Photoperiodism: The "T Experiment" as Evidence for an Hourglass Mechanism

Abstract. *"T experiments" demonstrate that the clock controlling termination of larval diapause in* Ostrinia nubilalis *is an hourglass mechanism that measures 8 hours of darkness. Circadian oscillations are not involved in photoperiodic time measurement in this species.*

Many insect species display a seasonal discontinuity in their life cycles (*1*). In temperate latitudes reproduction and development routes are characteristic of spring and summer months. A state of arrested development known as diapause, however, is entered during late summer and early fall, which enables the insects to withstand the environmental rigors of winter. When induction and termination of diapause are controlled by the daily alternation of light and dark in the environment, this is referred to as photoperiodism (*2*).

The central problem in the study of both animal and plant photoperiodism has been that of elucidating the nature of the clock that measures the duration of the day (or night). For 30 years, evidence has been accumulating that circadian clocks are somehow involved in photoperiodism. Circadian clocks are endogenous oscillations that have a period length of about a day when measured under constant conditions of light and temperature. They control the timing of many metabolic and behavioral events in eukaryotic organisms.

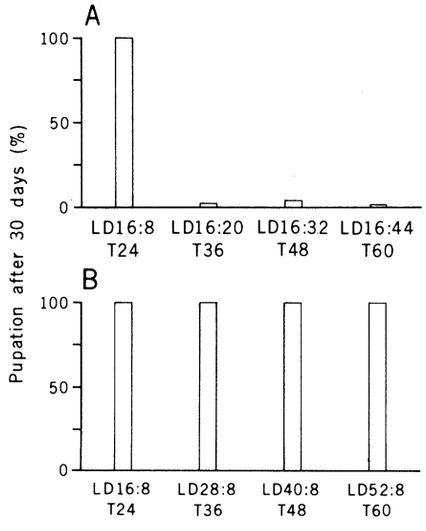

Fig. 1. Percentage of pupation 30 days after transfer from LD 12 : 12 to various T's. In (A), L was held constant at 16 hours and T was varied by extending D. In (B), D was held constant at 8 hours and T was varied by extending L. In both (A) and (B) the data from the four replicates in each light cycle are combined. The sample size is thus 100.

Although an extensive search for a common photoperiodic timing mechanism involving the circadian clock was made in the 1960's, Pittendrigh (*3*) has suggested that a diversity of mechanisms exist. Two different models of the photoperiodic clock, external and internal coincidence, do involve circadian oscillations. A third, however, views the clock as an hourglass or interval timer which measures the length of the night by the accumulation of some unknown metabolic product. Light destroys the product (resets the hourglass). This model of the clock is nonoscillatory in nature.

The experimental paradigm most commonly used to demonstrate that a circadian oscillation is involved in photoperiodic time measurement is referred to as a "T experiment," where T is the period of the light-dark cycle in hours. In a standard T experiment the organisms are exposed to cycles in which a fixed light phase is systematically coupled to dark phases of various durations, thus creating different T's (*4*). When photoperiodic induction rises and falls as a function of T, neither the length of the light phase nor that of the dark phase is responsible for the response. Although the rationale