QUANTITATIVE ESTIMATES OF PROTHROMBIN COMPONENTS

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CHARLES E. HIAR
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ABSTRACT

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by Charles E. Hiar

The formation of thrombin from prothrombin involves the interaction of Factors V, VII, X, and prothrombin. Physicochemical studies on these clotting factors have been published by various authors. Many of their results have not been in agreement. The work summarized in this report concerns the heat stability of the "prothrombin complex". Normal and artificially reconstituted plasmas were incubated at 45 C. for varying lengths of time. The effect of this incubation is evaluated. In addition, a method is presented by which the concentration of the heat labile Factor V can be determined by this incubation.

The presence or absence of the four factors are detected by the one-stage prothrombin time. Specially prepared plasmas and serums are used to vary the levels of the factors. These reagents, when used in conjunction with the automated prothrombin time procedure, provide a sensitive method of detecting differences in levels of the clotting factors. The study indicates that incubation at 45 C. can be used to measure the concentration of Factor V, while the other 3 factors are not adversely affected.

QUANTITATIVE ESTIMATES OF PROTHROMBIN COMPONENTS

By

Charles E. Hiar

A THESIS

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To Ruth Ann

for her constant encouragement
over the past two years

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INTRODUCTION

As candidates for the Master's Degree in Clinical Laboratory Science, we are mainly interested in the clinical aspects of pathology. One of our major goals is to devise easy and practical methods of solving problems arising in clinical laboratory procedures. As the field of clinical pathology advances, new methods are continuously being sought to augment existing ones. Procedures must also be found to measure substances which could previously not be assayed. Such is the problem encountered in oral anticoagulant therapy with respect to the four factors of the prothrombin complex.

The prothrombin time is universally used in the clinical laboratory to study hemorrhagic disorders in Stage II of the clotting mechanism. The test is simple in concept, easy to perform, and can be adapted to a variety of uses. It was originally thought that only prothrombin deficiencies could be detected by the test. It is now known that the prothrombin time may reflect deficiencies in a complex of interacting factors. These are the Factors V, VII, X, and prothrombin itself.

Factor V is probably the least understood of the four factors involved. No one is certain as to where it originates or exactly what role it plays in coagulation. The factor can be assayed by several methods. Each of these has its disadvantages, however. The relationship or ratio of Factor V to Factors VII, X, and prothrombin has been the prime concern of this work. A change in ratio of the Factor V

concentration to the others which are affected by coumarin therapy might indicate a more complete picture of the patient's progress.

Various investigators have tried to isolate the clotting factors and analyze them to determine their function, chemical, and physical properties. Varying results have been achieved. These have been, in large part, due to difficulties in reagent standardization, and the inherent instability of the clotting factors. Technical errors have also affected reproductibility and accuracy and have played a large part in leading to conflicting results.

The author was concerned with the unreliability of this physicochemical data presented on the factors of the prothrombin complex. With this in mind, the thermostability of these factors was reinvestigated.

The study was carried out using the one-stage prothrombin time adapted to an automated procedure. Standardized commercial reagents were used whenever possible. This eliminated, to a large degree, the technical difficulties encountered in testing, and allowed for greater sensitivity and reproductibility of results. The study was carried out with the purpose of measuring the thermal decay of the factors involved in the prothrombin complex. With this in mind we hoped to shed additional light on the physicochemical properties of these clotting factors. Knowledge of the extent of decay should give an estimation of concentration of the labile factors present. Our second purpose, then, was to devise a simple procedure for the detection of concentration of the labile Factor V using thermal incubation.

REVIEW OF LITERATURE

Since several prominent investigators have postulated mechanisms for the coagulation of blood, it would be impossible to review the prothrombin mechanism without first relating it to the overall coagulation scheme. The simple explanation by Morowitz was an accumulation of all the known facts about coagulation up to the end of the last century. His classical theory stated that:

$$\text{Thrombokinase} + Ca^{2+} + \text{Prothrombin} \longrightarrow \text{Thrombin}$$

This simple theory lay dormant until the development of the one-stage prothrombin time by Quick (1937) whereby the classical theory of Morowitz was put to practical use. Since then the Morowitz theory has become extremely inadequate, but the usefulness of his basic principles still prevails.

Seegers (1962) postulated a theory that prothrombin plays the central dominant role in the blood clotting scheme, and that all other factors except Platelet Co-factor I (Factor VIII) and Ac-globulin (Factor V), are derivatives of it.

A third scheme by Macfarlane (1964), called the "cascade" theory, and a similar one by Davie and Ratnoff (1964), the "waterfall" sequence, differs from that of Seegers. They postulated that clotting is initiated by the activation of Factor XII. The active Factor XII acts as an enzyme activating Factor XI. This in turn activates other factors in a sequence resulting in the formation of thrombin from prothrombin, and finally the formation of fibrin from fibrinogen.

Prothrombin

Seegers, Macfarlane, Davie and Ratnoff all agree on one fact in their proposed clotting mechanisms. They have found that prothrombin exists in only the free or readily activated state. Anderson and Barnhart (1964) found prothrombin in isolated microsomal and soluble fractions in bovine and canine liver parenchymal cells. The workers found subcellular prothrombin in these fractions using specific fluorescent antiprothrombin homologous with the species. Induced hypoprothrombinemia reduced or eliminated microsomal prothrombin. This led them to conclude that the site of synthesis of prothrombin is the microsomes of the liver cells.

Various techniques have been used to isolate and study prothrombin in a relatively pure state. Tishkoff and Williams (1967) isolated prothrombin as a barium complex containing protein 21.5%, barium 32%, and citrate 8.9%. They discovered prothrombin to be an α-2-glycoprotein found in a high degree of purity as a true prothrombin-metal complex. The molecule was stable in this form. This work agrees with other investigators to the extent that prothrombin is a glycoprotein.

Seegers (1940) observed the protein-carbohydrate complex to contain about 4% carbohydrate, while Tishkoff *et al.* (1960) found 10.4-14.7% in bovine prothrombin. Whether or not there is a species difference has not been conclusively determined.

Tishkoff and his co-workers (1960) found that sialic acid comprised the largest constituent in the carbohydrate fraction of their bovine preparations. Their electrophoretic studies on starch gel showed prothrombin to be the least mobile, when compared to Factors VII and X.

These other factors contained little protein but substantial quantities of sialic acid.

The conversion of prothrombin to thrombin is an undisputed fact. Whether or not the prothrombin itself converts autocatalytically to thrombin, or whether it is comprised of different subunits which interact to form thrombin, has not been decided. Seegers (1968) presented a paper which postulated this conversion. His version of the mechanism is as follows: (1) prothrombin dissociates to form mainly prethrombin and Autoprothrombin III; (2) Autoprothrombin III converts to Autoprothrombin C; (3) Autoprothrombin C converts prethrombin to thrombin. The Autoprothrombin C and III were isolated by Seegers and Marciniak (1965). This view is not shared by those who believe prothrombin itself is converted to thrombin in the classical theories of coagulation.

Physicochemical data published in Todd and Sanford (1963) state that prothrombin is precipitated from oxalated plasma by 50% saturated $(NH_4)_2SO_4$. It is adsorbed by the usual adsorbing agents $Mg(OH)_2$, $BaSO_4$, $Al(OH)_3$, and $Ca_3(PO_4)_2$. Seitz filtration will also remove it from plasma. The substance is relatively labile between 40 C. and 60 C. and inactivated at 85 C. It is a sulfur containing glycoprotein. Prothrombin is consumed almost completely in normal clotting. It is present in quantities of about 0.2 mg./ml. and represents 0.14% of the plasma proteins. It converts gradually upon standing with sodium citrate anticoagulant to thrombin. Seegers and Alkjaersig (1953) agree with the conversion and Marciniak and Seegers (1962) state that Autoprothrombin C is stable for 30 minutes at 56 C. As stated previously, this Autoprothrombin C may or may not be a component of prothrombin.

Prothrombin is affected by the common oral anticoagulant drugs of coumarin origin. These compounds are thought to compete with vitamin K in reactions leading to prothrombin synthesis. The relationship was first described by Waddell and Guerry (1939). The first congenital deficiency was reported by Rhoads and Fitz-Hugh (1941) and deficiency in the newborn by Brinkhous *et al.* (1937). Congenital defects, however, are rare, while those associated with liver disease or inability of the liver to produce the factor are common.

Factor V

Factor V, or Labile Factor, is also the Ac-globulin referred to by Seegers. This factor is believed to be involved in acceleration of the conversion of prothrombin to thrombin. Physicochemical data summarized in Todd and Sanford (1963) show that this factor is precipitated by 33% $(NH_4)_2SO_4$ and by a pH less than 4.0 and greater than 10.0. It is not adsorbed from plasma by the usual adsorbing agents $BaSO_4$, $BaCO_3$ or $Al(OH)_3$. The factor is readily inactivated by heat and also by storage even at 0 C. It decays much faster when oxalate is used as an anticoagulant rather than citrate. An antibody to Factor V has been found by Ferguson *et al.* (1958). The antibody acted as an inhibitor to Factor V, was non-dialyzable, and remarkably stable to temperature, pH, and fat solvents.

It has been postulated that Factor V interacts in some way with metal ions to enhance the coagulability of blood. Two studies have been carried out to this effect. Leikin and Bessman (1956) studied the effect of EDTA in lengthening the prothrombin time of normal plasmas. The workers found that the prolonged prothrombin time could be partially

or completely corrected by the addition of prothrombin-free human plasma. They concluded that the EDTA complexed some metal and that Ca^{2+} was not principally involved. In this study the Mn^{2+}, Ba^{2+}, or Zn^{2+} ions had no effect on the prothrombin time. Fe^{2+}, Fe^{3+}, or Ca^{2+} lengthened the time while Ni^{2+} seemed to protect the Factor V activity.

Other alkaline earth cations (Ba^{2+}, Sr^{2+}, Mg^{2+}) can also be substituted experimentally for calcium to achieve clotting. Ferguson *et al.* (1967) found that these ions in a concentration of 16-20 mM, at a pH of 6.2-7.3, may substitute for calcium. Factor V still converted prothrombin to thrombin in their presence. Their study also showed that Factor V showed no activation prior to its participation with thrombokinase (thromboplastin) in the final prothrombin conversion. Using an enzyme free prothrombin plus Ca^{2+}, and cephalin, they could achieve no conversion with Factor V until some thromboplastic enzyme was added. They concluded that the role of the cation is to complex with thrombokinase and thus determine its true prothrombin activator function.

In a study by Hougie (1957) indications were found of a sedimentable complex formed in the reaction of AHF (Factor VIII), PTC (Factor IX), Stuart factor (Factor X), and platelets. The Factor V was found to interact with this complex. If it was left out, the complex had very little activity. They found Factor V to enter the scheme subsequent to the coagulant formed by the other factors. This complex may be the thromboplastic enzyme mentioned by Ferguson and his co-workers (1967), and would support their findings.

The question has been asked whether or not an increase in amount of one factor of the prothrombin complex would effect a decrease in another factor. Since the discovery that the prothrombin time will detect deficiencies in any one of four factors involved, this poses an interesting question. Ferguson and Patch (1956) presented work in which they artificially reconstituted plasmas with various factors of the prothrombin complex. Their studies found that moderately low ranges of prothrombin and Factor VII can be compensated by high levels of Factor V. This level of Factor V was found to be in the range of 200-300% of normal. Factor VII in increased amounts at fixed concentrations of prothrombin and V exerted relatively minor effects. Increasing levels of prothrombin exerted little effects also, and only a large excess of Factor V offset any deficiencies of the other factors. No mention was made of the fourth factor (X), however.

Factor V, unlike prothrombin, VII, and X, is not affected by the coumarin anticoagulants. Shanberge (1956) found no change in Factor V levels during the entire course of Dicumarol therapy on his patients. Additional remarks published in Todd and Sanford (1963) say that Factor V levels in plasma are not affected by dietary lack of vitamin K, coumarin type medication, or biliary obstruction.

Factor VII

It is interesting to note that although Factor VII has been found deficient in certain hereditary hemorrhagic disorders, it is not included in the scheme of Macfarlane or by Davie and Ratnoff. The "cascade" or "waterfall" schemes make no allowance for it. Seegers, however, does claim that Factor VII (Autoprothrombin I) and Factor IX (Autoprothrombin

II) are both derivatives of prothrombin that reinforce enzyme activity for the production of thrombic activity.

A patient with a severe hereditary deficiency in Factor VII has a normal prothrombin time. Conversely, a patient with a severe hereditary hypoprothrombinemia often has normal levels of Factor VII. This was noted by Quick and Hussey (1962).

In vitamin K deficiency or after a vitamin K antagonist such as Dicumarol has been administered, both Factor VII and prothrombin decrease. This, according to Quick (1966), does not mean that one is a precursor to the other, but their site of synthesis is probably the same. In fact, several authors state that Factor VII is more sensitive to Dicumarol therapy than either prothrombin or Factor X. Alexander (1959) found Factor VII to be the first to decrease under therapy and that it responds more quickly to vitamin K than does prothrombin. This agrees with Georgatsos *et al.* (1958), who found Factor VII to be depressed to a greater extent than prothrombin after Dicumarol therapy.

The importance of Factor VII is not in agreement. Ackroyd (1956) says that, although prothrombin conversion is slower in Factor VII deficiencies, there is no incontrovertible evidence at present to show that it is needed in the coagulation of blood. This disagrees with Alexander (1959), who found only a slow or insignificant thrombin formation during *in vitro* studies of the factor, and agrees with Koller *et al.* (1951), who say that Factor VII increases the rate but not the amount of thrombin formed. These findings fail to explain the hemorrhagic symptoms found in congenitally deficient patients or why, in the presence of tissue thromboplastin, Johnston *et al.* (1959) and Johnston

and Hjort (1961) found increases in the level of 2-1/2 to 3 times that found before clotting. Factor VII must therefore have a physiological function however obscure it may be.

Todd and Sanford (1963) summarize physicochemical data showing Factor VII to be relatively stable. It is resistant to heat at 37 C. in serum for at least four hours and to 45 C. for at least six minutes. These findings agree in part with the results of this study (see discussion). Factor VII is also readily adsorbed by the usual adsorbing agents. It can be precipitated at a pH less than 5.0 and greater than 9.0. Hogenaur and Deutsch (1968) found that Factor VII probably consists of two polypeptide chains much like Factor X. Their adsorption techniques and fractionations on DEAE cellulose gave RF values of 0.54 and 0.70 for Factors VII and X, respectively. These values were obtained with polyacrylamide electrophoresis. The N and C terminal ends of both polypeptide chains were composed of glycine and serine. Fingerprints of the amino acid composition of the two factors gave similar results. These findings suggest that the two factors have similar chemical compositions and may account for the difficulty in separation of one from the other.

Factor X

The physicochemical properties of Factor X are very similar to those of Factor VII. According to data published in Todd and Sanford (1963), both are present in serum and in plasma and are adsorbed by the same agents. The stability of Factor X to heat is somewhat disputed. Deficiency of Factor X has been described in both hereditary and acquired conditions. Some investigators feel that it is involved in most

hemorrhagic complications of oral anticoagulant therapy. Factor X is believed to be a converter of prothrombin to thrombin. Studies by Lanchantin *et al.* (1969) show that inactive Factor X is converted to the active form. At a pH of 7.4, 0.01 M Ca^{2+}, and a temperature of 23 C., the activated Factor X converts prothrombin to thrombin, the reaction following first-order kinetics.

Hougie *et al.* (1956) and Telfer *et al.* (1956) found that Factor X is necessary in both the intrinsic and extrinsic systems, and is concerned with the formation of a prothrombin activator. Other work has been published in which Factor X has been consumed in blood activated by tissue thromboplastin, but not by blood activated merely by surface contact. Niemetz (1967) found that blood allowed to clot in siliconized glassware showed no activation of Factor X. This verifies the claim by the previous workers in that this factor is involved in the extrinsic system. Niemetz found it to be involved in the intrinsic system but only if a phospholipid or AHG (Factor VIII) was added. He stated that Factor X is consumed during *in vitro* coagulation only when surface activation, and either bovine AHG or phospholipid, were added. Neither surface activation, AHG, or phospholipid alone will result in a significant consumption of Factor X.

Factor X deficiencies can be separated from those of Factor VII by the action of Russell's Viper Venom. The observation that the venom restores blood clotting in Factor VII deficiencies, but not in Factor X deficiencies, has been observed by various workers. This thromboplastic activity in the presence of added cephalin makes a useful diagnostic test to differentiate the two clotting abnormalities.

MATERIALS AND METHODS

Preliminary Procedures

Studies were made on normal human subjects. Blood was drawn in Vacutainer* tubes containing sodium oxalate 0.1 M as an anticoagulant, and sorbic acid 0.2 mg./ml. as an antimycotic agent. The specimens were centrifuged immediately for 10 minutes at 2000 rpm. The plasma was separated from the cells and placed in clean test tubes.

Plasma specimens requiring incubation at 37 C. for more than 3 hours (aged plasma) were collected in an aseptic manner according to regulations described by the American Association of Blood Banks (AABB). The blood was drawn into autoclaved Vacutainer tubes. The plasma was then placed into sterile screw-topped tubes utilizing previously autoclaved disposable pipettes. The plasma specimens were then incubated at 37 C. until a one-stage prothrombin time greater than 32 seconds was obtained. This prothrombin time was checked at various intervals using aseptic technique to withdraw the plasma portions from the stock mixture. Total incubation times varied from 48 to 56 hours. Upon completion of the incubation, the plasma mixture was recentrifuged for 10 minutes at 2000 rpm. Gram stains and cultures on blood agar plates were made of the sediment to check for bacterial contamination.

Other plasmas and serums were specially prepared to be rich in clotting Factors V, VII, and X according to the methods of Eichelberger

*Becton, Dickinson, and Co., Columbus, Nebraska and Rutherford, N.J.

(1965) with minor modification. These methods are described in detail in Appendix I.

Prothrombin times were measured by the one-stage method of Quick (1957) as adapted for use on the Clot-timer.* All determinations were performed in duplicate or until the results agreed within ± 0.3 seconds. Exceptions were allowed when the time exceeded 35 seconds where deviations of ± 0.5 second were accepted. Simplastin** was used as the thromboplastin-calcium reagent and was diluted according to the manufacturer's instructions.

With each new batch of reagents a commercial control was included in the day's run to check the reliability of the reagents. Constant temperature heating blocks and water baths were used for incubation and their temperatures controlled to ± C.5 C. All plasma specimens were stored at 4 C. until ready for testing.

Experiments Concerning the Heat Stability of the Prothrombin Complex

Experiment I. This experiment was designed to find a temperature high enough to destroy rapidly any labile factors of the prothrombin complex, but low enough to prevent denaturation of any essential proteins. Seven normal plasmas were incubated for 30 minutes at 56 C. Prothrombin times were determined before and after incubation. The results are shown in Table 1.

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*Heller Laboratories, San Mateo, Calif.

**Warner-Chilcott Laboratories, Morris Plains, N.J.

Experiment II. To determine if any factors were labile to heat, 3 normal plasma specimens were used. They were incubated for 90 minutes at 45 C. At various time intervals from 0 to 90 minutes portions were withdrawn and their prothrombin times measured. The curve illustrating the effects of destruction are shown graphically in Figure 1.

Experiment III. To determine the effect of heat on Factors VII and X, 2 pooled plasma samples were utilized. They were heated at 45 C. for 30 minutes, during which prothrombin times were measured at various intervals. At each time interval, 2 aliquots of 0.4 ml. of plasma were removed. They were designated as Control and Test samples. On the first run 0.1 ml. aged serum (Appendix I) was added to the Test samples and nothing was added to the Control samples. On the second run, 0.1 ml. of physiologic saline was added to the Control samples and the aged serum added to the Test samples. This was done to equalize the dilution of the factors already present in the plasma. The results are presented in Table 2.

Experiment IV. To determine further the effects of heat on Factors VII and X, the following experiment was completed. Plasma samples from 4 normal individuals were pooled. They were collected aseptically and incubated at 37 C. until the prothrombin time was increased to 32 seconds (see preliminary procedures). After this incubation the pooled plasma was incubated at 45 C. for 60 minutes. At various time intervals, 0.4 ml. portions were removed and placed in each of 2 test tubes. To the Control tube was added 0.1 ml. saline and to the Test sample was added 0.1 ml. $BaSO_4$ adsorbed plasma. The results are summarized in Table 3.

Experiment V. This experiment was designed to determine whether or not prothrombin was labile to heating to 45 C. The same aged plasma as in Experiment IV was used. To 0.7 ml. of the aged plasma, previously incubated for 60 minutes at 45 C., was added 0.1 ml. aged serum and 0.2 ml. $BaSO_4$ adsorbed plasma. These reagents were added at various time intervals to portions removed from the stock supply. Their prothrombin times were determined and the results are given in Table 4.

Experiment Concerning Development of the Factor V Assay

Experiment VI. Results of the previous experiments indicated that Factor V was the only significantly labile factor in the prothrombin complex. This experiment was designed to devise a test whereby the concentration of Factor V could be estimated using thermal incubation. Dilutions of $BaSO_4$ adsorbed plasma were prepared ranging from an undiluted plasma (100%) to 6.25%. When added in the amount of 0.1 ml. to 0.4 ml. of plasma in the testing procedure, they represented Factor V concentrations of 20, 10, 5, 2.5, and 1.25%. The results are shown graphically in Figures 2 and 3 and in Tables 5 and 6.

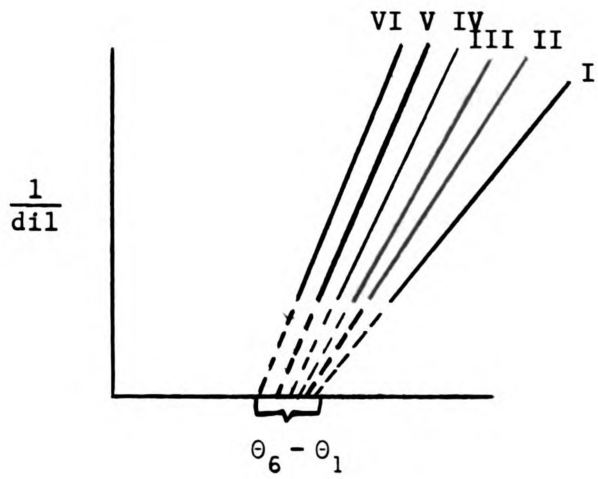
RESULTS

Figure 1 represents graphically the destruction of the coagulation factors over a period of time at 45 C. At this point it was not known which factors were destroyed. It was assumed that only Factor V was labile. The correction studies (Tables 2 through 4) showed this assumption to be true.

Experiment VI represents the major emphasis of this study. The explanation of Table 5 and Figures 2 and 3 may be found in the section devoted to the derivation of the formulas used in this work. Table 6 summarizes the statistical evaluation of the estimation of the concentration of Factor V as described by the formula: $[V] = 67.0 - 3.5\ \Theta$. The derivation of this formula and a proposed method of obtaining the relative concentration of the thermostable components are as follows.

1. A series of dilution curves were made of the incubated plasma (45 C.). To these were added varying concentrations of Factor V in the form of $BaSO_4$ adsorbed plasma. Table 5 lists the relative concentrations of the thermolabile and thermostable components. Figure 2 represents the true value of these lines as calculated by the method of least squares. Initial investigations relating concentration of Factor V to the change in prothrombin time after heating did not yield linear results.

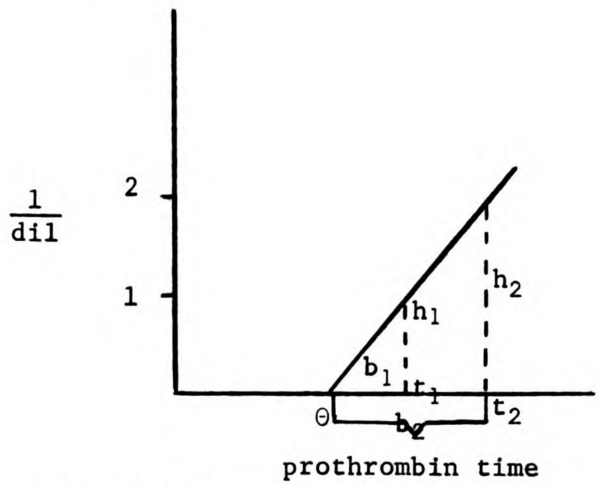
2. The data were treated in the following manner:



Lines I-VI represent additions of $BaSO_4$ adsorbed plasma to obtain concentrations of 0-20% (see Table 5).

3. The lines were extrapolated to yield Θ values. These are the most stable points on the line and eliminate effects due to hemodilution or changes in concentration of the thermostable component.

4. By taking only one line of the series we can see the following relationship:



(a) $\frac{h_1}{h_2} = \frac{b_1}{b_2} = \frac{t_1 - \Theta}{t_2 - \Theta} = \frac{1}{2}$

(b) $2(t_1 - \Theta) = 1(t_2 - \Theta)$

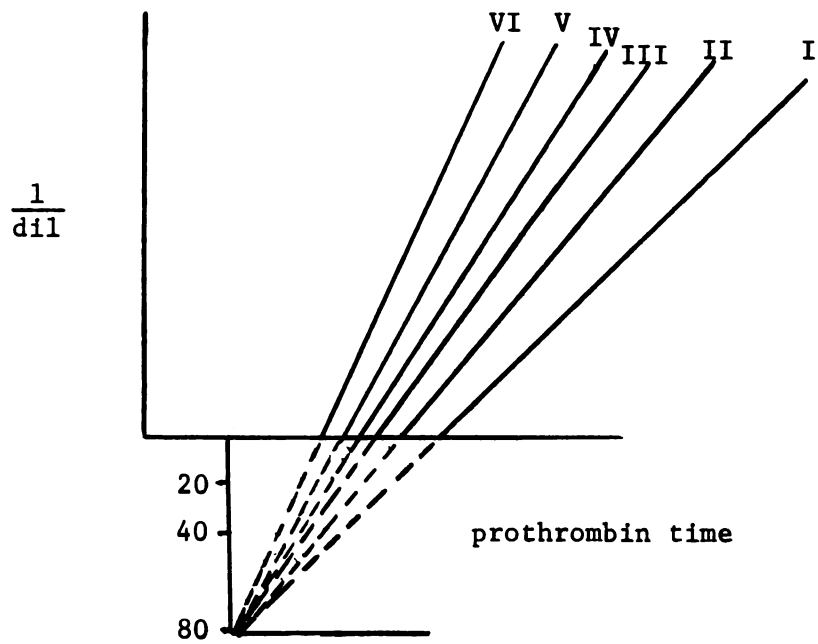
(c) $2t_1 - 2\Theta = t_2 - \Theta$

(d) $2t_1 - t_2 = \Theta$

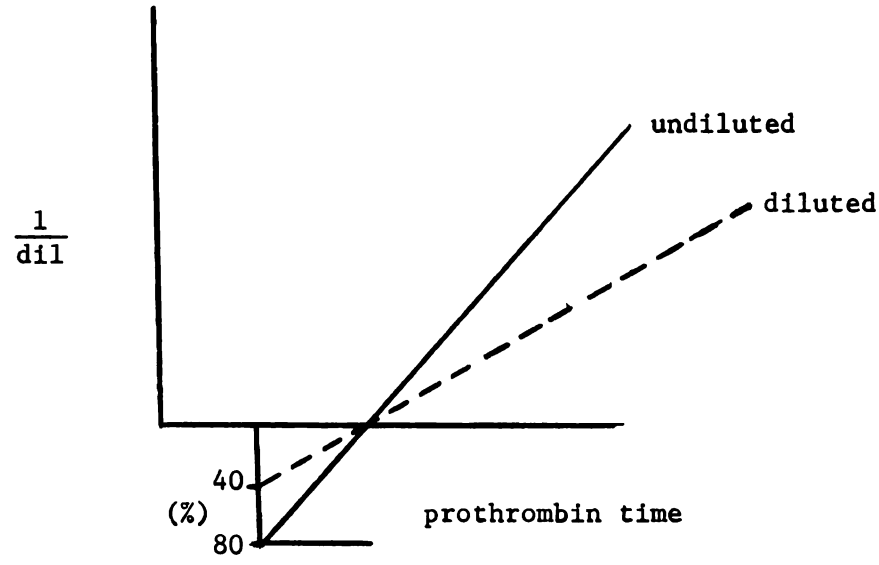
5. The values for Θ were plotted against Factor V concentration (see Figure 3). This results in completion of the formula having the form of $y = a - bx$.

The following is a proposed method of determining the thermostable component of the prothrombin complex. We can see from Figure 2 that a theoretical point of intersection can be calculated for all the dilution curves of Experiment 6 in which varying amounts of Factor V has been added. The proposed method is as follows:

1. A new set of axes is constructed, giving the intersection of the lines a value of 80% (see the concentration values in Table 5).



2. The lines above represent at least 80% activity of the thermostable component. Taking one line and diluting the stable component in half we have the following case:



This method can then be applied to all cases once the concentration of Factor V is known.

Table 1. Comparison of prothrombin times of normal human plasma before and after incubation for 30 minutes at 56 C.

Patient	Prothrombin times (sec.)	
	Before incubation	After incubation
1	14.0	> 70*
2	16.0	> 70*
3	15.6	> 70*
4	14.5	> 70*
5	13.6	> 70*
6	14.0	> 70*
7	14.6	> 70*
Control	13.0**	

*Dense flocculent precipitate

**Normal value for control = 11-15 seconds

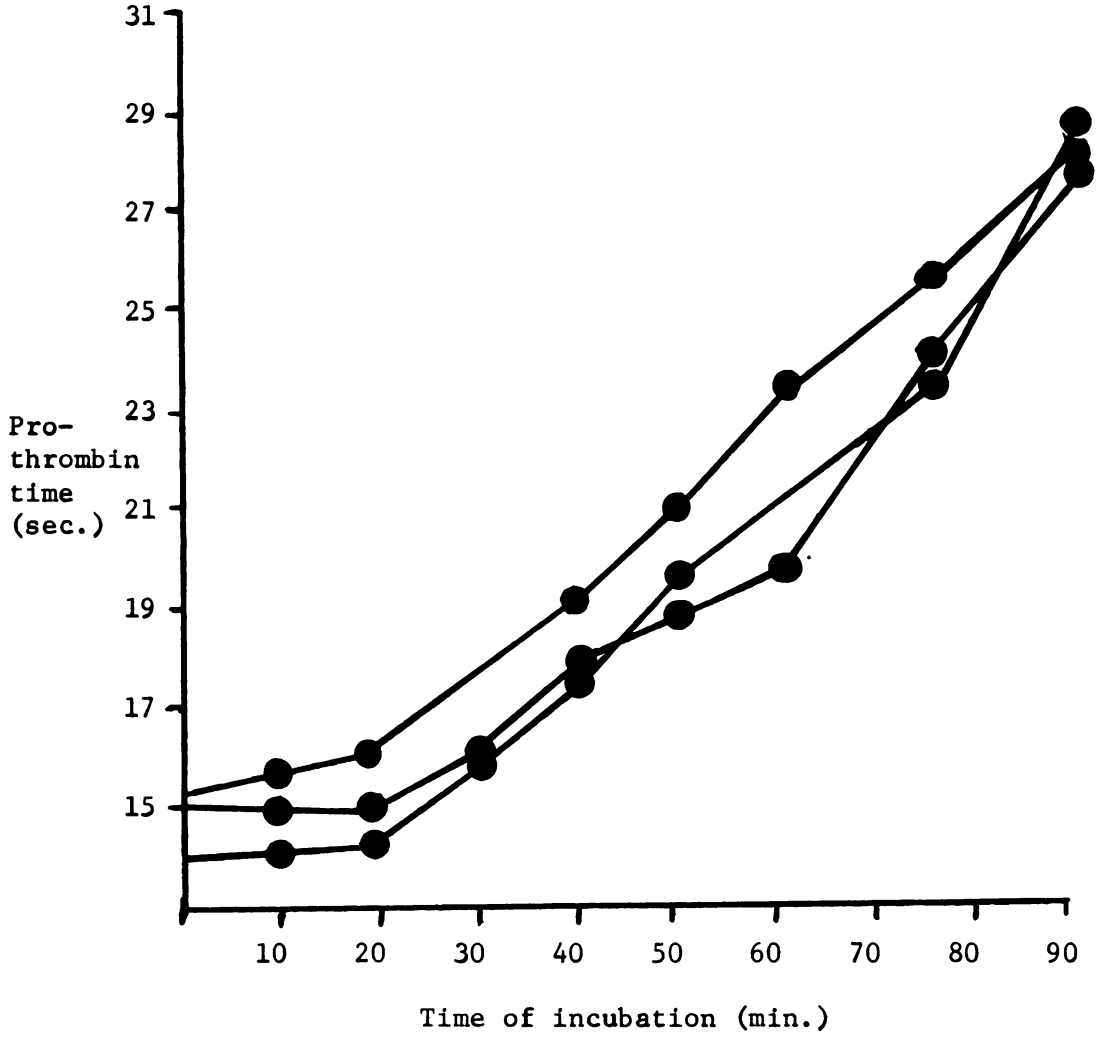


Figure 1. Destruction of the labile factors in the plasma of 3 normal subjects.

Table 2. Effect of incubation on Factors VII and X

	Time (min.) of incubation	Prothrombin times (sec.) Control	Test
(a)	0	15.0	13.5
	10	16.5	17.0
	20	17.5	18.0
	30	18.0	20.0
	Control = 12.5*		
(b)	0	16.6	13.5
	10	17.0	16.6
	20	19.8	19.0
	30	23.5	22.3

*Normal range for control = 11-15 seconds

Table 3. Effect of incubation on Factors VII and X

Time (min.) of incubation	Prothrombin times (sec.)	
	Control	Test
0	32.3	15.5
10	32.6	16.0
20	33.6	16.0
30	36.3	16.5
60	39.3	17.0
Control = 11.5*		

*Normal value for control = 11-15 seconds

Table 4. Effect of incubation on prothrombin

Time (min.) of incubation	Prothrombin time (sec.)
0	15.8
10	16.5
20	16.5
30	15.6
60	16.6
Control = 11.5*	

*Normal value for control = 11-15 seconds

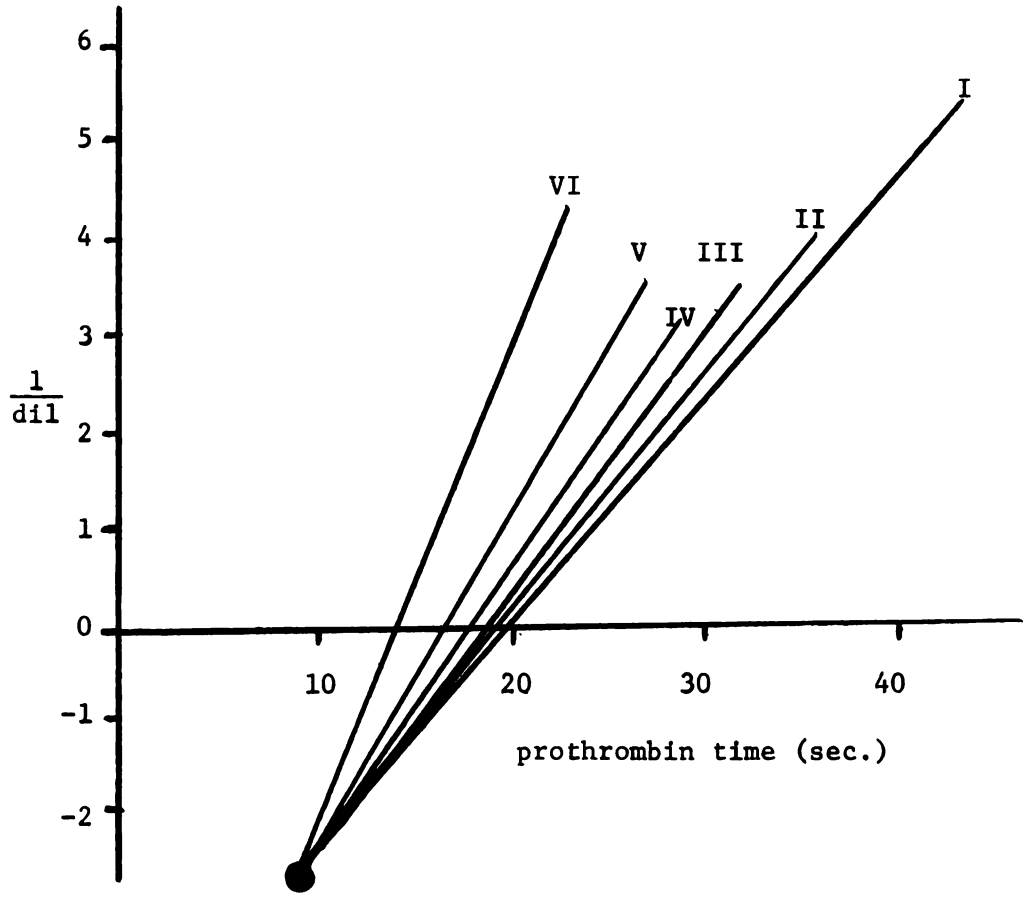


Figure 2. Demonstration of intersecting point of dilution curves. Lines were calculated by the method of least squares. Lines I-VI denote Factor V concentrations of 0, 1.25, 2.5, 5.0, 10.0 and 20.0%, respectively.

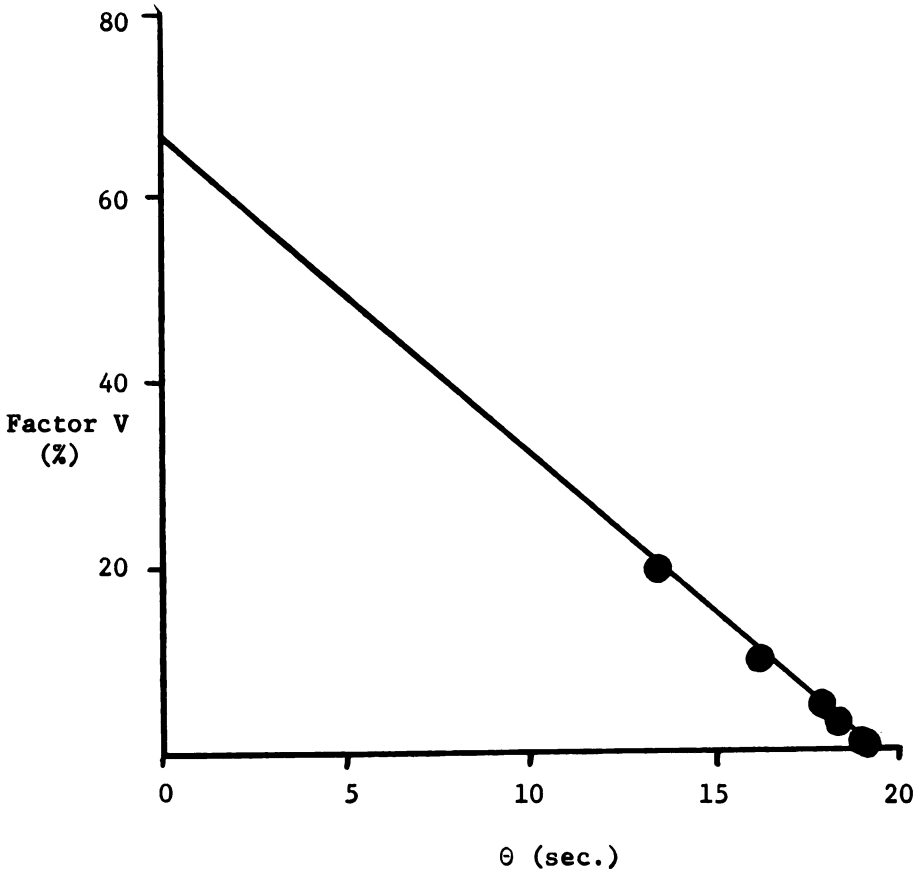


Figure 3. The linear relationship of Factor V concentration to Θ. Factor V conc. = 67.0-3.5 Θ.

Table 5. Concentration of Factor V restored to normal plasmas. Percentages represent final concentrations

Mixtures		Incub. plasma %	Prothrombin time (sec.)
Normal plasma %	$BaSO_4$ plasma %		
100	---	---	13.4
---	---	100	25.8
---	20	80	15.7
---	20	40	19.0
---	20	20	23.2
---	10	80	19.0
---	10	40	21.3
---	10	20	27.0
---	5	80	20.4
---	5	40	24.4
---	5	20	29.3
---	2.5	80	22.5
---	2.5	40	24.9
---	2.5	20	34.3
---	1.25	80	23.0
---	1.25	40	28.0
---	1.25	20	36.0
---	0.0	80	25.5
---	0.0	40	31.0
---	0.0	20	43.3

Table 6. Statistical evaluation of estimating the Factor V concentration in an unknown plasma

Θ	Conc. of V	Calculated conc.*
19.3	0.00	-0.55
19.0	1.25	0.50
18.1	2.50	3.65
17.8	5.00	4.70
16.2	10.00	10.30
13.5	20.00	19.75

S.D. = 0.70

*Calculated from: $[V] = 67 - 3.5\ \Theta$

DISCUSSION

The various data published on heat denaturation of proteins agree with the results of Experiment I. Each particular protein has its own denaturation temperature. In addition, human serum inactivated at this time and temperature for various serologic tests (VDRL, and heterophile) shows no precipitate. We must conclude that the precipitate found after incubation was indeed fibrinogen.

Experiment II agrees with other published work in that at least one factor of the prothrombin complex is labile to heat. We noted an increase in prothrombin times in the normal plasmas after incubation. The decay curve of Figure 1 reveals that at least one factor is being inactivated.

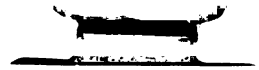
Table 2 reveals that factors other than V are stable during incubation at 45 C. The 2 runs differed only by the addition of 0.1 ml. saline to the control tubes in Part b, while none was added in Part a. In neither case did the addition of aged serum, rich in Factors VII and X, bring a large correction in the prothrombin time when compared to the controls. It appears from Experiment III that Factors VII and X are stable for at least 30 minutes at this temperature. Part of the experiment agrees with Hougie *et al.* (1957), who found Factor X to be stable up to 56 C. and at a pH range of 6-9 in a serum medium.

Experiment IV was also designed to test the stability of Factors VII and X. The addition of $BaSO_4$ adsorbed plasma corrected the prothrombin time to normal or near normal levels in the test samples. The purpose of aging the oxalated plasma was to destroy the labile Factor V.

Eichelberger (1965) presented the method used in this study, but due to bacterial contamination certain modifications had to be used. With these modifications it was found that Factor V was destroyed much more slowly than stated by Eichelberger. The experiment showed that if plasma is kept in a sterile, tightly stoppered container, Factor V is destroyed much more slowly than previously thought. The slight increase in the prothrombin times noted in Table 3 could be due to some destruction of the other factors because of the lengthy heating time involved, but the change is small when compared to differences in the Test and Control samples obtained by correcting the aged heated plasma with $BaSO_4$ adsorbed plasma.

Experiment V concerned the stability of prothrombin at 45 C. The results shown in Table 4, after reconstituting plasmas in all factors except prothrombin, show very small changes in prothrombin levels over the 60 minute incubation period. In addition, the oxalated aged plasma kept prothrombin at a stable level for a period of 24 to 56 hours at 37 C.

Experiment VI presents a method by which Factor V concentration can be estimated. As demonstrated, incubation at 45 C. is not necessary in the new method, but to develop this method the incubation and dilutions of the plasmas described in materials and methods were necessary. In addition, this method presents a way of estimating concentrations of Factors VII, X, and prothrombin at the same time. Various methods have been presented for estimating Factor V concentration. Eichelberger (1965) used the aged oxalated plasma described earlier as a substrate for the reaction and found the concentration from a Factor V dilution



curve. This is time consuming when one must incubate plasma at 37 C. for several days and is subject to bacterial and other types of contamination. Some methods estimate the severity of a defect in Factor V by the difference in the uncorrected and corrected prothrombin times after the addition of $BaSO_4$ adsorbed plasma. The method presented after the work in Experiment VI combines some features of both methods, resulting in a more useful and accurate determination.

By further consideration of the data of Experiment VI, several conclusions can be made. It has been shown that there is a linear relationship between the reciprocal of "prothrombin" concentration and clotting time after optimal amounts of Ca^{2+} and tissue thromboplastin have been added. The first analysis of the data was designed to test the hypothesis that the abscissal intercepts were a function of the labile factor concentration.

The slopes of the lines relating the reciprocal of concentration to "prothrombin time" are assumed to be mathematically related to the relative concentrations of labile and stable factors. If these hypotheses are correct, all the lines should meet at a single point when extrapolated.

The procedure for estimating the relative concentrations of the thermolabile and thermostable factors (i.e., Factor V and Factors VII, X and prothrombin) may be summarized as follows. Essentially the procedure consists of measuring two prothrombin times; one in the usual way and one on plasma diluted with an equal volume of saline. The time required for each of these specimens to coagulate after adding calcium and thromboplastin is recorded as t_1 and t_2, respectively.

First, it is necessary to complete the calculation of the concentration of Factor V as follows:

$$v = 67 - 3.5(2t_1 - t_2),$$

where v represents the concentration of Factor V expressed as a percent of normal levels.

The value of $2t_1 - t_2$ has been shown to be identical to the value Θ (see results).

It is emphasized that a theoretical method of obtaining the thermostable component is presented. Further work is necessary to obtain additional data and more accurate dilutions of this component are needed. It is believed, however, that this proposed mechanism will hold true in all cases.

SUMMARY AND CONCLUSIONS

The work presented here brings out several basic facts. The factor called prothrombin is relatively stable to incubation at 45 C., as are Factors VII and X. Factor V is indeed thermolabile as most workers believe, although it is reasonable to assume that under proper conditions its decomposition may be retarded. This will only hold true at 37 C. At 45 C. it is rapidly destroyed.

The last finding of this study is the principle by which the concentration of Factor V in an unknown plasma can be estimated. A theoretical method is also presented whereby the relative concentration of the thermostable factors can be found.

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APPENDIX I

Preparation of reagents rich in the clotting factors of the prothrombin complex

$BaSO_4$ Adsorbed Plasma

1. Collect blood in oxalate or citrate anticoagulant.
2. Immediately centrifuge for 10 minutes at 2000 rpm.
3. To each ml. of plasma add approximately 100 mg. of chemically pure $BaSO_4$.
4. Place in 37 C. water bath for 10 minutes. Invert tube several times during this incubation.
5. Centrifuge for 10 minutes at 2000 rpm.
6. Remove upper 2/3 of plasma. It should have a one-stage prothrombin time of over 1 minute.
7. Readsorption may be carried out until prothrombin time of over 1 minute is obtained.

Aged Plasma

1. Strict aseptic technique must be used throughout this procedure.
2. Separate the plasma from the cells by centrifugation at 2500 rpm for 10 minutes.
3. Using a sterile disposable pipette, transfer the plasma to a sterile screw-topped test tube.
4. Incubate at 37 C. for 24-56 hours or until the one-stage prothrombin time exceeds 32 seconds. If citrate anticoagulant is used, the incubation time may be longer.
5. Check prothrombin times at various intervals using aseptic technique when removing plasma samples from the stock mixture.
6. At the end of the 37 C. incubation, recentrifuge for 10 minutes at 2000 rpm. Remove plasma from any sediment formed.

7. Make a smear for Gram's stain of the sediment and inoculate a portion of it on a blood agar plate to check for bacterial contamination.

Aged Serum

1. Obtain serum samples after clotting of normal human blood.
2. Pool 3 or 4 of these samples.
3. Allow to stand at 4 C. for at least 48 hours before using.

VITA

The author was born in Petoskey, Michigan, on June 21, 1942. He graduated from Pellston Public High School in June, 1960, and enrolled at North Central Michigan College in September of the same year. Upon completion of 2 years of undergraduate work, he transferred to Central Michigan University and received a B.S. in biology and chemistry in June, 1964. He completed one year of internship at St. Mary's Hospital School of Medical Technology in June, 1965. In July 1965 he passed the registry for medical technologists given by the American Society of Clinical Pathologists.

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The author was married to Ruth Ann Nyp of Grand Rapids, Michigan, on October 8, 1966, and has a son 8 months old. He is a member of the Lansing, Michigan, and American Societies of Medical Technologists and is active in society functions at the state and local levels.

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