Techniques, Vol. I, Chromatography, 3rd ed, New York, N. Y., Interscience Publishers.
Stewart, J. M., and Young, J. D. (1969), Solid Phase Peptide Synthesis, San Francisco, Calif., W. H. Freeman and Co.
Stockigt, J. R., Collins, R. D., and Biglieri, E. G. (1971), *Circ. Res., Suppl. II 28* and *29*, 175.
Waldhäusl, W. K., Lucas, C. P., Conn, J. W., Lutz, J. H., and Cohen, E. L. (1970), *Biochim. Biophys. Acta 221*, 536.

Activation of the Coagulation Factor VII by Tissue Thromboplastin and Calcium†

Bjarne Østerud,‡ Åse Berre,§ Anne-Brit Otnaess, Eirik Bjørklid, and Hans Prydz*

ABSTRACT: Factor VII has been isolated in an activated state after incubation with tissue thromboplastin and calcium and subsequent destruction of all tissue thromboplastin activity by phospholipase C (EC 3.1.4.3). This activated state is dependent upon the presence of phospholipid bound to factor VII, since the activation is reversible by prolonged phospholipase C treatment. Binding of factor VII to tissue thromboplastin membranes is thus not necessary for the activation of factor X by factor VII provided factor VII previously has been exposed to tissue thromboplastin.

In the presence of calcium ions, tissue thromboplastin and factor VII form a complex which activates factor X. Whether thromboplastin activates factor VII to an enzyme, VII_a,[1] which alone can activate factor X, or the complex is necessary for the activation, is not definitely known. Pitlick *et al.* (1971) recently described a peptidase activity in purified tissue thromboplastin preparations. If the activation of factor VII in the extrinsic blood coagulation system were due to a limited proteolysis by this peptidase, the product of this activation, VII_a, might possibly be irreversibly activated and able to act alone as the extrinsic factor X activator.

We report experiments to decide between these alternative mechanisms for the first reaction of the extrinsic coagulation pathway. After interaction between thromboplastin, factor VII, and calcium ions, we tested the product for its ability to activate factor X following destruction of thromboplastin by phospholipase C. We also have separated factor VII_a from thromboplastin.

Materials and Methods

Cephalin from human brain was prepared and stored as described by Hjort *et al.* (1955). The stock was diluted 150-fold in barbital buffer (sodium diethyl barbiturate (11.75 g), sodium chloride (14.67 g), 0.1 N HCl to pH 7.3 (about 430 ml), and distilled water to 2000 ml) (Owren 1947) prior to use.

Tissue thromboplastin was a saline extract of human brain (Hjort, 1957) further purified as described by Hvatum and Prydz (1966, 1969).

Factor VII and factor X were purified from serum (Gladhaug and Prydz, 1970). The preparations were homogeneous in immunoelectrophoresis (Prydz and Gladhaug, 1971), disc electrophoresis (Gladhaug and Prydz, 1970), and analytical ultracentrifugation (to be published) and did not clot fibrinogen (Prydz, 1965; Gladhaug and Prydz, 1970). An antiserum against pure factor X was produced and treated with barium sulfate (Prydz and Gladhaug, 1971).

Coagulation Tests. One stage assays for prothrombin and factors VII, IX, IX_a and X were carried out as described by Østerud and Rapaport (1970), and tissue thromboplastin was measured as described by Hvatum and Prydz (1966) using both normal and factor VII deficient plasma as substrates.

The bentonite-adsorbed plasma used in the factor X assay contained 21% prothrombin. The final system had a buffer time about 120 sec. The test sample was always preincubated separately to avoid the effect of phospholipase C upon the cephalin of the test system.

The extrinsic factor X activator activity (VII_a) was measured in plastic clotting tubes with factor VIII deficient plasma as substrate, using a cephalin reagent without kaolin powder. Substrate (0.1 ml) and cephalin (0.1 ml) were incubated for 3 min at 37°, the test substance (0.1 ml) and calcium (0.1 ml of prewarmed 40 mM $CaCl_2$) were added and the clotting time was noted.

Barium sulfate eluates were prepared according to Hjort (1957), from plasma of patients congenitally deficient in factor VII, factor IX, factor X or factor XI, respectively. The anticoagulants used (one volume per nine volumes of plasma) were a citrate buffer (for factor VII, VIII, IX, and X deficient plasma samples) (Rapaport *et al.*, 1965) and a 2% solution of Na_2EDTA adjusted to pH 7.3 with 2 M NaOH (for factor XI and some samples of factor X deficiency plasma). One volume of the same EDTA solution was added to nine volumes of citrated plasma samples before $BaSO_4$ treatment. The plasma was made factor X deficient before or after the $BaSO_4$

† From the Institute of Medical Biology, University of Tromsø, Tromsø, Norway, and from the Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo, Norway.
‡ Research Stipendiate of the Norwegian Research Council for Science and the Humanities.
§ Research Fellow of the Norwegian Council on Cardiovascular Diseases.

[1] The subscript "a" is usually applied to the activated form of various coagulation factors (X_a, IX_a, etc.) which are assumed to be derived from the native form by a change in the primary, secondary or tertiary structure of the molecules. Since the activated form of factor VII described here apparently is a complex of factor VII and phospholipid, we suggest that it should be called VII_{pl}.

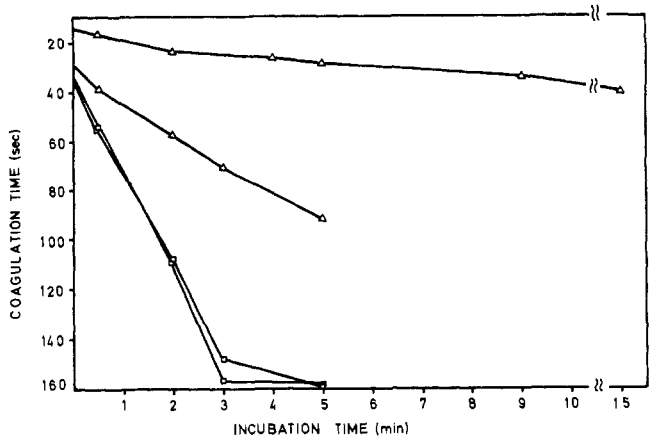

FIGURE 1: Decrease in tissue thromboplastin and factor VII_a activity during treatment with phospholipase C. $BaSO_4$ eluate (0.85 ml from factor X deficiency plasma dialyzed against barbital buffered saline), tissue thromboplastin (0.05 ml), and calcium (0.1 ml, 60 mM $CaCl_2$) were incubated for 5 min at 37°. Phospholipase C was added and samples were withdrawn at indicated time intervals for testing of factor VII_a. In the controls buffered saline was used instead of $BaSO_4$ eluate. The control samples were tested for tissue thromboplastin activity with the same substrate plasma as used in the factor VII_a test system. Duplicate experiments. (□) Tissue thromboplastin activity; (△) factor VII_a activity.

treatment and after activation with tissue thromboplastin, by incubation with specific antiserum against factor X (2 hr at 37°, then 12 hr at 4°), so that no factor X activity was detectable even after activation with tissue thromboplastin or Russel's viper venom. Plasma samples deficient in factors X and XI were kindly provided by Dr. S. I. Rapaport, Department of Medicine, University of Southern California, Los Angeles.

Phospholipase C (EC 3.1.4.3) was purified from *Bacillus cereus* ATCC 10987 culture supernatants to apparent homogeneity in disc electrophoresis, sodium dodecyl sulfate electrophoresis, and analytical ultracentrifugation (Otnaess *et al.*, 1972). When desired the action of the enzyme was stopped by cooling in ice water or addition of EDTA.

Results

Barium sulfate eluates from various deficiency plasmas were incubated at 37° with tissue thromboplastin and calcium to activate factor VII. Small residual amounts of factor X activity were neutralized with specific antibody, and tissue thromboplastin was destroyed with phospholipase C (Otnaess *et al.*, 1972). The phospholipase reaction was stopped after 3–5 min by cooling in ice water. The ability to activate factor X was estimated with factor VIII deficient plasma as substrate to exclude any activation mediated through the intrinsic pathway (Table I) (see Methods). Factor X activator activity (VII_a) was markedly reduced or abolished when tissue thromboplastin was destroyed with phospholipase C by prolonged incubation, in accordance with earlier studies (Otnaess *et al.*, 1972). A significant factor VII_a activity remained, however, when brief phospholipase C treatments were used. The activity was obtained with $BaSO_4$ eluates from factor IX or factor XI deficiency plasma (Table I), but not from factor VII deficient plasma. Factor X_a was not present in the reaction mixtures (column 3, Table I).

All samples were neutralized with a titrated amount of antifactor X serum before incubation with tissue thromboplastin and calcium. This incubation regularly caused a small increase in the residual activity of factor X. A small amount of antiserum was again added to neutralize this activity. The coagulation times given by the test samples were longer than the buffer values of the factor X system (column 3) due to this addition of a slight excess of antifactor X serum. This excess was, however, very small compared to the amount of factor X in the factor VIII deficiency plasma used as substrate in the factor VII_a test system and could not significantly influence this system. Factor X was thus completely neutralized with the antiserum before the activity of VII_a was tested.

In control incubations the procoagulant activity of tissue thromboplastin was completely destroyed by identical phospholipase C treatment (before treatment 30 sec, after treatment 137 sec, when tested in the VII_a system, 35 and 158 sec when tested with normal plasma).

Tissue thromboplastin is retained by filters with a pore size of 0.1 μ (Hvatum and Prydz, 1967). To exclude the presence of a tissue thromboplastin–factor VII complex resistant to phospholipase C, we passed the activator-containing mixture through such filters with or without prior enzyme treatment. The filtrate activated factor X to the same extent (Table II) while tissue thromboplastin or $BaSO_4$ eluate filtered separately was negative. When the filter was washed with 0.15 M NaCl in 0.01 M sodium citrate (pH 7) more VII_a activity was eluted. Such a filtrate from a reaction mixture containing $BaSO_4$ eluate from factor X deficient plasma gave 26 sec in the factor VII_a system.

Similar results were obtained when a reaction mixture (factor X deficient plasma treated with antifactor X serum to neutralize residual factor X activity, tissue thromboplastin, and calcium) was centrifuged at 100,000*g* for 1–2 hr. This treatment will cause essentially all tissue thromboplastin activity to sediment (1–2% remained in the supernatant, corresponding to coagulation times of about 70 sec in the controls without plasma). The supernatants gave clotting times in the factor VII_a assay of 29 sec.

The recovery of factor VII_a in the supernatant was less reproducible when barium sulfate eluates from the same factor X deficient plasma were used as source of factor VII. In the presence of bovine serum albumin (final concentration 13%), however, factor VII_a was reproducibly recovered from the supernatant in the presence or absence of free calcium ions during ultracentrifugation. With citrate added before centrifugation, the coagulation time in the VII_a system was 26 sec, without citrate 24 sec. Tissue thromboplastin sedimented less completely in the presence of albumin, and should therefore be spun down through albumin before use in the incubation mixture to exclude the fraction not sedimenting. The VII_a activity in these supernatants was susceptible to phospholipase C. Factor X assay of the reaction mixture showed no activity (buffer time or longer) in all ultracentrifugation experiments.

The above results might be explained by the formation of an activated factor VII in the reaction with tissue thromboplastin and calcium. Since prolonged incubation of the tissue thromboplastin–factor VII–calcium mixture with phospholipase C abolished the VII_a activity completely (Otnaess *et al.*, 1972), the mixture was submitted to phospholipase C after removal of tissue thromboplastin by filtration. Factor VII_a was fairly stable in the absence of phospholipase C, whereas no activity remained after the phospholipase treatment (Table III). After neutralizing the phospholipase C with EDTA (Otnaess *et al.*, 1972) cephalin, tissue thromboplastin or its isolated phospholipid fraction (fraction B,

TABLE I: Activation of Factor X by the Product of the Interaction between Tissue Thromboplastin, Ca^{2+}, and Factor VII.[a]

$BaSO_4$ Eluate from	Factor VII_a Act. after Phospholipase Treatment[b]		Factor X Act. after Phospholipase Treatment[b]
	3 min	10 min	
XI dp[c]	33		206
	40	141	
IX dp[c]	48		180
	45	189	
VII dp[c]	156		200
Tpl[d]	137		
Buffer	240		120

[a] $BaSO_4$ eluates (0.4 ml), from which factor X was removed by immunoprecipitation, were activated (37°, 3 min) with tissue thromboplastin (0.05 ml) and calcium (0.05 ml, 60 mM $CaCl_2$) and incubated with phospholipase C (6 μl) for 2 min at room temperature and 1 min at 37° or for 10 min at 37°. The phospholipase reaction was stopped by cooling in ice water. The samples were then added to a substrate mixture prepared by incubating factor VIII deficiency plasma (0.1 ml) and cephalin (0.1 ml) for 3 min at 37°. In rapid succession, a sample from the reaction mixture (0.1 ml) and 40 mM $CaCl_2$ (0.1 ml) were added and the clotting time was noted (columns 1 and 2). Factor X activity (column 3) was estimated as described in Methods. The mixtures did not contain thrombin. No clot was observed with plasma for 20 hr. [b] Coagulation time (sec). Average of three experiments. [c] Deficiency plasma. [d] Tissue thromboplastin, calcium and saline treated with phospholipase C.

TABLE II: Separation of Activated Factor VII from Tissue Thromboplastin by Filtration.[a]

$BaSO_4$ Eluate from	Act. in Filtrates of Reaction Mixture	
	Factor VII_a Act. (sec)	Factor X Act. (sec)
XI dp	41	106
X dp	50	134
IX dp	36	108
Normal plasma	40	140
Normal plasma[b]	260	

[a] After addition of sodium citrate (0.1 ml, 0.14 M) the mixtures of thromboplastin, calcium, and factor VII (prepared and incubated as in Table I, including neutralization with anti factor X and phospholipase C) were passed through 0.1 μ filters. [b] Incubated without thromboplastin.

Hvatum and Prydz, 1969) was added. Neither cephalin nor fraction B alone restored the factor VII_a activity (Table IV). When tissue thromboplastin was added the activity reappeared, indicating that factor VII itself was not destroyed by phospholipase.

To exclude that other factors took part in this activation, the experiments were repeated with pure factor VII preparations. In the presence of tissue thromboplastin and calcium a potent factor X activator was obtained, but in contrast to factor VII in $BaSO_4$ eluates, factor VII purified from serum stuck to the complex formed and no factor VII_a was found in the filtrate after citrate or phospholipase C treatment.

To study the kinetics of formation of factor VII_a, crude factor VII ($BaSO_4$ eluates of plasma from the various deficiency patients) or pure factor VII were mixed with tissue thromboplastin and calcium and incubated. Samples were pipetted into tubes containing sodium citrate and cooled in ice water. All samples were treated simultaneously with phospholipase C for 3 min, and subsequently tested. Factor VII_a was clearly demonstrable although control experiments indicated that all tissue thromboplastin activity was destroyed by this treatment (Figure 1). Purified factor VII was activated by tissue thromboplastin within 20 sec, while factor VII in a $BaSO_4$ eluate underwent a more time consuming reaction (Table V).

To demonstrate the different kinetics for destruction of tissue thromboplastin and factor VII_a by phospholipase C, various sources of factor VII were mixed with tissue thromboplastin and calcium and incubated for 5 min at 37°. Phospholipase C was added and subsamples were withdrawn after various intervals for testing of factor VII_a with factor VII, VIII or IX deficiency plasma as substrate. Essentially similar results were obtained with these different substrates. Tissue thromboplastin activity was tested in parallel incubations identically treated except that 0.15 M NaCl was substituted for factor VII. The results clearly demonstrated that the incubation mixtures retained their ability to activate factor X when all tissue thromboplastin activity had been destroyed (Figure 1).

Discussion

The activation of factor X by the extrinsic coagulation pathway is generally thought to be due to a tissue thromboplastin–factor VII–calcium complex (Hougie, 1959; Straub and Duckert, 1961; Williams and Norris, 1966; Nemerson, 1966). There is to our knowledge no direct evidence in the literature for a thromboplastin activated factor VII, VII_a, which can activate factor X in the absence of tissue thromboplastin. Our results (Table I and Figure 1) demonstrate that

TABLE III: Effect of Phospholipase C on Factor VII_a after Filtration.[a]

Addition	Clotting Time (sec) in Factor VII_a System after Incubation for	
	30 sec	600 sec
Phospholipase C	46	300
	33	168
Buffer	46	50

[a] Mixtures of thromboplastin, calcium, and factor VII (prepared and incubated as in Table I) were passed through 0.1-μ filters. Phospholipase C (3 μl) or buffer was added to the filtrates (0.4 ml) and incubated at 37° for 30 or 600 sec. Factor VII_a was determined.

TABLE IV: Restoration of Factor VII_a Activity after Inactivation by Phospholipase C.[f]

Factor VII Source	Activity[a] in Sample after				
	1. Filtration	Inactivation	Addition of Phospholipid	Addition of Tissue Thromboplastin	2. Filtration
X dp ($BaSO_4$ eluate)	26	181	171[c]	30	
	32	70	78[c]	31	36
XI dp ($BaSO_4$ eluate)	33	168	150[d]	41	
Normal plasma[b]	46	400		32	
Tissue thromboplastin	>180[e]				

[a] Coagulation time (seconds) tested in VII_a system except results in column 4 where a factor VII deficiency plasma was used as substrate. [b] Factor X removed by immunoprecipitation. [c] Cephalin (final dilution $^1/_{250}$ from stock). [d] Fraction B (final concentration about 60 μg/ml). [e] Filtered through 0.22-μ filter. [f] Incubation mixtures were prepared and incubated as in Table I, 0.1 volume of citrate anticoagulant was added, and the mixtures were filtered through 0.1- or 0.22-μ filters. After phospholipase C treatment and inactivation of phospholipase by EDTA (final concentration 0.2–0.4 mM), cephalin or fraction B was added and aliquots of the mixtures were tested in the VII_a system after 10 min at 2°. Aliquots were also tested by mixing with prewarmed tissue thromboplastin and factor VII deficient plasma (column 4) and immediate recalcification. Another aliquot was incubated with thromboplastin and calcium (3 min/37°) and tested in the factor VII system before filtration and in the factor VII_a system after filtration.

mixtures of factor VII, calcium and tissue thromboplastin still contain a factor X activator when all thromboplastin activity has been destroyed by phospholipase C. This activator was formed in $BaSO_4$ eluates from plasma samples deficient in factor VIII, IX, X, or XI, but not in factor VII deficient plasma. The presence of factor X or thrombin in the mixtures used to produce the activator was rigorously excluded. A similar activator was formed when factor VII preparations purified to electrophoretic homogeneity were used. We conclude that the factor X activator is an activated form of factor VII.

Factor VII_a passed through filters with pore size 0.1 μ (Table II). Such filters retain all tissue thromboplastin activity of preparations like those used here (Hvatum and Prydz, 1967). Similar results were obtained in the ultracentrifugation experiments. Under conditions where tissue thromboplastin sedimented, factor VII_a remained in the supernatant. The presence of tissue thromboplastin was therefore clearly not necessary for the activation of factor X by factor VII_a. On the other hand, factor VII_a was sensitive to further phospholipase C treatment (Table III). (This provided additional evidence that factor X_a was not present, since X_a is not sensitive to phospholipase C.) The enzyme did not affect factor VII itself, since the factor was reactivated by a second addition of tissue thromboplastin. The activator (VII_a) was thus probably a smaller complex of factor VII and phospholipid not including the protein part of tissue thromboplastin (Hvatum and Prydz, 1969), since this would require that the addition of factor VII to tissue thromboplastin provoked the major changes in the membraneous structure of thromboplastin necessary to solubilize the protein. The protein part was, however, necessary for the transformation of factor VII to VII_a, since neither cephalin nor the phospholipid component of tissue thromboplastin alone activated or reactivated factor VII (Table IV). Binding of factor VII to the complete tissue thromboplastin complex may cause conformational changes in factor VII, permitting its activation of factor X. The activated state may be maintained in the complex with phospholipid. The phospholipid may also serve to place factor VII and X in juxtaposition to facilitate their interaction.

In any case, the hydrophilic part of the phospholipid molecules must be of importance, since removal of the phosphorylated component reduced or abolished the activity of factor VII_a. No marked differences were seen in VII_a activity in the presence or absence of citrate. Our tentative conclusion was therefore that calcium was not necessary to maintain factor VII in the activated state.

In some experiments, filtration or ultracentrifugation caused a partial inactivation of factor VII_a, *i.e.*, the filtrate or supernatant contained factor VII that could be activated by tissue thromboplastin, although factor VII in the original solutions was maximally activated. The factor VII–phospholipid complex was therefore probably destabilized or split by these treatments, since it appeared to be reasonably stable in solution when left untreated. In some of the ultracentrifugation experiments albumin seemed to stabilize the complex.

It now appears that factor VII may be activated in at least three different ways. The intrinsic coagulation mechanism

TABLE V: Time Course of Appearance of Factor VII_a.[a]

Reagent	Clotting Time (sec) in Factor VII_a System after Incubation of Reaction Mixtures for		
	20 sec	60 sec	300 sec
Crude factor VII ($BaSO_4$ eluate)	75	59	37
Purified factor VII	26	27	27

[a] Factor VII reagent (0.85 ml) and 60 mM $CaCl_2$ (0.1 ml) were incubated with tissue thromboplastin (0.05 ml). Subsamples (0.1 ml) were withdrawn as indicated into tubes containing 0.14 M sodium citrate (0.05 ml). Phospholipase C was added and the samples were processed as in Table I.

causes the increase in factor VII activity observed in serum when plasma is left to clot spontaneously (Johnston and Hjort, 1961). The activation is accompanied by a decrease in the apparent molecular weight of factor VII from about 59,000 to about 45,000 (Gladhaug and Prydz, 1970). Factor VII activated in this way cannot activate factor X without prior exposure to tissue thromboplastin, and is therefore not equivalent to VII_a. The reduction of the molecular size probably gives the molecule a higher affinity for tissue thromboplastin or its phospholipid component since (1) factor VII purified from serum formed VII_a much more rapidly with tissue thromboplastin than factor VII partially purified from plasma (Table V), and (2) no VII_a was recovered in the filtrate of the reaction mixture, indicating a stronger binding of the pure factor VII to tissue thromboplastin. Alternative explanations are, however, possible. The second way of activation of factor VII may be through the kallikrein system (Gjønnaess and Stormorken, 1970, 1971[2]). The product of this activation remains uncharacterized. Thirdly, the activation by tissue thromboplastin seems to involve the formation of a phospholipid–factor VII complex without any irreversible changes in the factor VII molecule, since factor VII_a was inactivated by prolonged phospholipase treatment and could subsequently be completely reactivated by renewed exposure to tissue thromboplastin.

The physiological significance of these three ways of activation remains largely unknown. The possible circulation of factor VII_a after activation by exposure to tissue thromboplastin *in vivo* may be important in the pathogenesis of intravascular coagulation, since it provides a mechanism by which a local exposure of tissue thromboplastin may lead to a more generalized activation of the coagulation system.

[2] Personal communication.

References

Gjønnaess, H., and Stormorken, H. (1970), *Thromb. Diath. Haemorrh. 24*, 308–310.

Gladhaug, Å., and Prydz, H. (1970), *Biochim. Biophys. Acta 215*, 105–111.

Hjort, P. F. (1947), *Scand. J. Clin. Lab. Invest. 9, Suppl. 27*, 17.

Hjort, P., Rapaport, S. I., and Owren, P. A. (1955), *J. Lab. Clin. Med. 46*, 89–97.

Hougie, C. (1959), *Proc. Soc. Exp. Biol. Med. 101*, 132.

Hvatum, M., and Prydz, H. (1966), *Biochim. Biophys. Acta 130*, 92.

Hvatum, M., and Prydz, H. (1969), *Thromb. Diath. Haemorrh. 21*, 217.

Johnston, C. L., and Hjort, P. F. (1961), *J. Clin. Invest. 40*, 743–751.

Nemerson, Y. (1966), *Biochemistry 5*, 601.

Østerud, B., and Rapaport, S. I. (1970), *Biochemistry 9*, 1854.

Otnaess, A.-B., Berre, Å., Bjørklid, E., and Prydz, H. (1972), *Eur. J. Biochem. 27*, 238.

Owren, P. A. (1947), *Acta Med. Scand. 128, Suppl. 194*, 327.

Pitlick, F. A., Nemerson, Y., Gottlieb, A. J., Gordon, R. G., and Williams, W. J. (1971), *Biochemistry 10*, 2650.

Prydz, H. (1965), *Scand. J. Clin. Lab. Invest. 17, Suppl. 84*, p 78.

Prydz, H., and Gladhaug, Å. (1971), *Thromb. Diath. Haemorrh. 25*, 157.

Rapaport, S. I., Hjort, P. F., and Patch, M. J. (1965), *Scand. J. Clin. Lab. Invest. 17, Suppl. 84*, 88.

Straub, W., and Duckert, F. (1961), *Thromb. Diath. Haemorrh. 5*, 402.

Williams, W. J., and Norris, D. G. (1966), *J. Biol. Chem. 241*, 1847.