DEMONSTRATION OF CHANGES IN THERMOLABILE COAGULATION FACTORS DURING COUMARIN THERAPY

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ABSTRACT

DEMONSTRATION OF CHANGES IN THERMOLABILE COAGULATION FACTORS DURING COUMARIN THERAPY

by Leona Gielda

A method is proposed for the detection of the effect of coumarin therapy on the labile and stable factors of the "prothrombin complex". Clinical application of this method indicates that incubation at 45 C. can be used to measure the concentration of labile factor and that labile factor is decreased by coumarin drugs.

DEMONSTRATION OF CHANGES IN THERMOLABILE COAGULATION FACTORS DURING COUMARIN THERAPY

By

Leona Gielda

A THESIS

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To My Parents and Family

for their constant encouragement

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INTRODUCTION

The clinical laboratory is concerned not only with the diagnosis of disease but also with monitoring therapy. Coumarin drugs have been used since 1941 in the treatment of myocardial infarction, thrombophlebitis and pulmonary embolism. When dicoumarol is used, a readily identifiable change in the patient's coagulation mechanism occurs. This change in the coagulation mechanism is demonstrated by prolongation of the clotting time. A quantitative measure of this change is obtained with the Quick one-stage prothrombin test.

The prothrombin test was introduced in the early 1930's and was based on the "classical" coagulation theory of Morawitz. This theory postulated that prothrombin is converted to thrombin under the influence of "thromboplastin" and free calcium ions. The thrombin formed from this reaction then interacts with fibrinogen to produce fibrin. On this basis, Quick's test assumed that prothrombin was the only variable when an excess of tissue thromboplastin extract and optimum calcium ions were added to plasma.

The prothrombin time is used in the clinical laboratory to study hemorrhagic disorders in Stage II of the clotting mechanism, and as mentioned above, in monitoring coumarin therapy. It is now known that an altered one-stage prothrombin time can signify any of the following deficiencies: Factor II (prothrombin); Factor VII (PTC); Factor V (labile factor, Ac-globulin); Factor X (Stuart-Prower); Factor I (fibrinogen); or an increased fibrinolytic activity or the presence of circulating

anticoagulants. It is also known that coumarin drugs affect not only the production of Factor II, but also Factors VII, IX, and X.

Coumarin drugs have been in use for over 25 years, yet there is still debate as to the effectiveness of this therapy in thromboembolic states. If one searches the literature, numerous reports can be found which seem to demonstrate the effectiveness of coumarin therapy. However, there are about as many reports by equally competent observers that seem to show the ineffectiveness of coumarin drugs. It is possible that this disagreement exists because of variations in therapy levels. Therapy levels may vary because these observers rely almost exclusively on the prothrombin test to determine the extent of patient therapy. There are documented cases involving serious hemorrhage in patients whose prothrombin tests were within the normal therapeutic range. There is also the incongruity of the patient with a greatly prolonged prothrombin time resulting from coumarin drugs who shows no hemorrhagic tendency.

Because of the multiplicity of factors affected by coumarin, we hoped to modify the Quick prothrombin test so that coumarin therapy could be monitored more effectively. Most writers consider the factors affected by coumarin drugs to be the thermostable members of the prothrombin complex. Because there are reports of high levels of Factor V masking a decrease in Factors II and VII, we divided the factors of the prothrombin complex into 2 groups: thermostable and thermolabile.

First we reinvestigated the thermostability of the factors affecting the one-stage prothrombin test. The one-stage prothrombin test was adapted to an automated procedure. Standardized commercial reagents were used whenever possible, to allow for greater reproducibility of results. Knowledge of the extent of thermal decay should give an estimation of the concentration of labile factors present. Our second purpose was to

devise a simple procedure for detecting and evaluating the effect of coumarin drugs on labile and stable factors, using thermal inactivation.

REVIEW OF LITERATURE

Basic to the consideration of the conversion of fibrinogen to fibrin is the question of how the 12 known factors involved in coagulation interact in such a way as to provide a predictable result. There appear to be roughly 2 schools of opinion: one, which is by far the larger group, holds that all these factors are involved in an ordered sequence of reactions. The "cascade" theory of Biggs and Macfarlane, and the "waterfall" theory of Davie and Ratnoff are examples of this opinion. These groups postulate that clotting is initiated by the activation of Factor XII. Active Factor XII then acts as an enzyme activating Factor XI. Active Factor XI in turn activates other factors in a sequence. According to this school, both enzyme and substrate through these reactions are proteins. The second school, represented by Seegers, holds that all factors other than prothrombin, Platelet Co-Factor I (Factor VIII) and Ac-globulin (Factor V), are nonspecific, pro- or anti-coagulants.

Prothrombin

Various techniques have been used to isolate and purify prothrombin. It was already known at the beginning of the century that prothrombin would be precipitated by bubbling CO_2 through diluted plasma. Laki *et al.* (1954) isolated bovine prothrombin by adsorption with $Mg(OH)_2$. By using ion-exchange chromatography this group identified 18 amino acids and hexosamine. Their isolate also contained 14.7% nitrogen, 5% carbohydrate, and a reducing sugar content of 6.5%, in terms of glucose. These

workers found no resemblance between the known amino acid composition of serum albumin and prothrombin, even though these 2 proteins are closely related with regard to their physicochemical characteristics in many respects. Ultracentrifugal and diffusion studies showed that their prothrombin preparations were homogeneous, yet on electrophoretic analysis, there were 2 peaks. They believe that this second peak is derived from prothrombin itself. They support this explanation by the fact that during electrophoresis, the second peak appears at the same time that prothrombin loses its property of being able to transform to thrombin.

Tishkoff *et al.* (1960) purified bovine prothrombin by adsorption on $BaSO_4$, elution with sodium citrate, and fractionation with ammonium sulfate. The total carbohydrate for their samples ranged from 10.4 to 14.7%, neuraminic acid ranged from 3.2 to 7.2%, hexosamine content ranged from 1.5 to 4.1%. Their product was contaminated with Factors VII and X. Their product could be separated electrophoretically from Factors VII and X and produced a single peak.

Aronson (1966), working with human prothrombin, $BaCl_2$ precipitation, and DEAE-cellulose chromatography, found the nitrogen content of the human prothrombin to be 14.5%. The sialic acid content of his product was 2.6%. The elution profile of his purified prothrombin showed 2 major peaks, both of which contained prothrombin activity. When Aronson modified his procedure, 3 chromatographic peaks were obtained from his purified prothrombin. He feels that the peak I prothrombin is the *in vivo* prothrombin and that the other prothrombins represent derivatives of prothrombin. The peak III prothrombin was found to mimic active thrombin. Aronson's laboratory also found that thrombin has 2 N-terminal amino acids and he concludes that the final step in the conversion of prothrombin to thrombin may be the cleavage of a peptide bond resulting in the formation

of a "double chain" protein without any peptide (or amino acid) loss.

Harmison and Mammen (1967), part of the Seegers group working with bovine prothrombin, found that their purified product had a molecular weight of between 68,000 and 68,500. In their titration of prothrombin with and without urea, they found that 4 disulfide groups were available for titration, and that 4 other disulfide groups were well protected in the interior of the molecule. The bovine prothrombin was analyzed for carbohydrate content and 3 hexoses were found: galactose, mannose and fucose, in the amounts of 3.06, 1.53 and 0.09%. The total carbohydrate in their product was 11.18%.

Lanchantin *et al.* (1968) isolated human prothrombin both by DEAE-cellulose column chromatography and by polyacrylamide disc electrophoresis. These workers obtained an average value of 14.9% nitrogen, which compares with the 14.5% obtained by Aronson. This group found that 90% of the weight of the human prothrombin molecule is composed of amino acid residues, and that the remainder consisted of carbohydrate; 4.1% hexose, 3.4% neuraminic acids and 2.4% glucosamine. A molecular weight of 68,700 $\pm$ 200 was calculated. This group found that human and bovine prothrombin had similar patterns for amino acid composition and carbohydrate content, the exception being tryptophan.

Casillas *et al.* (1969) have adapted DEAE-cellulose for purification of trace proteins and report the isolation of Factors I, II, V, VII, IX, and X. From their observations, their system does not perceptibly modify the physical and chemical properties of plasma or its derivatives. Their method gives an unusually good recovery of activity of these factors and appears to eliminate activation and contamination of the final products. Because a portion of their purification is carried out under sterile conditions, they are presently testing the clinical application of these products.

Physicochemical data published in Todd and Sanford (1969) state that prothrombin is a glycoprotein present in normal plasma in the amounts of 10 to 15 mg./100 ml. It is readily removed from plasma by insoluble alkaline earths such as: $Mg(OH)_2$, $BaSO_4$, $Al(OH)_3$, and $Ca_3(PO_4)_2$. The activity can be recovered from the precipitated material by elution with sodium citrate. Prothrombin has an electrophoretic mobility between albumin and alpha globulins. It represents 0.14% of the plasma proteins and is almost completely consumed in normal clotting. By the use of fluorescent antibody technique, prothrombin was found to be associated with the microsomal and soluble fractions of the liver parenchymal cells.

The divergent schools of thought on coagulation do agree that prothrombin does change to thrombin. The disagreement centers around whether or not prothrombin itself converts autocatalytically to thrombin, or whether other factors, namely: activated X, V, phospholipid and Ca^{++} convert prothrombin to thrombin. Seegers' (1967) version of the prothrombin conversion mechanism is as follows: (1) prothrombin dissociates to form mainly prethrombin and Autoprothrombin III; (2) Autoprothrombin III converts to Autoprothrombin C + peptide (s); (3) Autoprothrombin C converts prethrombin to thrombin + peptide (s). This view is not shared by those who believe in the "waterfall" or "cascade" theory of coagulation.

Prothrombin is affected by the common oral anticoagulant drugs of coumarin origin. Vitamin K is required, possibly as a coenzyme for the synthesis of prothrombin by the liver. It is now believed that coumarin drugs act as competitive inhibitors which prevent the utilization of vitamin K by one of the enzymes involved in the synthesis of prothrombin.

Factor V

Factor V was discovered independently in 4 laboratories during the communications breakdown of World War II. These discoveries were made in 3 different contexts. In 1943, Owren treated a patient with a congenital defect, that he called parahemophilia. This patient had a prolonged prothrombin time that could be corrected by the addition of prothrombin-free plasma. Quick (1947) noted that the prothrombin time of stored, oxalated plasma increased on standing and postulated that prothrombin was composed of a labile and stable component. This labile component, he called component A of prothrombin.

Ware, Guest and Seegers (1948) noted that prothrombin lost its ability to convert to thrombin as it was purified. They discovered that prothrombin-free plasma contained a substance which promoted the conversion of purified prothrombin to thrombin. They noted that a quantitative relationship existed between prothrombin activation rate and "accelerator-globulin" activity. They found that Ac-globulin comprises 0.7% of the total plasma proteins in bovine plasma. This group found that 98% of the bovine Factor V was destroyed by incubation at 45 C. for 30 minutes.

The Australians, Fantl and Nance (1946) also discovered the same accelerator by studying the activation of purified prothrombin.

Most workers agree that Factor V is involved in accelerating the conversion of prothrombin to thrombin. Quick (1957) noted that Factor V is not adsorbed with $Ca_3(PO_4)_2$; is precipitated by 38% saturated $(NH_4)_2SO_4$; is unchanged in vitamin K deficiency, but diminished in liver damage, and is unchanged with coumarin therapy.

Aoki *et al.* (1963) partially purified bovine Factor V and obtained a 15% yield. They found that in the absence of salts, Factor V is insoluble at pH 5.0. They found that their product was stable for 1 month at -60 C. in a 50% glycerol solution that included 0.1 M $CaCl_2$. Their product deteriorated while they attempted to obtain physical and chemical data. The molecular weight was calculated from the amino acid analysis, and was estimated at 98,000. They found the concentration of Factor V in bovine plasma to be 9 mg./100 ml. The concentration of Factor V in human plasma was 1 mg./100 ml. They found 1.4 g. of hexosamine/100 g. of protein. Their amino acid analysis followed the pattern of prothrombin to a certain extent. Eighteen amino acids were observed and the Factor V had a higher methionine and phenylalanine, but a lower arginine and tryptophan than prothrombin. They were able to produce antibodies to Factor V by injecting rabbits with their product.

Esnouf and Jobbin (1967) prepared purified Factor V from bovine plasma and estimated a molecular weight of 290,000. Their product was considered a homogeneous protein, as judged by the criteria of a single component on sedimentation, and a single band after immunoelectrophoresis. Their work also implies that the structural integrity depends on the presence of a bivalent metal ion.

In an earlier work, Lewis (1964) estimated the molecular weights of 10 coagulation factors by gel filtration and determined the molecular weight of Factor V to be in excess of 200,000.

Physicochemical properties summarized in Todd and Sanford (1969) show that this factor is present in human plasma, but deficient in human serum. This is a trace protein, and the activity can be salted out with the globulin fraction by 33 to 50% $(NH_4)_2SO_4$ saturation and is precipitated from dilute plasma at pH of 5 to 5.5. Factor V is thermolabile and destroyed by trypsin.

In a study by Hougie (1957) and in earlier work by Hörder and Sohal, indications were that Factor V interacts with a sedimentable complex formed by the reaction between Factors VIII, IX, X and platelets. Hougie's work showed that if any of the following: VIII, IX, X, or V is omitted from the preparation of the sedimentable coagulant, it will have comparatively little activity. Subsequent addition of the coagulation factor originally omitted did not result in a further increase of coagulant activity, except in the case of Factor V. They concluded that Factor V acts subsequent to the formation of the complex.

Miller (1965) considered the disappearance rates of the "consumable" coagulation factors in a congenital Factor V deficiency. He studied Factors II, V, and VIII. He assumed that he could predict that the 3 factors should be consumed in the order in which they entered the coagulation sequence. According to the coagulation sequence, Factor VIII should be consumed first, then Factor V, and finally prothrombin. In the absence of Factor V, he expected to find a normal rate of consumption of Factor VIII, but a delayed consumption of prothrombin, which follows Factor V. He found this to occur in a patient with near total absence of Factor V.

In more recent work, Ferguson and Ennis (1969) postulate that Factor V has a dual role. First, they confirm that Factor V is one of the determinants of prothrombin-converter enzymatic activity. Secondly, they postulate that it appears to serve somewhat like an amboceptor, to bring the enzymatic activator complex (thrombokinase, + Ca^{++} + V) into steric juxtaposition to the reactive groups of prothrombin.

The influence of hyponormal levels of factors detected by the one-stage prothrombin test are heavily documented. A study concerning hypernormal values was conducted by Ferguson and Patch (1956). They noted

that in an earlier study by Fresh and Ferguson on newborn infants, there was a lack of bleeding disorders, with a normal "prothrombin time" test, despite significantly low levels of prothrombin and Factor VII. In this earlier study, they noted that Factor V in the infants' bloods was hypernormal, with values up to 300% of standard adults' levels. Using artificial plasma systems, Ferguson and Patch found that excessively high (200-300% of normal) Factor V levels can compensate for moderately low prothrombin and Factor VII levels. The importance of this finding in clinical application has yet to be evaluated.

There is disagreement as to the effect of coumarin drugs on Factor V. Fahey *et al.* (1948) measured the effect of dicoumarol on Factor V levels in both dogs and humans. They found only a moderate reduction in Factor V concentration, rarely below 65% of normal. The drop in Factor V generally began on the day following the first dose and usually returned to normal within 2 weeks. They noted that in dogs, the Factor V level extended to values above the original levels, in one instance going as high as 150%. This effect did not occur in the human patients studied. Nowicki *et al.* (1966) also showed a moderate drop in Factor V during treatment of patients with dicoumarol. Most other authors do not mention this finding.

Factor VII

Synonyms for Factor VII (Koller) include proconvertin (Owren), SPCA--serum prothrombin conversion accelerator (deVries, Alexander), and stable factor (Stefanini). The action of Factor VII was described by Alexander *et al.* (1948). They noted that this substance was found in serum and the evolution of this substance was closely related to prothrombin consumption. Alexander (1959) noted that Factor VII activity

is demonstrable in human, bovine, rabbit and canine serum. This factor accelerates the conversion of prothrombin to thrombin in the presence of tissue thromboplastin, Factor V and Calcium ions. A deficiency in this factor results in retarded prothrombin conversion and a prolonged one-stage prothrombin time.

In a communication from the International Committee on Nomenclature of Blood Clotting Factors (1959), it was noted that Factor VII deficiency can be either congenital or acquired. The acquired deficiency may occur in liver disease, in vitamin K deficiency, in the immediate neonatal period, and after the administration of prothrombinopenic agents. They also noted that excesses of Factor VII have been found in certain states associated with a high incidence of thromboembolism.

Some of the biochemical properties of Factor VII as noted by Alexander (1959) are: Factor VII is readily adsorbed from oxalated and nonoxalated plasma and serum by $BaSO_4$, $CaCO_3$, Seitz asbestos filters, alumina and $Ca_3(PO_4)_2$. In serum, this factor can withstand incubation at 37 C. for at least 4 hours. This factor is not precipitated with the globulins from serum or plasma at pH 5.0 to 5.2.

Tishkoff *et al.* (1960) obtained complete separation of prothrombin, Factors VII and X by means of starch gel electrophoresis and found substantial amounts of neuraminic acid in all 3 factors. They also noted that this acid can be cleaved from all 3 factors without proportionate loss of biologic activity.

Prydz (1965) purified Factor VII from normal human serum. His product contained about 51% carbohydrates; the bulk were hexoses and 1% was methyl-pentose. He found no sialic acid in his purified Factor VII. The molecular weight of Factor VII obtained from plasma was 63,000, while

the molecular weight of the purified serum factor was estimated to be about 48,000.

The Seegers group (1967) does not consider Factor VII as a separate clotting factor; rather, this factor is called Autoprothrombin I_p. This group noted that their purified prothrombin corrects abnormal coagulation tests of patients who are deficient in this factor and conclude that an abnormal prothrombin molecule is the basis for the abnormal coagulation in these patients.

Leavell and Thorup (1968) describe Factor VII as one of the essential factors necessary for the development of thromboplastin activity from a tissue source. They note that Factor VII behaves as a beta globulin on electrophoresis and that it does not play any role in the production of thromboplastin activity by the intrinsic system.

Kazmier *et al.* (1965) note that during anticoagulant therapy, Factor VII is the first factor diminished by coumarin drugs, and is also the first factor to return to normal levels when medication is discontinued.

Factor X

The use of serum from various pathological conditions in the thromboplastin generation test led to the conclusion that there is another factor besides the serum factors already known (Factor VII and Factor IX). Duckert *et al.* (1955) were able to separate Factor X from Factors VII and IX because of its greater affinity for $BaSO_4$. Their studies showed that Factor X is present in normal serum and in hemophilia B-serum; absent in hepatitis serum and in serum of coumarin treated patients.

Hougie *et al.* (1957) described some of the properties of Factor X. They found that Factor X in serum is stable for 30 minutes at 37 C. in the pH range from 6 to 9.

Tishkoff *et al.* (1960), in their experiments on the electrophoretic separation of prothrombin, Factor VII and Factor X on starch gel, found that Factor X moved the most rapidly toward the anode. They found that the Factor X they separated had very little protein, although neuraminic acid was present in significant quantities. They postulated that Factor X was either nonprotein in nature or that its biologic activity might be associated with a trace protein of high neuraminic acid content.

Esnouf and Williams (1962) purified bovine Factor X and by DEAE-cellulose separation estimated the molecular weight of Factor X to be 86,000. Lewis (1964), using Sephadex gel filtration, found the molecular weight of Factor X to be between 50,000 and 100,000.

Physicochemical data in Todd and Sanford (1969) describes Factor X as an alpha globulin that migrates ahead of prothrombin and Factor VII. It can be precipitated from plasma at between 55 and 65% $(NH_4)_2SO_4$ saturation.

In most theories that follow the "waterfall" or "cascade" sequence of coagulation, Factor X appears to play the pivotal role in prothrombin activation. This factor is activated by the extrinsic, intrinsic and venom systems (Leavell and Thorup, 1968).

In the Seegers system (1967), Factor X is autoprothrombin III.

Coumarin

In 1924, it was noted that the feeding of improperly cured sweet clover produced serious hemorrhage and even death in cattle. Quick (1936) noted that the feeding of toxic sweet clover to cattle produced a drop in plasma prothrombin (as tested by the Quick one-stage prothrombin test). Campbell and Link (1941) isolated and crystallized the hemorrhagic agent from toxic sweet clover and found the empirical formula to be

$C_{19}H_{12}O_6$. This compound was found to be 3,3'-methylene-bis(4-hydroxy-coumarin).

Clinical trials of coumarin drugs began in 1941 to test their use as therpauetic agents in human thromboembolic diseases. The mode of action as described by Douglas (1955) is related to the inability of the patient to form thromboplastin.

Kazmier *et al.* (1965) performed experiments to show that the coumarin drugs interfere with the production of Factors II, VII, IX, and X. The mode of action of coumarin drugs has not been fully elucidated. However, their structural resemblance to vitamin K suggests that they competitively interfere in an enzyme system involved in the formation of the above-mentioned coagulation factors. It was shown by Douglas (1955) that the administration of vitamin K_1 prevents or reverses the action of these drugs.

Work by Weiner *et al.* (1950) shows that coumarin drugs are erratically absorbed from the gastrointestinal tract, and that the drug is extensively attached to proteins of plasma and other body tissues. These workers also showed that the rate of transformation of the drug is widely divergent in different subjects. This rate also depends on the dose, and they found that for each subject there is a threshold plasma level of coumarin which must be reached before there is a detectable response in the prothrombin test.

Goodman and Gilman (1956) give the minimum structural requirements for anticoagulant activity of the coumarin drugs. These minimum requirements are an intact 4-hydroxycoumarin molecule or a 4-hydroxy-coumarin structure with a 3 substituent, having a keto group in 1,5 spatial relationship to the enolic OH of 4-hydroxycoumarin.

MATERIALS AND METHODS

Preliminary Procedures

Preliminary studies were made on normal human subjects. At first the Vacutainer system* was used for collecting blood, but a precipitate formed in the incubated specimens of patients on coumarin therapy. It is not known whether this was caused by an antimycotic agent in the tubes. We then prepared our own anticoagulants.

In the preliminary studies, blood samples were mixed with 0.1 M sodium oxalate. The sodium oxalate was distributed in 0.5 ml. amounts and 4.5 ml. of whole blood was added to each tube. The specimens were centrifuged immediately for 10 minutes at 2000 rpm. The plasma was removed from the cells and placed in clean test tubes and refrigerated.

Plasma specimens that required incubation at 37 C. for more than 3 hours (aged plasma) were collected aseptically. After centrifugation, this plasma was then placed into sterile screw-topped tubes with sterile disposable pipettes. The plasma specimens were then incubated at 37 C. as described by Hiar (1969). The specimens were incubated until a one-stage prothrombin of greater than 32 seconds was obtained. Using aseptic technique, the prothrombin time from this stock mixture was checked at various intervals. Total incubation time was approximately 56 hours.

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*Becton, Dickinson & Co., Division of BioQuest, Cockeysville, Maryland.

Upon completion of the incubation, this plasma 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. The specimens that were free from contamination were then divided into aliquots and frozen.

Other plasmas and sera rich in clotting factors V, VII, and X were prepared according to the methods of Eichelberger (1965) with modifications by Hiar (1969). These methods are described in detail in Appendix I.

Prothrombin times were measured by the one-stage method of Quick (1936), but adapted for use on the Fibrometer.* Determinations were performed in duplicate or until the results agreed within 0.3 second on the times shorter than 30 seconds, and 0.5 second agreement on times longer than 30 seconds. Simplastin** was used as the source of thromboplastin and calcium ions. Dilutions were made according to the manufacturer's instructions.

For each new batch of reagents and at the beginning of each daily run, a commercial control was used to check the reliability of the reagents. Heating blocks and water baths that maintained a constant temperature $\pm$ 0.5 C. were used. Plasma specimens were stored at 4 C. until they were ready for testing.

Experiments Concerning the Heat Stability of Factors Involved in the Prothrombin Test

Experiment I. To determine at what temperature the coagulation factors would be destroyed by heat, 10 plasma specimens were obtained from

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*Fibrometer System; BD, Division of BioQuest, Cockeysville, Md.

**Simplastin and Verify Normal; General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, N.J.

patients who had no known bleeding dyscrasias. These specimens were incubated for 30 minutes at 56 C. Preincubation prothrombin times were determined and the determinations were repeated at the end of the incubation period. The results of these determinations are shown in Table 1.

Experiment II. To determine whether any factors were labile to heat at lower temperatures, 10 plasma specimens were obtained from patients admitted to a hospital. These specimens were incubated for 90 minutes at 45 C. At varying intervals from 0 to 90 minutes, portions of these plasmas were withdrawn, and prothrombin times were determined. The results of these determinations are shown in Table 2.

Experiment III. To determine the effect of heat on Factors VII and X, 10 pooled plasma samples were used. These samples were heated at 45 C. for 30 minutes. Prothrombin times were determined at 10-minute intervals. At each time interval, 2 aliquots of 0.4 ml. of plasma were removed. To the sample marked Test, 0.1 ml. of aged serum (Appendix I) was added. To the sample marked Control, 0.1 ml. of physiologic saline was added. Prothrombin times were determined on 0.1 ml. samples of both Test and Control plasmas. The results of this experiment are presented in Table 3.

Experiment IV. To further determine the effect of heat on Factors VII and X, plasma samples from 5 normal individuals were pooled. These samples were collected aseptically and incubated at 37 C. until the prothrombin time was increased to over 32 seconds (see preliminary procedure). After this incubation, a portion of this aged plasma was incubated at 45 C. for 60 minutes. At various time intervals during this incubation, aliquots of 0.4 ml. plasma were removed. To the sample marked Test, 0.1 ml. of $BaSO_4$ adsorbed plasma was added. To the Control sample, 0.1 ml.

of physiologic saline was added. Prothrombin determinations were run on 0.1 ml. amounts of these samples. These results are summarized in Table 4.

Experiment V. This experiment was designed to determine if prothrombin was labile to heating at 45 C. Aged plasma, as in Experiment IV, was used. Aliquots of the aged plasma were incubated at 45 C. for 60 minutes. During this time, at various intervals, 0.7 ml. of plasma was removed. To this plasma was added 0.1 ml. of aged serum and 0.2 ml. of fresh $BaSO_4$ adsorbed plasma. The prothrombin times of 0.1 ml. of these mixtures were determined and the results appear in Table 5.

Experiment VI. From the results of the previous experiments, it appeared that the factor remaining in the $BaSO_4$ adsorbed plasma was the only significantly labile factor in the prothrombin complex. The purpose of this experiment was to determine whether the concentration of this labile factor could be estimated using thermal incubation. Blood was obtained from 5 normal volunteers. Before pooling, a one-stage prothrombin time was performed on the individual plasmas. Only those plasmas having a prothrombin time of less than 14 seconds were used in the pool.

The source of labile factor was a portion of the normal pool. Plasma from the normal pool was adsorbed with 100 mg. of $BaSO_4$/ml. This plasma-$BaSO_4$ mixture was incubated at 37 C. for 10 minutes, with occasional gentle agitation during the incubation period. The mixture was then centrifuged for 10 minutes at 2000 rpm and the upper 3/4 of the supernatant plasma removed. A prothrombin time was determined on the adsorbed plasma. If the prothrombin time was shorter than 60 seconds, the specimen was readsorbed with fresh $BaSO_4$ for another 10 minutes. This portion of the sample was kept refrigerated at 4 C. while the next step was carried out.

The pooled fresh plasma was incubated at 45 C. for 1 hour. After incubation, physiologic saline was used to make the following dilutions of prothrombin activity in percent:

a = 100%(undiluted)	c = 50%	e = 16%
b = 75%	d = 20%	f = 10%

A portion of the labile factor plasma was also incubated for 1 hour at 45 C. and was the source of the 0% specimen used in the next step.

The refrigerated plasma containing labile factor was diluted with physiologic saline to give the following percentage of activity:

A = 100%(undiluted)	C = 25%	E = 6.25%
B = 50%	D = 12.5%	F = 0%(undiluted, incubated labile-factor plasma)

Six rows of 6 tubes each were prepared in the following manner: 0.4 ml. of undiluted incubated normal plasma (a) was added to tube 1 of every row; 0.4 ml. of (b) was added to tube 2 of every row; 0.4 ml. of (c) was added to tube 3 of every row; 0.4 ml. of (d) to tube 4 of every row; 0.4 ml. of (e) to tube 5 of every row; and 0.4 ml. of (f) to tube 6 of every row.

Next, 0.1 ml. of undiluted labile factor (A) was added to every tube in row 1. Then, 0.1 ml. aliquots of the mixtures in row 1 were removed and one-stage prothrombin times were performed until the results agreed within 0.3 second. One-tenth milliliter of labile factor (B) was added to tubes in row 2, and prothrombin times were determined on 0.1 ml. aliquots as mentioned above. Rows 3 through 6 were set up in a similar manner, using dilutions C through F. The final concentrations of labile factor and incubated plasma/tube are included in Table 6.

These dilution curves were then plotted on graph paper using the method of least squares. The results of this plot are in Figure 1.

Experiment VII. Because it appeared from the plots of Experiment VI that the concentration of labile factor could be quantitatively determined, this experiment was designed to devise a method for investigating the corrective properties of $BaSO_4$ adsorbed plasma when it was added to incubated plasma. This experiment further investigated the possibility of a quantitative diminution of a single clotting factor.

Blood was obtained from 4 normal volunteers. The anticoagulant used for this procedure was 0.5 ml. of 3.8% sodium citrate/4.5 ml. of whole blood. The source of $BaSO_4$ adsorbed plasma was a portion of this pool. A portion of the normal pool was incubated for 1 hour at 45 C. A portion of the $BaSO_4$ adsorbed plasma was also incubated for 1 hour at 45 C.

After incubation, 3 rows of 7 tubes each were prepared in the following manner: 0.5 ml. of physiologic saline was added to all tubes in rows 2 and 3. Varying combinations of incubated and unincubated plasmas were added to the tubes in row 1:

1 = 1.0 ml. of unincubated plasma (normal)
2 = 1.0 ml. of incubated normal plasma
3 = 0.8 ml. of incubated plasma and 0.2 ml. of unincubated $BaSO_4$ plasma
4 = 0.5 ml. of incubated plasma and 0.5 ml. of unincubated $BaSO_4$ plasma
5 = 0.8 ml. of incubated plasma and 0.2 ml. of incubated $BaSO_4$ plasma
6 = 0.5 ml. of incubated plasma and 0.5 ml. of incubated $BaSO_4$ plasma
7 = 0.2 ml. of incubated plasma and 0.8 ml. of unincubated adsorbed plasma

Next, 0.5 ml. from each tube in row 1 was removed and placed in the tubes containing the physiologic saline in row 2. These specimens were then mixed with a pipette and 0.5 ml. removed and placed in the tubes containing saline in row 3. This provided concentrations of 100%, 50%, and 25%. These dilution curves were then plotted on graph paper using the method of least squares. The results are in Figure 2.

Experiment VIII. This experiment was designed to determine whether $BaSO_4$ adsorbed plasma was needed to correct the loss of labile factor after inactivation. The plasma from 4 normal persons was used. Prothrombin times were determined before the plasmas were pooled. A portion of this plasma pool was incubated at 45 C. for 1 hour. Three rows, of 6 tubes each, were used for this experiment. The following combinations of incubated and unincubated plasmas were used in row 1:

1 = 1.0 ml. of unincubated plasma
2 = 0.8 ml. of unincubated plasma and 0.2 ml. of incubated plasma
3 = 0.5 ml. of unincubated plasma and 0.5 ml. of incubated plasma
4 = 0.25 ml. of unincubated plasma and 0.75 ml. of incubated plasma
5 = 0.2 ml. of unincubated plasma and 0.8 ml. of incubated plasma
6 = 1.0 ml. of incubated plasma

Fifty and twenty-five percent concentrations were made from these tubes and prothrombin times were determined. These dilution curves were then plotted on graph paper using the method of least squares. The results are in Figure 3.

Experiments on Clinical Patients

Experiment I. Two baseline prothrombin curves were plotted for each patient before he was placed on coumarin therapy. The first curve consisted of unincubated plasma in dilutions of 100, 50 and 25% activity. The second curve was determined from a portion of the same plasma that was incubated at 45 C. for 1 hour. These results can be found in Figure 4. The results of the changes in intercept can be found in Chart I.

Experiment II. Specimens were obtained from the patients studied in Experiment I on the preceding page, after they had been placed on coumarin therapy. The same 2 types of curves were plotted on the treated plasma: unincubated and incubated. The effect of coumarin and heating on slope and intercept can be found in Figure 5. The results of the changes in intercepts after coumarin therapy, and the length of therapy, are included in Chart II. A total of 50 patients was studied.

RESULTS

Table 1 represents the rapid destruction of the coagulation factors over a period of time at 56 C. Table 2 represents the destruction of 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. Correction studies in Tables 3 through 5 appear to verify this assumption.

Experiments VI through VIII represent correction studies of labile factors. Because of these studies, the 2 clinical experiments were carried out and evaluated.

Charts I and II represent the results of the clinical studies.

TABLE 1. Rapid destruction of coagulation factors in the plasma of 10 hospital subjects

Patient No.	Prothrombin Time in Seconds	
	Before Incubation	After Incubation at 56 C. for 30 minutes
1	13.2	> 70
2	13.5	> 70
3	13.8	> 70
4	13.0	> 70
5	12.3	> 70
6	12.6	> 70
7	11.3	> 70
8	12.8	> 70
9	12.5	> 70
10	11.7	> 70
Control 11.8 sec.*		

*Normal value for control = 11-15 seconds

TABLE 2. Destruction of the labile factors in the plasma of 10 hospital subjects

Patient No.	Prothrombin Times in Seconds Time (min.) of Incubation at 45 C.							
	0	10	20	40	50	60	80	90
1	12.8	14.3	16.3	18.5	19.0	20.0	23.0	24.0
2	12.3	13.7	14.7	17.1	18.3	19.5	24.6	26.3
3	12.4	13.0	14.0	15.6	17.0	18.3	21.8	23.8
4	14.0	15.7	16.5	19.7	20.0	22.0	23.7	25.0
5	14.2	15.7	16.7	19.8	21.6	22.8	24.3	26.5
6	14.0	16.3	18.0	19.5	21.2	23.9	29.6	33.6
7*	13.7	18.1	20.2	23.2	29.1	37.6	42.0	> 70
8**	13.2	18.0	18.5	21.2	23.5	26.0	31.9	41.7
9	12.5	14.6	15.0	18.2	18.3	20.0	22.5	24.5
10	12.7	16.6	18.0	18.2	19.0	20.5	22.5	23.5
Control 11.8 sec.***								

*19-year-old epileptic, on medication for 1 year. Bleeding time and Partial Thromboplastin Test were normal as was a routine one-stage prothrombin.

**50-year-old alcoholic from alcoholism therapy unit. Liver function studies showed some liver damage.

***Normal value 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	12.8	11.9
10	14.0	13.8
20	16.5	15.0
30	16.9	16.8

TABLE 4. Effect of incubation on Factors VII and X using aged plasma

Time (min.) of Incubation	Prothrombin Times (sec.)	
	Control	Test
0	41.9	18.8
10	43.7	18.5
20	44.8	19.5
30	47.5	19.2
60	50.6	19.8

TABLE 5. Effect of incubation on prothrombin

Time (min.) of Incubation	Prothrombin Time (sec.)
0	18.3
10	18.5
20	18.8
30	18.8
60	18.9
Control = 12.3 sec.*	

*Normal value for control = 11-15 seconds.

TABLE 6. Final concentration in each tube in percent of labile factor per percent of heated pooled plasma

row 1 A:a-f	20/80	20/60	20/40	20/20	20/16	20/10
row 2 B:a-f	10/80	10/60	10/40	10/20	10/16	10/10
row 3 C:a-f	5/80	5/60	5/40	5/20	5/16	5/10
row 4 D:a-f	2.5/80	2.5/60	2.5/40	2.5/20	2.5/16	2.5/10
row 5 E:a-f	1.25/80	1.25/60	1.25/40	1.25/20	1.25/16	1.25/10
row 6 F:a-f	0/80	0/60	0/40	0/20	0/16	0/10

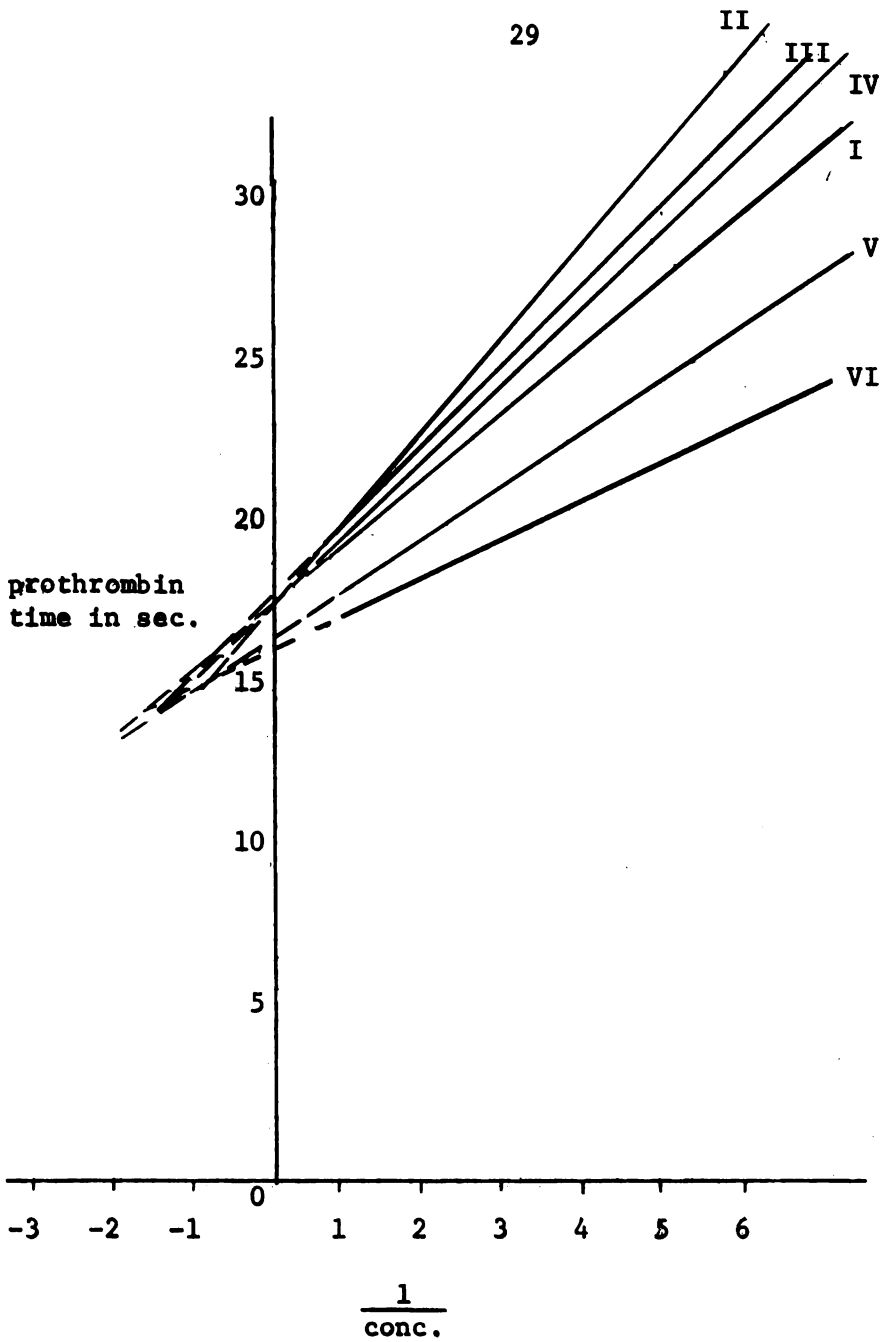

Figure 1. Demonstration of intersecting point of dilution curves. Lines I-VI denote labile factor concentrations of 0, 1.25, 2.5, 5.0, 10.0 and 20.0%, respectively.

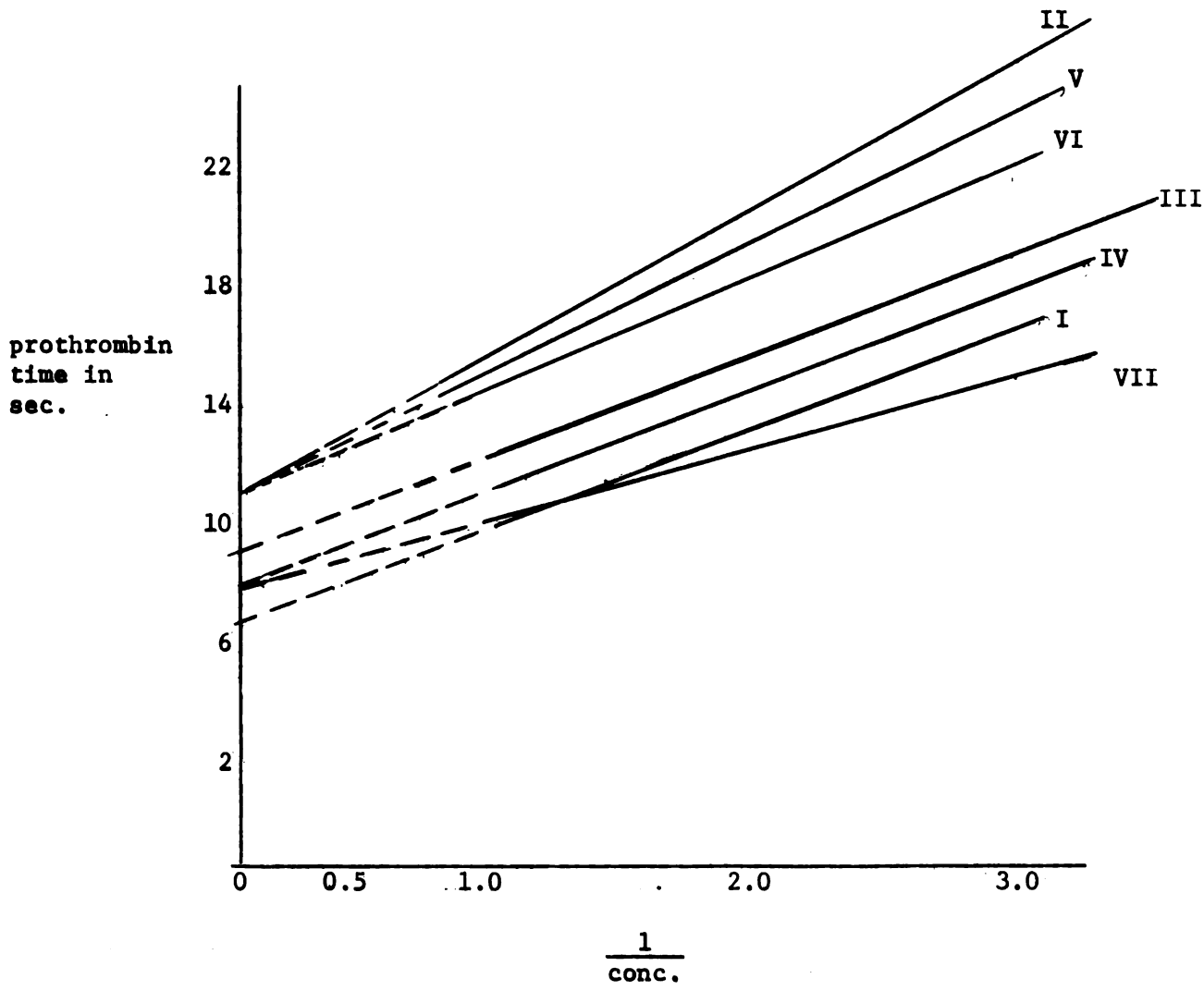

Figure 2. Correction curves of citrated plasma with $BaSO_4$ adsorbed plasma.

I = pooled, unincubated plasma

II = pooled, incubated plasma

III = 0.8 ml. incubated pool, 0.2 ml. unincubated $BaSO_4$ adsorbed plasma

IV = 0.5 ml. incubated pool, 0.5 ml. unincubated $BaSO_4$ adsorbed plasma

V = 0.8 ml. incubated pool, 0.2 ml. incubated $BaSO_4$ adsorbed plasma

VI = 0.5 ml. incubated pool, 0.5 ml. incubated $BaSO_4$ adsorbed plasma

VII = 0.2 ml. incubated pool, 0.8 ml. unincubated $BaSO_4$ adsorbed plasma

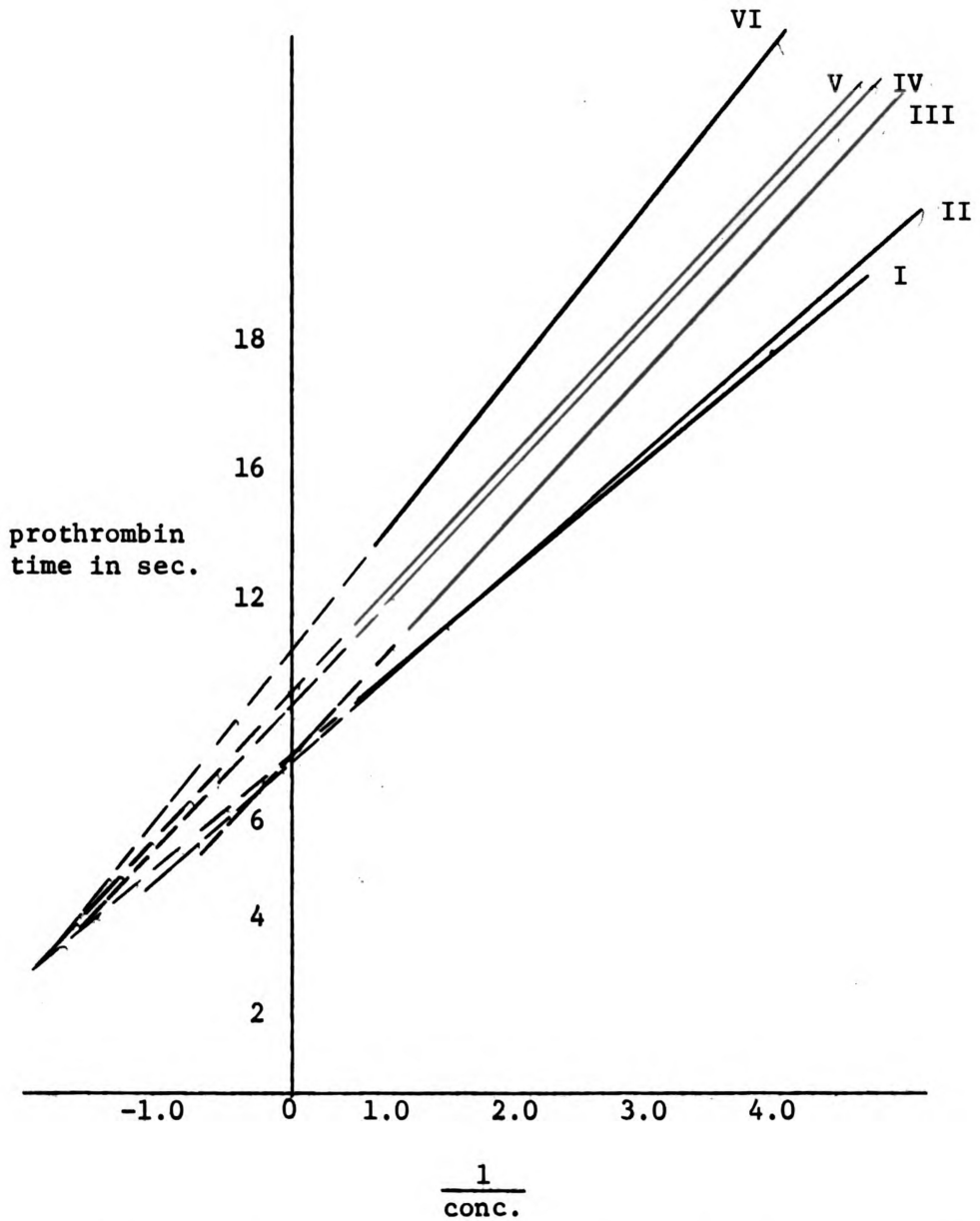

Figure 3. Changes in intercept due to incubation. Citrated plasma was used. No $BaSO_4$ adsorption was performed.

I = pooled, unincubated plasma.

II = 0.8 ml. unincubated pool, 0.2 ml. incubated pool.

III = 0.5 ml. unincubated pool, 0.5 ml. incubated pool.

IV = 0.25 ml. unincubated pool, 0.75 ml. incubated pool.

V = 0.2 ml. unincubated pool, 0.8 ml. incubated pool.

VI = pooled, incubated plasma.

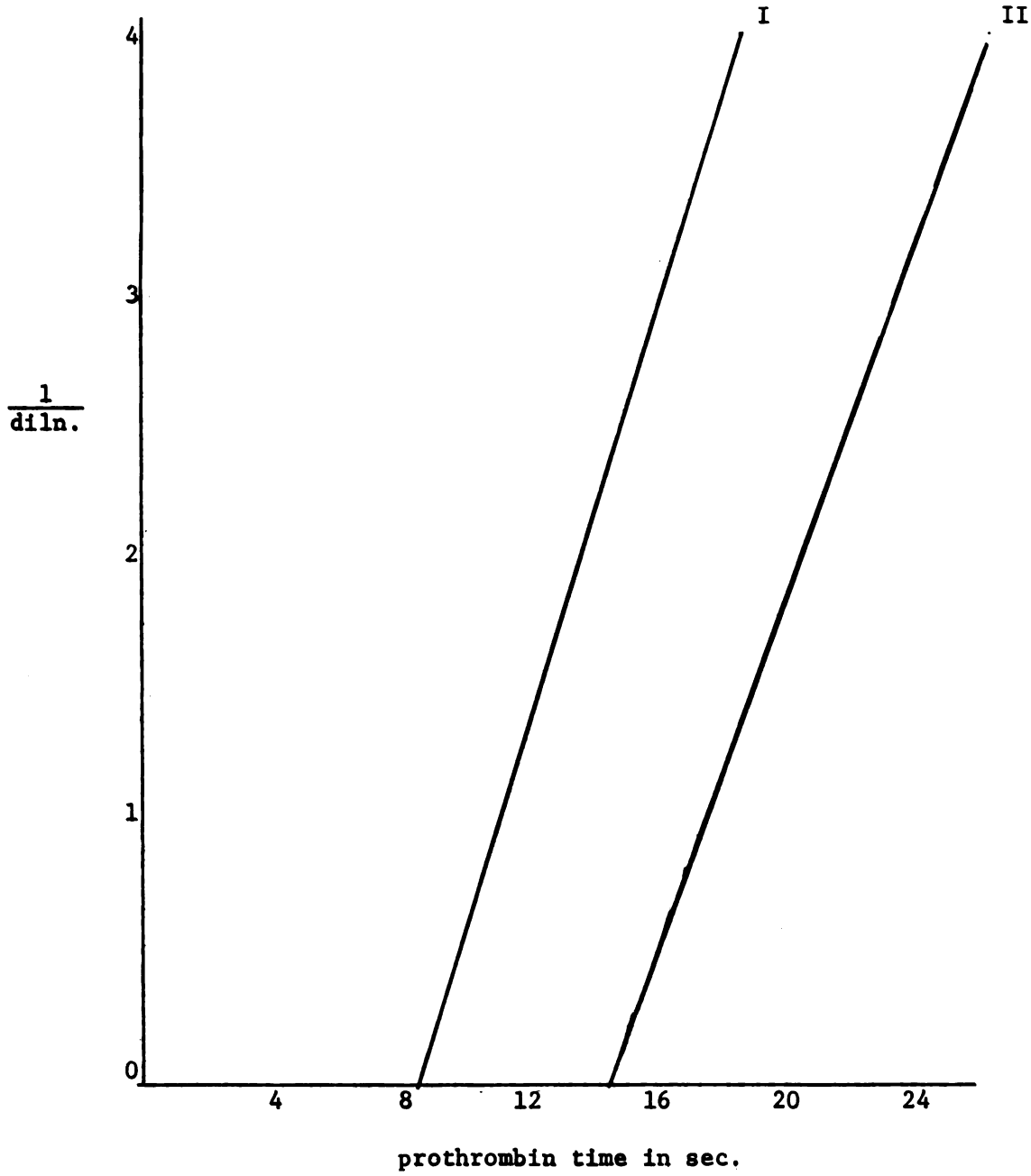

Figure 4. I = baseline prothrombin

II = portion of same plasma incubated for 1 hour at 45 C.

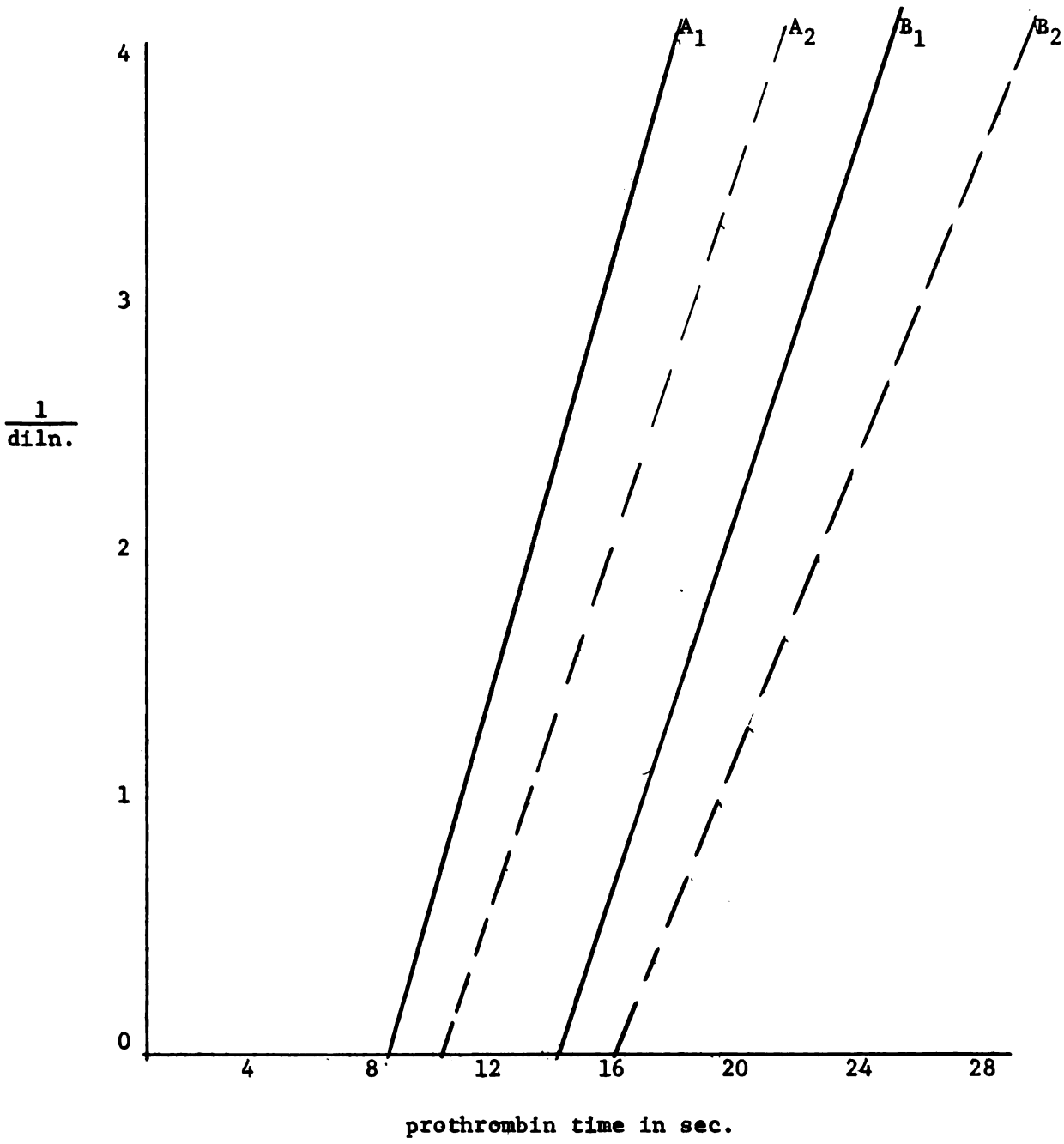

Figure 5. Effect of heating and coumarin therapy on citrated plasma.

A_1 = Baseline prothrombin, unincubated plasma

B_1 = Baseline prothrombin, incubated plasma

A_2 = Prothrombin after 1 day of therapy, unincubated plasma

B_2 = Prothrombin after 1 day of therapy, incubated plasma

CHART I. Change in intercept due to incubation at 45 C. for 1 hour, on normal patients. θ = Intercept

θ_1	θ_2	$\triangle\ \theta$
7.94	11.16	3.22
8.32	11.63	3.31
8.66	13.97	5.31
8.94	13.73	4.79
8.51	13.89	5.38
8.63	11.54	5.10
10.08	14.02	2.91
8.54	12.36	3.94
8.09	12.73	3.83
7.44	10.64	4.64
8.74	12.16	3.20
9.07	13.76	3.42

$\triangle\ \bar{\theta} = 4.13$

s.d. = 0.88

$\triangle\ \bar{\theta} \pm t_{(2.18)}$ (s.d.)$_{(0.88)}$ = range 2.21 - 6.05

CHART II. Change in intercept due to incubation at 45 C. for 1 hour, after treatment with coumarin compounds. θ = Intercept

θ_1	θ_2	$\Delta\theta$	% proth.	Duration of therapy in days	Comments
13.52	13.25	-0.27	24.0	6	
24.35	24.37	0.02	10.5	3	
16.15	17.84	1.68	19.6	6	
9.86	11.56	1.70	24.7	8	
15.99	17.79	1.80	95.0	120	
13.47	17.78	3.31	23.0	3	
23.68	27.41	3.73	10.8	2	
12.48	16.79	4.30	27.0	720	
13.65	18.13	4.48	17.4	4	
13.78	18.23	4.50	17.4	4	
10.49	15.06	4.57	100.0	60	
19.90	25.00	5.10	11.5	7	
10.51	16.36	5.85	51.2	2	
16.51	22.87	6.36	17.8	5	
17.01	23.80	6.79	15.8	210	
27.09	33.89	6.80	<10.0	4	
16.87	24.64	7.77	18.0	6	
14.94	27.29	13.34	12.3	540	bruising

DISCUSSION

Table 1 represents the rapid destruction of coagulation factors at 56 C. Various data published on heat denaturation of proteins agree with the results of Experiment I. Human serum inactivated at this same time and temperature for various serologic tests shows no precipitate. A precipitate formed in the bottom of each experimental tube that was incubated, and it is assumed that this precipitate was fibrinogen.

Table 2 represents the destruction of coagulation factors over a period of 1-1/2 hours. It was not known at this point which of the factors were destroyed. Patient #7 was an epileptic who had been on medication for convulsions for approximately 1 year. She had no complaints of bruising or bleeding. The screening tests for bleeding disorders were all within normal limits. This patient was not available for further testing during the clinical experiments carried out later in this paper. Patient #8 in this same experiment was an alcoholic admitted to the alcoholism therapy unit of a hospital. A possible point for further study might be an evaluation of alcoholics to see if the change in intercept and slope recorded in the clinical tests might be a screening test for liver damage.

Tables 3 and 4 reveal that factors other than Factor V are stable during incubation at 45 C. The addition of aged serum in Experiment III did not bring about a large correction in the prothrombin time when compared to the controls. This serum contained Factors VII and X;

therefore, these factors were not being destroyed at this incubation time and temperature.

The addition of $BaSO_4$ adsorbed plasma in Experiment IV corrected the prothrombin time to near normal levels in the test samples. It is likely that the aging process involved in the first portion of this experiment destroyed some of the stable factors, because the process took an average of 56 hours. The purpose of aging the plasma prior to incubation at 45 C. was to destroy the labile Factor V.

Experiment V was concerend with the stability of prothrombin at 45 C. The results shown in Table 5 reflect very small changes in prothrombin levels over the 60-minute incubation period.

Experiment VI was an attempt to estimate quantitatively the amount of labile factor destroyed by thermal incubation by adding "known" amounts of labile factor to plasma that had been incubated. Figure 1 is the plot of these dilution curves. Except for line I, the lines follow a typical concentration plot. It is assumed that line I is out of sequence because it represents an undiluted specimen which may contain more fibrinogen than the other specimens. Initial investigations estimating the concentration of labile factor did not give as clear intercept lines as later studies.

Experiment VII represents further studies on correction of heated plasma with $BaSO_4$ adsorbed plasma. This procedure used 3.8% sodium citrate as the anticoagulant. The slopes obtained are not as steep as those with the oxalate anticoagulant. This agrees with the claim of some authors that citrate appears to have some stabilizing effect on thermolabile components. Lines II, V, and VI represent varying dilutions of stable factor. It can be seen that the intercept does not change; however,

there is a change in slope. It was therefore assumed that the destruction of the labile factors in human plasma can be represented by a change in intercept.

Experiment VIII also represents correction studies on heated plasma; however, no $BaSO_4$ adsorbed plasma was used. Because of this fact, each tube is assumed to contain 100% of the stable factors. There does appear to be an extrapolated point of intersection of these lines, thus further confirming the fact that the amount of labile factor present in plasma can be qualitatively determined by examining a change in intercept.

Experiment I on clinical patients involved the plotting of a baseline prothrombin curve, and also a prothrombin curve to demonstrate the effect of incubation on the normal prothrombin curve. These studies demonstrate that thermal incubation affects both the slope and intercept. The major change was in the intercept. Figure 3 and Chart I are examples of the baseline studies.

Experiment II on clinical patients represents the major emphasis of this study. Figure 4 represents the contrast of the baseline prothrombins and the prothrombin plots after 1 day of therapy. Chart II represents the changes in intercept observed in various patients. The duration of therapy ranged from 1 day to several years. It can be seen from Chart II that coumarin drugs exert a greater effect on labile factor than most authors expect. Many authors do not mention a change in labile factor during coumarin therapy. Some authors state that there is a drop in labile factor during the first few days of therapy. The results of clinical Experiment II bear out this fact. The fact that some patients show a much lower decrease in labile factor after a few days of therapy shows that there is the possibility of a "rebound". The fact that there is a higher concentration of labile factor in some of the patients after

a few days of therapy brings to mind the question of how much of an effect the concentration of labile factor has on the prothrombin times of patients with hemorrhagic complications. Chart II also demonstrates that there is no correlation between the percent prothrombin concentration of the patient's plasma and the concentration of labile factor present in the plasma. It is not known at this time whether the last patient on Chart II with the 13.34 second change in intercept reflects a change in labile factor or a change in both labile and stable factors.

Since percent prothrombin concentration does not correlate well either with bleeding tendency or the estimate of labile factor from changes in intercept, anticoagulant therapy should be monitored with both methods until one or the other measurement becomes established.

SUMMARY AND CONCLUSIONS

The work presented here brings out several basic facts. The factor called prothrombin as well as Factors VII and X are relatively stable to incubation at 45 C. for at least 1 hour. At 45 C. Factor V is rapidly destroyed. If citrate is used as an anticoagulant, this destruction is retarded.

The last finding of this study is the fact that contrary to most authors, labile factor (Factor V and possibly others) is indeed affected by coumarin drugs. A theoretical method for determining the effect of coumarin drugs on both labile and stable factors is presented.

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APPENDIX

APPENDIX I

Preparation of Reagents Rich in the Clotting Factors of the Prothrombin Complex

Source of Fresh Plasma

1. Collecte blood in 0.1 M sodium oxalate or 3.8% sodium citrate* (0.5 ml. of anticoagulant/4.5 ml. of blood).
2. Immediately centrifuge specimen for 10 minutes at 2000 rpm.
3. Remove the upper 3/4 of plasma and store at 4 C. until ready to use.

*The author switched to citrate because of problems with precipitates in the heated specimens.

$BaSO_4$ Adsorbed Plasma

1. Collect blood in oxalate or citrate anticoagulant.
2. Centrifuge immediately for 10 minutes at 2000 rpm.
3. Add 100 mg. of chemically pure $BaSO_4$ to each ml. of plasma to be adsorbed.
4. Place in a 37 C. water bath for 10 minutes. Agitate the tube several times during this incubation.
5. Centrifuge for 10 minutes at 2000 rpm.
6. Remove upper 3/4 of the plasma. A one-stage prothrombin test should be performed on this plasma and should be longer than 3 minutes.
7. If the prothrombin time is not longer than 3 minutes, readsorption with fresh $BaSO_4$ should be performed.

Aged Plasma

1. Strict aseptic technique should be maintained throughout this procedure. Tubes containing anticoagulant should be autoclaved.
2. Centrifuge the plasma for 10 minutes at 2500 rpm.
3. Transfer the plasma aseptically to a sterile screw-topped test tube with a sterile disposable pipette.
4. Incubate the plasma at 37 C. for 24-56 hours or until the one-stage prothrombin test exceeds 32 seconds. If citrate is used as the anticoagulant, the incubation time may be longer.
5. Using aseptic technique, check the prothrombin time at various intervals by removing a portion of the plasma from the stock mixture.
6. At the end of the 37 C. incubation, recentrifuge the plasma for 10 minutes at 2000 rpm. Remove plasma from any sediment that formed.
7. Make a smear for Gram's stain of the sediment and also inoculate a portion of the sediment on blood agar to check for bacterial contamination. If the specimen is contaminated, a new stock plasma must be prepared.

Aged Serum

1. Obtain serum samples after the clotting of normal human blood.
2. Pool 3 or 4 of these samples.
3. Allow these samples to stand at 4 C. for at least 48 hours before using. It may be necessary to recentrifuge these samples if there is any precipitate.

VITA

The author was born in Bay City, Michigan, on November 23, 1937. She graduated from St. Stanislaus High School in June of 1955. During the summer of 1955 she attended Northeastern School of Commerce in Bay City. In September of that same year, she entered Bay City Junior College on a medical technology program. After one year of undergraduate work, she transferred to Madonna College, Livonia, Michigan. She received a B.A. in biology from Madonna College in June, 1959.

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The author is a member of the Lansing, Michigan, and American Societies of Medical Technologists and, prior to entering graduate school, was active in society functions on the local and state levels.