RICE UNIVERSITY

STUDY OF PROTHROMBIN ACTIVATION BY TAIPAN SNAKE VENOM
USING LIGHT BEATING SPECTROSCOPY

by

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ABSTRACT

STUDY OF PROTHROMBIN ACTIVATION BY TAIPAN SNAKE VENOM USING LIGHT BEATING SPECTROSCOPY

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In this study, the recently developed technique of light beating spectroscopy has been used for the first time to measure the dynamic properties of prothrombin and its activation kinetics via Taipan Snake Venom, micellar phospholipid and Ca\(^{++}\).

The correlation times (\(\tau_c\)) on the samples of human prothrombin, which were prepared by membrane filtering techniques, were measured as a function of scattering angle to determine the particle size uniformity. \(I^2\) vs \(K^2\) plot was found to be linear for one sample at 10°C, but non-linearity was seen for other two samples of human prothrombin at 20°C, indicating some aggregation. The translational diffusion coefficient was measured on more than 20 samples at 2 mg/ml. The average value of diffusion coefficient (\(D_{20,W}\)) was measured to be 4.72 \(\times\) \(10^{-7}\) cm\(^2\)/sec with a standard deviation of 0.23 \(\times\) \(10^{-7}\) cm\(^2\)/sec. The diffusion coefficient value for a sample of 5 mg/ml human prothrombin was 4.51 \(\times\) \(10^{-7}\) cm\(^2\)/sec. These values are in agreement with the reported values of 4.80 \(\times\) \(10^{-7}\) cm\(^2\)/sec and 4.60 \(\times\) \(10^{-7}\) cm\(^2\)/sec at the respective concentrations. Sedimentation coefficients (\(S_{20,W}\)) determined from sedimentation velocity experiments were 5.37S and 6.70S at 2 mg/ml and 5 mg/ml. These were higher than the reported values of 4.80S and 5.20S for the corresponding concentrations, which could be attributed to aggregation at very low ionic strength and low pH.
The molecular weights of human prothrombin calculated from sedimentation and diffusion coefficients data were found to be 92,000 and 120,000 at 2 mg/ml and 5 mg/ml concentrations, respectively. From a sedimentation equilibrium experiment on a sample of first lot human prothrombin diluted to 0.2 mg/ml, the sample was found to be homogeneous species having 69,000 molecular weight.

In solution, the conversion of prothrombin was observed by monitoring the change of translational diffusion coefficient during its activation process. A single clipped autocorrelation function at low counts per sample interval (<1) and clipping level k = 0, is approximately equal to \( \langle I(t) I(t+\tau) \rangle \). Consequently, the autocorrelation function is proportional to the second power of M and c and therefore very sensitive to the presence of big particles in the solution. To satisfy this requirement nonphysiological activator Taipan Snake Venom (oxyuranus Scutellatus) and micellar phospholipid, Dihexanoyllecithin were used which gave negligible contribution to the autocorrelation function. An unusually long time of 3-5 days was observed for the complete conversion of first lot of human prothrombin. This slow rate could be due to possible conversion of prothrombin to Intermediate I and inhibitory fragment 1 prior to the activation reaction. These products retard the activation rate by an order of magnitude. Other factors such as the low surface area per unit mass of the micellar phospholipid (compared to the commonly used bilayer phospholipid), the low concentration of \( \text{CaCl}_2 \) (0.3mM) set by its precipitation threshold and the use of low temperatures to avoid aggregation may also contribute to the slow conversion.

For a sample of second lot human prothrombin 18% change in diffusion coefficient was observed at 20°C in less than an hour. Complete conversion study could not be done as the solution aggregated. This supports our hypothesis that a prior conversion of prothrombin of first lot had taken place.
ACKNOWLEDGEMENTS

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NOMENCLATURE

RVV           Russell's Viper Venom
Intermediate 3 = Fragment 1
Intermediate 4 = Fragment 2

Thrombokinase, fibrin ferment and thrombase are synonymous with thromboplastin.

\( i_s \)     Scattered Radiation Intensity
\( I_s \)     Average Total Scattered Intensity
\( I_0 \)     Intensity of Incident Radiation
\( \lambda \)  Wavelength of Incident Radiation in Vacuum
\( \alpha \)   Polarizability
\( \bar{\alpha} \) Average Polarizability
\( \theta \)   The angle between plane of polarization and the line joining the point of observation.
\( r \)       The distance between radiation center and point of observation.
\( N \)       Number of volume elements or number of macromolecules per cubic centimeter.
\( N' \)      Avogadro's Number
\( N_A \)    Number of Coherence Area
\( T \)       Temperature in °K
\( P \)       Pressure
\( c \)       Concentration of Macromolecules in gm/cc
\( \bar{c} \)  Average Concentration
\( \tilde{n}, n \) Refractive Index of Solvent
n(τ) The number of photo counts per unit time at τ.
n₀(τ) The number of clipped photon counts per unit time at τ.
n₀ The number of photon counts per unit time at τ=0.
k Clipping Level

Κₒ, Κₛ Wave Vectors of the incident and scattered light respectively.
Κ Scattering Vector (cm⁻¹)
cₑ(τ) Electric field autocorrelation function.
cᵢ(τ) Current autocorrelation function.
cₐ(τ) Amplitude autocorrelation function.
cₚ(τ) Phase autocorrelation function.
B, C Virial coefficients of second and third order respectively.
Κ Optical Constant
R0 Rayleigh Ratio

i(t) Photoelectric Current

E(t), E*(t) Complex electric field and its conjugate respectively.
g⁽¹⁾(τ), g⁽²⁾(τ) First and Second order autocorrelation function respectively.

φ Phase Angle

Pᵢ(ω) Power spectrum of the photocurrent.

τc Coherence Time
Γ Decay time constant (Γ = 1/τc).

w⁽¹⁾(t) Probability per unit time of photoelectron emission by the field.
\( W^{(2)}(t+\tau) \) Joint probability that one photoelectron will be emitted at time \( t \) (per unit time) and another at time \( t+\tau \) (per unit time).

\[ \begin{align*}
\text{\( d \)} & \quad \text{Stokes diameter of a particle.} \\
\text{\( \eta \)} & \quad \text{Viscosity in poise} \\
\text{\( D \)} & \quad \text{Translational diffusion coefficient.} \\
\text{\( \bar{\nu}_1 \)} & \quad \text{Partial specific volume of solvent.} \\
\text{\( \bar{\nu}_2 \)} & \quad \text{Partial specific volume of solute.} \\
\text{\( \delta_1 \)} & \quad \text{Degree of solvation (gm/gm).} \\
\text{\( \sigma \)} & \quad \text{Quantum Efficiency}
\end{align*} \]
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1. **SCOPE OF THIS WORK**

The conversion of prothrombin to thrombin is one of the most important reaction in the blood coagulation mechanism. The purpose of this investigation is to study the activation of pure prothrombin in the presence of Taipan Snake Venom, Ca\(^{++}\) and micellar phospholipids using light beating spectroscopy. According to a proposed mechanism prothrombin is cleaved sequentially to a thrombogenic molecule of M.W. of 65,000 (Intermediate 1) and an inhibitory Fragment 1. Intermediate 1 is further cleaved to form two single chain molecules with M.W. of 39,000 (Intermediate 2) and 24,000 (Fragment 2). Intermediate 2 is the single chain precursor of the first, most active thrombin.

Light beating spectroscopy has been used to study for the first time in solution the conversion of prothrombin. This was done by measuring the translational diffusion coefficients for monodisperse solution of pure prothrombin and prothrombin in presence of Taipan Snake Venom, Ca\(^{++}\) and micellar phospholipid. The activation of prothrombin to thrombin which results in a substantial change in molecular weight and size of prothrombin molecule is directly related to changes in translational diffusion coefficient of the activated protein.
2. THEORETICAL BACKGROUND:

In this chapter theoretical background on the theory of blood coagulation, mechanism of prothrombin activation, conventional light scattering, light beating spectroscopy and autocorrelator will be given.

A. The Theory of Blood Coagulation:

The sol-gel transformation (commonly known as 'clotting') that blood can undergo is properly considered one of the important ways in which blood loss can be limited, when there is a break in the barriers within which blood is ordinarily confined. Additionally, the process is important for wound healing and plays a role in numerous pathological conditions. Justifiably, much research has been done in the last few decades to clarify the mechanism of blood coagulation.

The simple equation of Morawitz:

\[ \text{Thrombokinase} + \text{Ca}^{++} + \text{Prothrombin} \rightarrow \text{Thrombin} \]

(Thrombokinase which is a synonym for thromboplastin was assumed to be supplied by cellular damage) epitomized nearly all the important knowledge that had accumulated concerning the coagulation of blood up to the end of the last century. It may, therefore, very well be called the classical theory. Yet strangely, this concept lay fallow for three decades. The tremendous expansion of knowledge concerning the coagulation of blood since 1935 has made the classical theory quite inadequate but its basic underlying principles that made it a useful guide remain.

The simple equation by Morawitz could not explain discrepancies observed in measurement of Quick's time\(^{(1)}\). Quick's one stage prothrombin time test was based on the literal acceptance of the four-factor theory
(three of the above factors and fibrinogen makes the above mechanism a four factor theory) and the assumption that if three factors thromboplastin, fibrinogen and calcium ion were present in adequate amounts, the clotting time of the system would reflect the concentration of the remaining factor, prothrombin. The circumstance which made this test obligatory in almost every clinical laboratory was the use of the dicoumarin drugs in the prevention and treatment of thrombosis. These drugs, by antagonizing the action of vitamin K were supposed to reduce the blood level of prothrombin, and careful laboratory control by the prothrombin time method was required to regulate dosage between the extremes of clinical inadequacy and disastrous haemorrhage. In the main, the test achieved its objective but puzzling discrepancies began to be reported which could not be explained on the basis of the four-factor theory.

The first of these discrepancies was reported by Quick (1935) himself. He observed that, on storage, plasma develops a prolonged prothrombin time, suggesting a decay in prothrombin. This prolongation, however, could be corrected by the addition of fresh plasma, from a patient being treated with dicoumarin, which itself had a long prothrombin time. Quick, therefore, suggested that there is a labile factor which is destroyed by storage, and a stable factor, which is reduced by dicoumarin. He did not, however, suggest that there is an additional clotting factor, but that there are two components of prothrombin, A and B.

In 1947, Owren\(^{(2)}\) published the details of work first described in 1944 which gave the first clear indication of an additional
clotting factor. Owren was able to concentrate this factor from plasma and showed that, unlike prothrombin, it was relatively labile, and not adsorbed by inorganic gels. It was not present in normal serum. He called it factor V, being the fifth clotting factor.

In 1948, Owren and Bollman found that normal serum would accelerate the thrombin generation with thromboplastin by the plasma of patients being treated with dicoumarin. Since normal serum contains little or no prothrombin or factor V, this finding suggested that the serum was supplying something else which was deficient in the patient's plasma, in other words that dicoumarin produces a defect which is not simply prothrombin deficiency. Owren (1950) on the basis of studies of the accelerating effect of serum, postulated the existence of an additional factor which he named 'proconvertin'. This resembled prothrombin in being stable, adsorbed by inorganic gels, and reduced in vivo by dicoumarin drugs but unlike prothrombin, it was present in serum. Finally, Koller et al. (1951-52) made experiments on a substance which they called factor VII, the term factor VI having been applied by Owren to a hypothetical derivative of factor V. This factor VII seemed to be identical with Owren's 'proconvertin' and the numerical term was adopted in International Nomenclature for blood clotting factors.

In 1953, therefore, the theoretical position was uneasy, to say the least. Two new factors V and VII had to be wedged somehow into the classical theory. This maneuver was made possible by the reasonable supposition that they were accelerators of one of the basic reactions as shown on the following page.
The 'Four-Factor' theory expanded to accommodate factors V and VII (1953).

The apparent paradox lay in the findings that, though the coagulation time of haemophilic blood is normally grossly prolonged as measured in glass tubes by the Lee-White method, the prothrombin time by the one-stage method was normal. This implied that all the mechanism set in motion by added tissue thromboplastin which might be at fault in haemophilia was ruled out by the finding that haemophilic tissue extract was as active as normal as a source of thromboplastin both with haemophilic and with normal blood. Thus it came to be realized that normal plasma contains a factor which is lacking in haemophiliac blood and which did not correspond to any of the factors of the classical theory, or even, when they were subsequently discovered, to the additional factors V and VII.

Unlike factor V and VII, this plasma factor, which came to be known as 'antihaemophilic globulin' (AHG) could not, by any stretch of the imagination, be wedged into the existing classical
theory, since it seemed to play no part in the thromboplastin-prothrombin reaction, or later reactions.

Development of a clinical test, called the 'thromboplastin generation test' allowed a more detailed analysis and localization of clotting defects than had been possible before. By applying this test, deficiency in factor V or AHG and factor VII can be detected.

In 1952 on a series of patients it was found that their plasma component was normal and the serum abnormal. This finding would, at first, suggest a defect in factor VII, however, the finding of a normal one stage prothrombin time in these patients precluded this explanation. The inference was that another serum factor existed which was required for intrinsic thromboplastin generation, but not for prothrombin activation by tissue extract, and that a proportion of patients suffering from clinical haemophilia lacked not AHG, but this new serum factor. On the basis of naming this factor after the first patient whose investigation led to its discovery, it was called 'Christmas Factor'. Now it is also known as 'Plasma Thromboplastin Component' (PTC) and Factor IX.

Following identification of 'Christmas Factor', the investigation of puzzling or anomalous clotting defects gained impetus, and coagulation theory became loaded with additional factors, real or imaginary. In 1953, Rosenthal, Dreskin and Rosenthal described a haemorrhagic state which differed in its inheritance and in the nature of the defect both from haemophilia and Christmas disease. They postulated the deficiency of a factor which became known as plasma thromboplastin antecedent (PTA). In 1955, Ratnoff and Colopy described
a defect due to deficiency of a factor which normally initiates a series of changes in the blood following glass contact. These changes include activation of the clotting mechanism, and also of the kinin system. The factor concerned was called Hageman factor (XII) after the name of the first patient investigated. A strange and still inexplicable finding is the fact that patients with Hageman deficiency have little or no haemostatic dysfunction.

The next development of importance was the study of Tefler, Denson and Wright (1956) and by Hougie, Barrow and Graham (1957) of two patients whose plasma gave prolonged one-stage prothrombin times both with R.V.V. and with brain thromboplastin. Deficiency of factor VII would not account for the prolongation of the clotting time with venom, and prothrombin deficiency was excluded by two-stage assay. Moreover, the serum from these patients was defective in the thromboplastin test, but contained the normal amount of factor IX. The apparently inescapable conclusion was that yet another serum factor had been discovered. This was named the 'Stuart-Prower Factor' (X) after both patients. Like the other serum factors, and prothrombin, it was relatively stable, and adsorbed by inorganic precipitates.

The last new factor which needs to be mentioned here is also the last to operate in the clotting sequence. It was noticed by Robbins (1944) that plasma contains a factor which, in the presence of calcium, causes freshly formed fibrin to become insoluble in Urea, whereas without this factor it remains soluble. This factor, which was studied by Laki and Lorand (1948), Lorand (1951) and Lorand and Jacobson (1958) became known as the 'fibrin stabilizing factor'. Its action is to form
stronger bonds between the fibrin molecules, and its deficiency can cause a peculiar haemorrhagic diathesis.

In 1957, therefore, current coagulation theory which ten years before had admitted to four factors was now faced with the very probable existence of at least twelve. No coherent account could be given of the way in which most of them might interact. They might combine in various ways to form active derivatives, they might act indirectly by removing inhibitors; the possible combinations and permutations were endless.

Though the terminology, and even the identity, of 'thromboplastin' is still unresolved, a dramatic resolution took place in the terminological confusion which entangled all other clotting factors in addition to the classical four during the years 1957-59. An International Committee under the chairmanship of Dr. I. S. Wright was to decide on an acceptable and universal system of nomenclature. The final choice was the Roman Numerical System begun in effect, by Owen when he used the terms Factor V and Factor VI, and continued by Koller et al. with their use of the term Factor VII.
# Table 1

The Roman Numerical Nomenclature of blood clotting factors, together with some common synonyms.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Synonyms</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>II</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>III</td>
<td>Thromboplastin; tissue extract</td>
</tr>
<tr>
<td>IV</td>
<td>Calcium</td>
</tr>
<tr>
<td>V</td>
<td>Accelerator globulin, proaccelerin</td>
</tr>
<tr>
<td>VI</td>
<td>This number is not in use as it was found to be a hypothetical derivative of V</td>
</tr>
<tr>
<td>VII</td>
<td>Proconvertin; Autopthrombin I</td>
</tr>
<tr>
<td>VIII</td>
<td>Antihaemophilic factor; Antihaemophilic globulin (AHG)</td>
</tr>
<tr>
<td>IX</td>
<td>Plasma thromboplastin Component (PTC); Christmas Factor</td>
</tr>
<tr>
<td>X</td>
<td>Stuart-Prower Factor</td>
</tr>
<tr>
<td>XI</td>
<td>Plasma Thromboplastin Antecedent (PTA)</td>
</tr>
<tr>
<td>XII</td>
<td>Hageman Factor</td>
</tr>
<tr>
<td>XIII</td>
<td>Fibrin stabilizing factor</td>
</tr>
</tbody>
</table>
These changes had taken effect by 1963. The idea of an extrinsic (tissue activated) system and intrinsic (contact-activated) system each forming a final activator had become firmly established, and the factors involved in both could be set out in the new nomenclature with greater clarity (Fig. 1). This scheme which makes no statement about the way or even the order in which the factors may interact, shows that factors V and X are common to both systems and to these might be added phospholipid since this is known to be a component both of tissue thromboplastin and of the platelets.

Information on the way in which the factors of the extrinsic system might interact emerged later. Straub and Duckert (1961) found that factor VII behaves like an enzyme, whereas factor V and X and tissue extract seemed to behave like substrates. From this work, it appeared that an early reaction involving tissue factor, factors VII, X and calcium resulted in the formation of a product which then reacts with factor V to form prothrombin activator.

Further information came from a renewed study of the coagulant activity of Russell's viper venom (RVV). From the observations of a number of different workers, it was shown that the venom required the presence of factors V and X, phospholipid and ionized calcium in order to activate prothrombin. In a system containing venom, factors V and X, phospholipid and Ca²⁺ it was found that venom reacts with factor X to form a new product which then reacts with factor V and phospholipid to form prothrombin activator, the action of which is not affected by inactivation of the venom.
Fig. 1. The factors concerned in fibrin formation.
The observation that factors V and X and phospholipid were also required for the formation of the intrinsic prothrombin activator naturally prompted the speculation that factor X might be activated in a similar way by the various plasma factors concerned with the reactions following contact activation.

In mixtures containing factors VIII, IX, and X and 'contact product' (a mixture of factors XI and XII activated by surface contact) there was evidence that the amount of Xa formed and inversely, the amount of residual inactivated X remaining in the serum was determined by the amount of Factor VIII. With high factor VIII concentration, all of factor X could be activated, without any residual that could be further activated by R.V.V. It was supposed, therefore, that factor X was activated in a similar manner both by the intrinsic plasma system and by RVV or tissue thromboplastin. With normal levels of Factor VIII, however, this activation was incomplete, possibly because factor VIII is rapidly destroyed during clotting and before all the substrate factor X is activated. It was therefore tentatively suggested that three different activation systems for factor X had been demonstrated and that the common product was the enzyme Xa as follows:
The Theory of Seegers:

During the years in which the sequential theory has gradually crystallized from a mass of seemingly amorphous and disconnected parts, another view has been developing which at first seemed in direct opposition. This is the theory of Seegers and his many collaborators based on mainly biochemical studies and expressed in a specific terminology which has been sometimes difficult to relate to the numerical system. The central object of study by this group is prothrombin, and a most important contribution is the convincing demonstration that thrombin can be derived quantitatively from prothrombin without the incorporation of any other protein in its molecules. (Seegers 1962). Until quite recently, sequential reactions between clotting factors prior to the activation of prothrombin were denied. Prothrombin, it was suggested, had the inherent ability to form thrombin, and this was enhanced by a number of relatively non-specific 'procoagulants' including the
physiological tissue thromboplastin and platelet derivatives. The additional clotting factors VII, IX and X were thought by Seegers to have no separate identity in the plasma, but to be derivatives of prothrombin itself, and were called by him autoprothrombin I, II and III, respectively. This theory recognized that these prothrombin derivatives can enhance prothrombin activation and that their deficiency can cause defective clotting, but supposed that they did not take part in any reactions leading up to prothrombin activation; they only augmented it autocatalytically once it had begun. There was much to support this view of the origin of these factors, particularly the similarity in physical and chemical properties between prothrombin and factors VII, IX and X and the dependence on vitamin K for the synthesis of all of them.

In the past few years, the discovery of activated factor X has been reflected in Seegers theory by recognition of 'autoprothrombin C' which was identified as an enzyme capable of causing thrombin generation from prothrombin in the presence of Ac globulin (factor V) and phospholipid. Thus there is now agreement with the sequential theory on the existence of a reaction prior to activation by tissue thromboplastin of autoprothrombin III. The next development has brought the two theories even closer. Seegers now recognized an immediate precursor of thrombin, 'prothrombin' which is part of the prothrombin complex. This prothrombin complex also includes 'co-thromboplastin' (factor VII) renamed for autoprothrombin I. It is emphasized by him\(^4\) that there are three basic reactions in clotting, the formation of autoprothrombin C, the formation of thrombin, and the formation of fibrin. The activation of autoprothrombin III can be brought about by an extrinsic system, which includes
tissue thromboplastin and co-thromboplastin (factor VII) or by an intrinsic system which includes the platelets and platelet cofactor (factor VIII). This scheme is illustrated in Fig. 2. The factor IX of the cascade theory is not recognized in this scheme, which seems to give no clear explanation of the clotting defect in Christmas disease.

Notation:

- Autoprothrombin I = Factor VII
- Autoprothrombin II = Factor IX
- Autoprothrombin III = Factor X
- Autoprothrombin C = Activated factor X
- Ac Globulin = Factor V
- Co-thromboplastin = Factor VII
- Platelet Cofactor = Factor VIII

By a series of modifications on both sides these two theories, once apparently irreconciable, are approaching agreement on most essential points.

It is clear from both of these proposed blood clotting mechanisms that prothrombin conversion to thrombin is one of the most important and crucial reactions in blood clotting. Justifiably much of the research has been done on it in past recent years. The most acceptable molecular mechanism of prothrombin activation has come up mostly by work of two groups Mann et al. and Esmon et al. A brief review of this prothrombin activation mechanism will be given here.
Fig. 2. A blood coagulation scheme proposed by Seegers.
B. **Prothrombin Activation Mechanism:**

Complete activation of prothrombin by activated factor X, factor V, phospholipid and Ca\(^{++}\) results in the formation of thrombin and two activation fragments (Fragment 1 and Fragment 2 called by Owen\(^8\) and associates; Intermediate 3 and Intermediate 4 referred by Mann et al.\(^5,6\)) which cannot be activated to thrombin. Partial activation of prothrombin with activated factor X allows the isolation of two products which can be further activated to thrombin. These products are designated Intermediate 1 and Intermediate 2. Incubation of prothrombin with thrombin results in the formation of Intermediate 1 and Fragment 1 (Intermediate 3). Intermediate 1 activation requires two proteolytic cleavages. The first cleavage of Intermediate 1 by activated factor X results in the formulation of Intermediate 2 and Fragment 2 (Intermediate 4). The second cleavage by activated factor X forms the A and B chains of thrombin. The sequential formation of thrombin from prothrombin is shown diagrammatically.

![Schematic representation of prothrombin, fragments and α-thrombin.](image-url)
Regardless of the nature of the activator of Factor X (that is, via the intrinsic pathway through IXa or through the extrinsic pathway mediated by Factor VII - tissue factor) and regardless of whether the Factor X is activated to Factor Xa by snake venom or trypsin, these four fragments of prothrombin are produced during activation. Direct prothrombin activation by means of tiger snake venom or Taipan viper venom results in the production of the same fragments.

**Chemical Role of Lipids, Factor V and Calcium in Prothrombin Conversion**

The pioneering work of Seegers provided most of our present insight into the requirements for maximal stimulation of prothrombin activation. In the 1940's and 1950's, Seegers and associates clearly demonstrated that maximal rates for prothrombin activation could be achieved if Factor Xa (autoprothrombin C), Ca++, phospholipid (platelet factor 3), and Factor V (Ac-Globulin) were present in the activation mixture. Milstone demonstrated that factor Xa alone can activate prothrombin and that the other components serve to enhance the overall rate of the Factor Xa - catalyzed reaction. Papahadjopoulos and Hanahan subsequently demonstrated that Ca++ binding served as the 'glue' that maintained the integrity of the prothrombin-activating complex. These workers showed that Factor V and phospholipid form a complex in the absence of Ca++ but that the binding of Factor Xa to the complex is totally dependent on the presence of Ca++. Furthermore, Cole and associates demonstrated that the complexing of Factor Xa to phospholipid could occur in the absence of factor V but not in the absence of Ca++. The work of Stenflo and Carrot and Nelsestuen and Suttie has demonstrated that
prothrombin binds Ca\(^{++}\) and that, interestingly, this Ca\(^{++}\) binding ability appears to be absent in the abnormal prothrombin that is elaborated in the plasma of dicoumarol-treated animals. Extensive equilibrium dialysis studies in Mann et al.\(^7\) laboratory indicate that prothrombin has 10 or 11 Ca\(^{++}\) binding sites. These Ca\(^{++}\) binding sites are distributed in the 'pro' fragments that are not on the pathway to thrombin – namely, Fragment 1 (Intermediate-3) and Fragment 2 (Intermediate-4). Fragment 1 has six sites with a Log Kassoc of 3.7; Fragment 2 has four or five sites having a Log Kassoc of about 2.5. Intermediate 2, the COOH-terminal segment of the prothrombin molecule and the immediate precursor of thrombin, has no affinity for Ca\(^{++}\).

The significance of these Ca\(^{++}\)-binding sites can be seen in studies of the kinetics of activation of prothrombin and the two precursors, Intermediate-1 and Intermediate-2. The addition of Ca\(^{++}\) to a Factor Xa-mediated activation of prothrombin increases the rate of prothrombin activation by 40 fold. The addition of phospholipid to this activation mixture increases the rate another 2-1/2 times or to about 100 times the rate of prothrombin activation in the presence of Factor Xa alone. In contrast, activation of Intermediate 1 and Intermediate 2 in the presence of Ca\(^{++}\) or Ca\(^{++}\) and phospholipid resulted in no enhancement of the rate. Thus, the deletion of the Fragment 1 (Intermediate-3) segment of prothrombin molecule eliminates the efficacy of Ca\(^{++}\) and subsequently that of phospholipid in enhancing the rate of prothrombin activation. The results suggest that the Fragment 1 segment is the site of phospholipid binding via Ca\(^{++}\).

The implication of Fragment 1, the NH\(_2\)-terminal 'pro' fragment of prothrombin, as the segment of the molecule responsible for binding
it to the Ca\textsuperscript{++}-phospholipid matrix is further strengthened by the observation that Fragment 1 is a potent inhibitor of prothrombin activation in purified systems and also in such complex systems as those measured by the plasma clot time, the partial thromboplastin time, the prothrombin time and the two stage activation of prothrombin. Direct studies of phospholipid binding to Fragment 1 have been provided by Gitel and associates who showed that Fragment 1 binds phospholipid in the presence of Ca\textsuperscript{++}.

As far as correlation between physical and chemical properties of phospholipid and rate of prothrombin activation is concerned, no conclusive evidence is available. Work reported by Rouser and Schlored and by Wallach et al. in 1958 and 1959 established that the colloidal properties of phosphatidylethanolamine were important for the function of these lipids in blood clotting. Bangham demonstrated the importance of surface charge on the colloidal particles by correlating electrophoretic mobility with lipid thromboplastin activity. An extensive study of the relationship of lipid electrophoretic mobility and clot promoting activity by Papahadjopoulos et al. not only confirmed the relationship between surface charge and biological activity which Bangham had proposed, but also showed that an electrophoretic mobility-activity optimum exists. This optimum appeared to be independent of the chemical structure of the phospholipid. The explanation of this optimum electrophoretic mobility-activity relationship is still not known. The data of Papahadjopoulos et al. indicated further that some mixtures of phospholipids (at the optimum electrophoretic mobility) were more active per mole of phospholipid, suggesting further that neither charge alone nor surface charge density
could completely account for the lipid's ability to accelerate clotting. Daeman et al., using synthetic phospholipid, confirmed the conclusion of the previous investigators.

The significance of the weak Ca\textsuperscript{++}-binding sites present in Fragment 2 (Intermediate-4) can be seen in studies of complete prothrombinase activation of Intermediate-1 and Intermediate 2. If Factor V is added to the Ca\textsuperscript{++} phospholipid-Factor Xa system, enhancement of the rate of activation of Intermediate 1 is observed. In the absence of Ca\textsuperscript{++}, Factor V has no stimulatory influence on the rate of Intermediate 1 activation. The rate of activation of Intermediate 2 is not enhanced by the addition of Ca\textsuperscript{++}, Ca\textsuperscript{++}-phospholipid or Ca\textsuperscript{++}-phospholipid-Factor V. Thus, the sensitivity to Factor V acceleration is lost when Fragment 2 segment of the molecule which contains the weak Ca\textsuperscript{++}-binding sites is deleted.

Just as Fragment 1 is a potent inhibitor of the prothrombinase activation of prothrombin, Fragment 2 also shows the properties that suggest it may play a regulatory role in blood coagulation. This fragment binds to thrombin and accelerates the thrombin-catalyzed hydrolysis of synthetic substrates. Fragment 2 can be competitively displaced from thrombin by Intermediate 2, indicating that Fragment 2 can also form a complex with Intermediate 2. When Intermediate 2 is complexed with Fragment 2 (which contains the weak Ca\textsuperscript{++}-binding sites in prothrombin), the stimulatory influence of Factor V on the Factor Xa-Ca\textsuperscript{++}-phospholipid mediated activation is restored. These studies reinforce the hypothesis, suggested by the studies with Intermediate 1, that the weak Ca\textsuperscript{++}-binding sites on Fragment 2 play a prominent role in the binding of Factor V to this segment of the molecule.
C. **Light Scattering Phenomena:**

If one passes a beam of light through an aerosol or through a colloidal solution, one sees that a part of the light is scattered sideways. However, in a crystalline solid where individual particles are rigidly fixed in space relative to one another, there is destructive interference between light scattered from individual particles and there is no resultant light scattering. The reason for this is that all the particles in the crystal can always be paired off in such a way that the light paths from the two particles in each pair to an observer at any particular viewing angle $\theta$ will differ essentially exactly one-half wavelength.

Pure liquids are intermediate between crystals and gases. They are ordered but not completely ordered, and, as a result, they give rise to some scattering. The problem has been treated by the method of fluctuations by Smoluchowski. The principle of the method can be seen if we consider two small equal volume elements of liquid (dimensions $\ll \lambda$) separated by just the right distance so that the light paths to an observer differ by half a wavelength. If they were volume elements of a crystal, they would possess the same number of scattering particles and complete destruction of the scattered light would occur. In a liquid, however, the number of particles in each volume element is the same only when averaged over a period of time. At any given instant the partially random motion of the particles in the liquid gives rise to fluctuations in the density at any particular point so that the number of particles in one volume element differs
from that in the other volume element. There is thus an excess scattering of light from one element which is not cancelled by that from the other element.

In a solution of macro-molecules, because of fluctuations in concentration, light scattering is orders of magnitude higher than in pure solvents. Using Rayleigh formula for scattered light, a quantitative relation between scattered intensity and concentration fluctuation can be derived as given below.

For a particle much smaller than \( \lambda \), scattered intensity for a plane polarized incident light is given by Rayleigh's formula:

\[
\frac{i_s}{I_0} = \frac{16\pi 4\alpha^2 \sin^2 \theta}{\lambda^4 r^2}
\]  

(1)

To calculate the time average of \( i_s/I_0 \) for \( N \) volume elements per cubic centimeter, we need to consider fluctuation in polarizability due to concentration fluctuation. Let \( \bar{\alpha} \) and \( \bar{c} \) be the average polarizability and average concentration of macro-molecule solution and \( \delta \alpha \) and \( \delta c \) be the fluctuations in them respectively.

From equation (1), the light scattered from any one volume element at a particular instant is

\[
\frac{i_s}{I_0} = \frac{16\pi 4\bar{\alpha}^2 + 2\bar{\alpha} \delta \alpha + (\delta \alpha^2)}{\lambda^4 r^2} \sin^2 \theta
\]  

(2)
Now if we average out the right hand side for N volume elements per cubic centimeter Nα will be zero, and since the contribution of each element is $\alpha^2$ so it cancels out and we are left with $N\alpha^2$, hence equation becomes

$$\frac{i_s}{I_0} = \frac{16\pi^2 N \sin^2 \theta}{\lambda^4 r^2} \delta \alpha^2$$  \hspace{1cm} (3)

If we let temperature, pressure and solute concentration be the independent variables affecting α in a volume element, the fluctuation $\delta \alpha$ may be expressed in terms of fluctuations in these variables i.e.,

$$\delta \alpha = \left( \frac{\partial \alpha}{\partial P} \right)_{T,c} dP + \left( \frac{\partial \alpha}{\partial T} \right)_{P,c} dT + \left( \frac{\partial \alpha}{\partial c} \right)_{T,P} dc$$  \hspace{1cm} (4)

The first two terms on the right-hand side of this equation are assumed for a dilute solution to be the same as the corresponding terms leading to scattering from a pure solvent and are thus ignored.

We can express $\frac{\partial \alpha}{\partial c}$ in terms of the readily measurable quantity $\frac{\partial n}{\partial c}$ by the relation

$$\frac{\partial n}{\partial c} = 4\pi N \alpha$$

$$(\frac{\partial \alpha}{\partial c})_{T,P} = \frac{\tilde{n}}{2\pi N} (\frac{\partial \tilde{n}}{\partial c})_{T,P}$$  \hspace{1cm} (5)
From the relation (3), (4) and (5) we have,

\[
\frac{i_s}{I_o} = \frac{4\pi^2 \tilde{n}^2 (\frac{\partial \tilde{n}}{\partial c})^2 \sin^2 \theta}{\lambda^4 r^2 N (\delta c^2)}
\] (6)

From statistical thermodynamics considerations it can be shown that

\[
\delta c^2 = \frac{kT}{(\frac{\partial^2 F}{\partial c^2})_{T,P}}
\] (7)

where, \(k\), is Boltzmann constant
\(F\), is free energy of solution.

Further,

\[
(\frac{\partial^2 F}{\partial c^2})_{T,P} = \frac{1}{\overline{v}_1 N} \left( \frac{\partial \mu_1}{\partial c} \right)_{T,P}
\] (8)

where, \(\overline{v}_1\), is partial molar volume of solvent
\(\mu_1\), is chemical potential of solvent.

Combining (6), (7), and (8)

\[
\frac{i_s}{I_o} = \frac{4\pi^2 \tilde{n}^2 (\frac{\partial \tilde{n}}{\partial c})^2 \sin^2 \theta c}{\lambda^4 r^2 [-\frac{1}{\overline{v}_1 kT} (\frac{\partial \mu_1}{\partial c})_{T,P}]} \] (9)
The dependence of the chemical potential of solvent on the concentration of solute may be expressed as,

\[- \frac{1}{V_1 kT} \left( \frac{\partial \mu_1}{\partial c} \right)_{T,P} = N \left( \frac{1}{M} + 2Bc + 3Cc^2 + \ldots \right) \quad (10)\]

where \( N \) is Avogadro number, \( M \) is molecular weight of solute and \( B, C \) are virial coefficients.

Combining (9) and (10)

\[
\begin{align*}
\frac{i_\theta}{I_0} &= \frac{4\pi^2 n^2 \left( \frac{\partial n}{\partial c} \right)_{T,P} \sin^2 \theta \ c}{N' \lambda^2 r^2 (1/M + 2Bc + 3Cc^2 + \ldots)} \\
&\equiv \frac{r^2 i_\theta}{2I_0 \sin^2 \theta} \\
K &= 2\pi^2 n^2 \left( \frac{\partial n}{\partial c} \right)^2 / N' \lambda^4 = \text{optical constant}
\end{align*}
\]

Let \( R_\theta = \frac{r^2 i_\theta}{2I_0 \sin^2 \theta} = \text{Rayleigh Ratio} \)

and

\[
\frac{K_c}{R_\theta} = \frac{1}{M} + 2Bc + 3Cc^2 + \ldots
\]

By drawing Zimm Double Extrapolation plot, the molecular weight and virial coefficients can be determined. The case considered here is for small (dimension \( a \leq \frac{\lambda}{20} \)) isotropic particles, but if the particle dimension is larger than \( \lambda/20 \), shape factor \( P(\theta) \) comes in. If the particle is anisotropic dissymmetry measurements are needed to get some idea about anisotropicity. These conventional methods of light scattering provides static properties such as molecular weights, shapes and dimensions of macromolecules.
But with the advent of laser and consequent development of light beating spectroscopy technique, kinetic properties such as translational and rotational diffusion coefficients and electrophoretic mobility can be determined. The technique may be applied to molecules as small as 25Å and is more rapid than conventional light scattering techniques. The autocorrelation function, or its Fourier transform frequency spectrum, may be obtained in less than 10 minutes under favorable circumstances;

D. **Light Beating Spectroscopy** \(^{(13,14,15)}\)

Consider a large volume (filled with a solvent) which contains \(N\) identical scatterers. The volume is illuminated with a monochromatic plane wave of frequency \(w_0\) polarized perpendicular to the scattering plane, and light scattered at an angle \(\theta\) is observed at a distant point \(R_0\) (see Fig. 3).

The field observed at \(R_0\) due to jth scatterer will be,

\[
\bar{E}_j = A_j(t) \ e^{i\varphi_j} e^{-iwo} \tag{13}
\]

where the amplitude \(A_j\) may depend on the orientation of the scatterer. If we let the position of jth scatterer be \(\vec{r}_j\) and we choose the phase angle \(\varphi = 0\) for a scatterer at the origin, then

\[
\phi_j \approx (\vec{k}_o - \vec{k}_s) \cdot \vec{r}_j = \vec{k} \cdot \vec{r}_j \tag{14}
\]

If \(\nu c\), \(\vec{k}_o \approx \vec{k}_s\) so that

\[
|\vec{k}| = 2|k_o| \sin \frac{\theta}{2} = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2} \tag{15}
\]
FIG-3. GEOMETRY OF A LIGHT SCATTERING EXPERIMENT

\[ \overline{K} = \overline{K}_0 - \overline{K}_s \]
where $n$ is refractive index of the solvent. For the total scattered field at $R_0$ we have

\[ E_s = \sum_{j=1}^{N} E_j = \sum_{j=1}^{N} A_j(t) \exp\{i \vec{K} \cdot \vec{r}_j(t)\}\exp\{-i\omega_0 t\} \]

(16)

The average total scattered intensity is given by

\[ I_s = \langle |E_s|^2 \rangle \]

where the angular brackets denote a time average. Since the scatterers are not correlated, all cross terms average to zero, whence

\[ I_s = \langle \sum_j |A_j|^2 \rangle = N\langle |A|^2 \rangle \]

(17)

Before we proceed further we define first and second order correlation functions $g^{(1)}(\tau)$ and $g^{(2)}(\tau)$ for homodyning or self-beating spectroscopy technique.

When an electric field illuminates photocathode the probability per unit time of photoelectron emission by the field is

\[ W^{(1)}(t) = \sigma E^*(t) E(t) \]

(18)

where $E^*(t) E(t)$ is defined as instantaneous intensity $I(t)$ and $\sigma$ is defined as quantum efficiency.
The $r$ dependence has been suppressed in view of the assumed spatial coherence. The photoelectric current $i(t) = eW^{(1)}(t) = e\sigma E^*(t) E(t)$ while the joint probability that one photoelectron will be emitted at time $t$ (per unit time) and another at time $(t + \tau)$ (per unit time) is

$$W^{(2)}(t, t+\tau) = \sigma^2 E^*(t) E(t) E^*(t+\tau) E(t+\tau)$$

(19)

Averages of $i(t)$ and $W^{(2)}(t, t+\tau)$ will also be needed, which for stationary fields are:

$$<i(t)> = e <W^{(1)}(t)> = e\sigma <E^*(t) E(t)>$$

$$= e\sigma <I>$$

(20)

$$<W^{(2)}(t, t+\tau)> = \sigma^2 <E^*(t) E(t) E^*(t+\tau) E(t+\tau)>$$

$$= \sigma^2 <I>^2 g^{(2)}(\tau)$$

(21)

where,

$$g^{(2)}(\tau) = \frac{<E^*(t) E(t) E^*(t+\tau) E(t+\tau)>}{<E^*(t) E(t)>^2}$$

(22)

The power spectrum $P_i(w)$ of the photocurrent is given by Wiener-Khintchine theorem

$$P_i(w) = \frac{1}{2\pi} \int_{-\infty}^{\infty} e^{i\omega \tau} c_i(\tau) d\tau$$

(23)
where the current autocorrelation function is

\[ c_i(\tau) = \langle i(t) i(t+\tau) \rangle = e^2 \langle W^{(1)}(t) W^{(1)}(t+\tau) \rangle \] (24)

and \[ c_i(\tau) = c_i(-\tau). \]

For distributed spectra (spectra with non-zero bandwidth) centered at \( \omega = 0 \), \( P(\omega) \) in equation (23) is multiplied by 2 for \( \omega > 0 \) and \( P(\omega) \) is set equal to zero for \( \omega < 0 \).

Now the photocurrent \( i(t) \) consists of a series of discrete pulses which will be assumed to be infinitely narrow. Therefore \( c_i(\tau) \) has two distinct contributions:

If the electrons at \( t \) and \( t+\tau \) are distinct,

\[ W^{(1)}(t) W^{(1)}(t+\tau) = \langle W^{(2)}(t, t+\tau) \rangle \]
\[ = \sigma^2 \langle I^2 \rangle g^{(2)}(\tau) \]

while if the same electron occurs at \( t \) and \( t+\tau \),

\[ \langle W^{(1)}(t) W^{(1)}(t+\tau) \rangle = \langle W^{(1)}(t) \rangle \delta(\tau) = \sigma \langle I \rangle \delta(\tau) \]

Therefore,

\[ c_i(\tau) = e^2 \sigma \langle I \rangle \delta(\tau) + e^2 \sigma^2 \langle I^2 \rangle g^{(2)}(\tau) \]
\[ = e\langle I \rangle \delta(\tau) + \langle I^2 \rangle g^{(2)}(\tau) \] (25)
For monochromatic light scattered by a dilute solution of macromolecules, the case we are considering, the Gaussian statistics of the scattered field follows from the central limit theorem.

The field is characterized by an autocorrelation function

\[ c_E(\tau) = \langle E^*(t) E(t+\tau) \rangle = \langle I \rangle \ g^{(1)}(\tau) \]  

(26)

For random Gaussian fields, the second order correlation function \( g^{(2)}(\tau) = 1 + |g^{(1)}(\tau)|^2 \)

whence

\[ c_I(\tau) = \langle I \rangle \delta(\tau) + \langle I \rangle^2 (1 + |g^{(1)}(\tau)|^2) \]  

(28)

Using these concepts of autocorrelation function, the autocorrelation function of the scattered optical field from eq. (16) and eq. (26) is,

\[ c_E(\tau) = \sum_{j=1}^{N} A^*_j(t) \ [\exp{-iK \cdot r_j(t)}] |\exp{i\omega_0}]X \sum_{m=1}^{N} A_m(t+\tau)[\exp{iK \cdot r_m(t+\tau)}] |\exp{-i\omega_0(t+\tau)}]] > \]  

(29)

The statistical independence is invoked to eliminate the cross terms \( j \neq m \) i.e., the statistical independence of position and orientation to factor amplitudes and phases, and finally the fact that the \( N \) scatterers are identical so that each must have the same autocorrelation function. Thus eq. (29)
is reduced to,

\[
c_E(\tau) = N \exp(-i\omega_0 \tau) \left< A^*(t) A(t+\tau) \right> \times
\]

\[
\left< \left[ \exp \{-i\mathbf{K} \cdot \mathbf{r}(t) \} \right] \left[ \exp \{i\mathbf{K} \cdot \mathbf{r}(t+\tau) \} \right] \right>
\]

\[
= N \exp(-i\omega_0 \tau) c_A(\tau) c_\phi(\tau)
\]

(30)

where,

\[
c_A(\tau) = A^*(t) A(t+\tau)
\]

\[
c_\phi(\tau) = \left< \left[ \exp \{-i\mathbf{K} \cdot \mathbf{r}(t) \} \right] \left[ \exp \{i\mathbf{K} \cdot \mathbf{r}(t+\tau) \} \right] \right>
\]

For stabilized laser and isotropic scatterer

\[
c_A(\tau) = |A|^2
\]

(31)

The phase angle autocorrelation function can be computed by introduction of the self-part of the Van Hove's space-time correlation function \( G(\Delta \mathbf{r}_n, \tau) \) (16). This is the probability per unit volume that the center has moved \( \Delta \mathbf{r}_n \) during the time interval \( \tau \). For scatterers undergoing translational diffusion characterized by diffusion coefficient \( D_T \), this function is given by

\[
c_\phi(\tau) = \exp \{-D_T |\mathbf{K}|^2 \tau \}
\]

(32)
Combining (30), (31) and (32) the resulting equation is

\[ C_P(T) = N|A|^2 e^{-i\omega_0 \tau} e^{-D_T|K|^2/2} \tau \]

\[ = I e^{-i\omega_0 \tau} e^{-D_T|K|^2/2} \tau = I g^{(1)}(\tau) \quad (33) \]

where I is the total intensity.

Substituting for \( g^{(1)} \) in equation (28), the autocorrelation function of photocurrent can be obtained which is directly measured by a digital correlator.

\[ c_i(\tau) = \langle i \rangle \delta(\tau) + \langle i \rangle^2 (1 + e^{-2D_T|K|^2/2}) \quad (34) \]

as

\[ |g^{(1)}(\tau)| = |e^{-i\omega_0 \tau} e^{-D_T|K|^2/2} \tau| \]

\[ = e^{-D_T|K|^2/2} \tau \]

The photocurrent spectrum associated with this field is found by applying Wiener-Khintchin theorem to \( c_i(\tau) \),

\[ P_i(w) = \frac{e}{2\pi} \langle i \rangle + \langle i \rangle^2 \delta'(w) + \langle i \rangle^2 \frac{2 D_T|K|^2/\pi}{w^2 + 2 D_T|K|^2} \]

\[ (35) \]

The photocurrent autocorrelation (eq. (34)) and its Fourier transform spectrum (eq. (35)) contain three components. The first,
e\langle i \rangle \delta(\tau) or \frac{e\langle i \rangle}{\pi} is the usual shot noise term. The second, \langle i \rangle^2 or 
\langle i \rangle^2 \delta^\perp(\omega) is the d.c. component. The third term is exponential decay or 
Lorentzian of half width \Delta \omega_{1/2} = 2D|K|^2 and total power centered at 
\omega = \omega_0. The third component is the light beating spectrum.

In development of equations (34) and (35) a simplifying assumption that the scattered radiation field is spatially coherent over the area of 
the photodetector is made. If this assumption is relaxed the equations 
(34) and (35) are modified as follows:

\[ c_i(\tau) = e\langle i \rangle \delta(\tau) + \frac{\langle i \rangle^2}{N_A} (1 + e^{-\Gamma \tau}) \]  
(36)

where

\[ \Gamma = 2D_T|K|^2 \]

and

\[ P_i(\omega) = \frac{e\langle i \rangle}{2\pi} + \frac{\langle i \rangle^2}{N_A} \delta'(\omega) + \frac{2\langle i \rangle^2}{N_A} \frac{\Gamma/\pi}{\omega^2 + (\Gamma)^2} \]

where,

\[ N_A \approx \{ \begin{array}{l} 1 \quad A<<\lambda^2/\Omega \\
\text{Number of} \quad A/(\lambda^2/\Omega) \quad A>>\lambda^2/\Omega 
\end{array} \] 

Coherence Area)
E. Digital Clipped Correlator:

In recent years the methods of Doppler (heterodyne) and intensity fluctuation (homodyne) spectroscopy, well known in the radar field, have, with the advent of the laser, been applied successfully in the optical region of the spectrum. Most of these measurements have been made using a scanning electrical filter or analog correlator. But it is now possible to simplify the instrumentation, at a small cost in experimental time, by the use of clipped signals for forming the autocorrelation. Jakeman and Pike\(^{(17)}\) have recently shown that for Gaussian radiation sources the autocorrelation of a clipped signal is simply related to that of the unclipped signal as follows:

Let \( n \) be the number of photon counts, \( k \) the clipping level, and \( \tau \) the sample interval. If one channel is clipped so that

\[
\begin{align*}
n_k(\tau) &= 1 \quad \text{if} \quad n(\tau) > k \\
n_k(\tau) &= 0 \quad \text{if} \quad n(\tau) < k
\end{align*}
\]

and the resultant cross-correlated with the original signal, the clipped second-order correlation function is given by

\[
g_{\text{clipped}}^{2}(\tau) = \frac{\langle n(\tau) n_k(0) \rangle}{\langle n(0) n_k(0) \rangle} = 1 + \frac{1 + k}{1 + \langle n(0) \rangle} |g^{(1)}(\tau)|^2
\]

(38)
By comparison of equations (28) and (38) it can be seen that clipping at the mean count rate \( k = \bar{n} \) produces a form identical with the unclipped autocorrelation function. For low mean rate clipping may be performed at \( k = 0 \) as it is done in our laboratory.
3. EXPERIMENTAL

A. Instrumentation for Light Beating Spectroscopy

To make light scattering measurements the following instruments were used:

(i) **Monochromatic Light Source**: A spectra physics model 165 Ion Laser was used as a source of incident vertically polarized monochromatic light. A wavelength of 4579Å was used for these experiments at a power level of 140 mW - 180 mW.

(ii) **Projecting Optical System**: The laser beam was focused using a set of mirrors, apertures and a lens as shown in Fig. 4.

(iii) **Solution Cell and Temperature Control**: For making light scattering measurements on the protein solution, the sample needs to be confined in a container which is unavoidably placed in the path of the intense primary beam. The interface in the path of incident beam becomes a serious source of stray light. Moreover, for accurate and effective temperature control of the protein solution, we need to keep the cell in direct physical contact with a heat exchanging medium, the temperature of which is to be regulated constant over the desired temperature range. To minimize the stray light, a cell (0.4 cm X 1 cm X 4 cm) made of special optical glass (HELLMA CELLS INC.) was used in conjunction with an aperture. For temperature control a well-insulated cooling chamber along with LAUDA circulator (k 4 R model) was used. It can control temperature to ± 0.05°C. The temperature measurements were made with a Chromel-Alumel thermocouple using Leeds and Northrup model #8662 potentiometer (+ 0.05°C).
FIG-4. EXPERIMENTAL SETUP
(iv) **Receiving Optical System and Detecting Optical Design:** The scattered light was collected over a wide range of angles (20° - 90°) through a series of pin holes and impinged on the surface of a cooled ITT PW130 photomultiplier (PM). The dark count of the PM tube is approximately 1 count/sec at -20°C. A polarizer was used just before the receiving end of PM tube, to cut down excess intensity. The signal from PM tube was amplified and passed through a discriminator to shape the pulses while at the same time preserving the time displacement between them.

The whole set up was placed on the top of vibration-isolated table to avoid any kind of disturbance in our measurements.

(v) **Autocorrelator:** The photocurrent from discriminator was analyzed on a single clipped digital correlator which was built at Rice. It has a sample time interval range of $1 \times 10^{-7}$ sec - 5 sec and a duration range of $1 \times 10^0$ to $5 \times 10^{11}$ with increment factors of 1, 2 and 5. High sensitivity of this instrument allows use of low concentrated protein solution without much loss in accuracy. It has 60 channels for registering the correlation and separate special counters to measure total counts and clipped counts.

B. **Sample Preparation Technique**

The removal of small particles of extraneous matter and/or protein aggregate from the solvents and solutions required for scattered light measurements is a formidable experimental problem peculiar to this technique. The major source of uncertainty within a given set of data results from the practical difficulties in preparing the clear solution.
The aspects of this practical problem to which consideration should be given will be discussed under three main headings: (i) Preparation of the solution including the solvents and the cleaning of the apparatus, (ii) clarification of the solution for which there are two principal techniques, filtration and centrifugation, (iii) judgment of the contribution made to the scattered light by the presence of extraneous material. The avoidance of degradation of proteins (which are generally labile in character and prone to aggregation by small disturbances in the system such as pH change, heat, mechanical stress in filtering etc.\(^{(21)}\)) during the preparation and clarification is essential when the solution is to be representative of the system from which it was prepared.

(i) **Preparation of the protein solution and the cleaning of the apparatus**: Purification and characterization of human prothrombin was done in Dr. Walter Kisiel's laboratory\(^{(20)}\) in the University of Arizona College of Medicine and purified samples sent to us. Characterization of two lots of human prothrombin sent to us was as follows:

<table>
<thead>
<tr>
<th>Human Prothrombin Lot #1:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer:</td>
</tr>
<tr>
<td>Absorbancy at 280nM A(_{280}):</td>
</tr>
<tr>
<td>Concentration:</td>
</tr>
<tr>
<td>Activity:</td>
</tr>
<tr>
<td>Specific Activity:</td>
</tr>
<tr>
<td>Contaminating Factor X Activity:</td>
</tr>
<tr>
<td>Factor Xa activity</td>
</tr>
</tbody>
</table>
Assuming a Factor X specific activity of 100 ortho U/mg,
the Factor X Conc. is approximately: 0.20 µg/ml

**Human Prothrombin Lot #2:**

**Buffer:** 0.1M Tris - HAc/5mM Benzamidine-HCl (PH 6.0)

**Absorbancy at 280 nm280:** 6.62

**Concentration:** 5 mg/ml

**Activity:** 40 ortho U/ml

**Specific Activity:** 8.0 ortho U/mg

Activity of Factor X and its activated form is negligible.

The buffers used for the protein is on the basis of its stability, with respect to denaturation and degradation(21).

Taipan snake venom(TSV) was supplied by Dr. E. A. Natelson of Baylor College of Medicine. It was used in dilution of 1:200 in our activation study of prothrombin. Dihexanoyllecithin (DHL), phospholipid in micellar form, was obtained from Dr. J D. Morrisett's laboratory at Baylor College of Medicine. It was used in varying concentrations in activation study of prothrombin. Both proteins were quite pure and were dissolved in the same buffer human prothrombin was in. Both of these molecules are several times smaller than prothrombin in size.

The avoidance of contamination, as far as possible, during the solution preparation is necessary. Particles, greases and surfactants all interact with proteins so that once introduced they can seldom be removed without some effect on the solution. Particles of extraneous material adhere tenaciously to the walls of the cells used for the light
scattering measurements and these besides contaminating the solution, enhance the problems arising from stray light. Therefore, all the apparatus components were washed thoroughly using clean laboratory detergent solution. After that they are rinsed several times with tap water. Finally, they were rinsed at least 5 times using deionized distilled water and then dried with dry nitrogen gas. The scattering cell was rinsed with absolute pure ethyl alcohol before it was washed with detergent in order to remove protein particles from previous solution which might be adhering to the walls.

(ii) Clarification of the solution: Though the solution of protein sent to us was quite pure and essentially in monomer form, yet it contained too many large particles for our measurements. These particles were either small aggregates of protein or debris from other components of the material from which it was extracted. There are two principal means of solution clarification, namely filtration and centrifugation. The centrifugation technique is more cumbersome and time-consuming compared to membrane filter technique.

The membrane filtering technique was judiciously used in our laboratory to remove big and small extraneous particles and/or protein aggregates without causing any damage to protein solution. An early example was the use of this type of filter by Goring and Johnson (1958)\textsuperscript{18} to remove aggregates from protein solutions prior to scattered light measurements. They demonstrated the superiority of membrane filters over sintered glass filter or any other kind of filtration by examining the angular dependence of the light scattered by buffer solutions as well as their protein solutions. After membrane filtration the
normalized intensities of the scattered light from the protein solutions were independent of the angle of scatterer, whereas it increased sharply at low angles when other methods like sintered glass filtration had been used.

There exist now a number of commercially available suppliers of membrane filters (e.g., Gelman, Millipore, Sartorious, etc.). After experimenting with different kinds, Millipore filters with the appropriate filter holders were used for the best results in our laboratory. Human prothrombin solution was first prefiltered through 0.22 μ filter to remove relatively large particles without causing any mechanical stress on protein molecules. The 0.22 μ filtered solution was filtered through a 0.1 μ filter directly into a clean and dry cell.

A single microfiltration was not sufficient to guarantee clear protein solution in the sense of light scattering measurements. After filtration the solution was checked under microscope by shining the laser beam through it. If the laser beam in solution is fairly diffused without any shiny dots, the solution is good; otherwise it is filtered again until free of particles.

(iii) Extraneous Scattering: Whilst the fulfillment of the above requirement for a good solution is necessary, this in itself does not constitute proof of adequate clarification. There are some more guidelines to check the 'goodness' of the solution while taking actual measurements. Before taking photoelectron counts on autocorrelator, the photocurrent reading is taken on photometer. If variations in photocurrent are more than ± 10% and erratic there is a high probability that
the protein solution has big particles and/or protein aggregates in it. Special care is taken in analyzing the autocorrelation data and in plotting graphs. A typical correlation function was measured in 100 sec-500 sec at a clipping level $k = 0$. The data were analyzed by fitting to a single exponential between 2-3 correlation times ($\tau_c = 1/T$) using a least square two-parameter fit\(^{(19)}\).
4. RESULTS

(i) Diffusion Coefficients of Pure Human Prothrombin: The correlation time ($\tau_c$) on the samples of Lot #1 human prothrombin which were prepared by the technique discussed earlier, were taken as a function of scattering angle ($20^\circ$-$90^\circ$) to determine the particle size uniformity. For a solution of uniform particle size, a linear relationship exists between the decay time constant ($\Gamma = 1/\tau_c$) and the square of the scattering wave vector ($|K|^2$). The angle runs were taken on three different samples of human prothrombin. For one sample at 10°C vs $|K|^2$ plot was linear (see Fig. 5) and for other two samples at 20°C little aggregation was observed at low angles as clearly seen from Fig. 6(a) and Fig. 6(b). The translational diffusion coefficient was determined for all of these samples. The single clipped autocorrelation function obtained from human prothrombin decayed exponentially between 2-3 correlation time as seen in Fig. 7. The translational diffusion coefficient obtained for 22 different human prothrombin samples is tabulated in Table 2. The average value of the diffusion coefficient ($D_{20,w}^*$) was $4.72 \times 10^{-7}$ cm$^2$/sec with standard deviation of $\pm 0.23 \times 10^{-7}$ cm$^2$/sec. The relative error of our measurement was $\pm 5\%$ as compared to an uncertainty of 10% from Sedimentation Boundary Curves(24,25). The diffusion coefficient value ($4.72 \pm 0.23$) $\times 10^{-7}$ cm$^2$/sec of pure human prothrombin at a 2 mg/ml concentration agreed well with the diffusion coefficient ($D_{20,w}^*$) $4.80 \times 10^{-7}$ cm$^2$/sec for bovine prothrombin at the same concentration reported by Cox and Hanahan(22).

*See Appendix B
FIG-5. $\Gamma$ vs $1k^2$ for a sample of human prothrombin @~10°C
FIG-6(a). $\Gamma$ vs $1\bar{K}^2$ FOR A SAMPLE OF HUMAN PROTHROMBIN @~20°C
FIG-6(b). $\Gamma$ vs $I_{KI}^2$ for a sample of human prothrombin @ 20°C
FIG-7. CLIPPED AUTO CORRELATION FUNCTION FOR SCATTERED LIGHT FROM A UNIFORM SAMPLE OF HUMAN PROTHROMBIN

SAMPLE INCREMENT (τ) = 1 μSec
COHERENCE TIME (τ_c) = 18.000 μSec
TEMPERATURE (T) = 20° C
Table 2

Diffusion Coefficient Data on Human Prothrombin

<table>
<thead>
<tr>
<th>Date</th>
<th>Run No.</th>
<th>τ (μ sec)</th>
<th>τc (μ sec)</th>
<th>D_{20,w} (x 10^{-7} cm^{2}/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Jan., 27</td>
<td>1</td>
<td>0.5</td>
<td>11.160</td>
<td>4.6458</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>11.029</td>
<td>4.7010</td>
</tr>
<tr>
<td>(2) Feb., 5</td>
<td>1</td>
<td>0.5</td>
<td>14.135</td>
<td>4.6565</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>14.242</td>
<td>4.6215</td>
</tr>
<tr>
<td>(3) Feb., 14</td>
<td>1</td>
<td>0.5</td>
<td>18.826</td>
<td>4.3639</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>19.192</td>
<td>4.2807</td>
</tr>
<tr>
<td>(4) Feb., 17</td>
<td>1</td>
<td>0.5</td>
<td>16.869</td>
<td>4.8710</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>16.762</td>
<td>4.9024</td>
</tr>
<tr>
<td>(5) Feb., 26</td>
<td>1</td>
<td>0.5</td>
<td>14.524</td>
<td>4.7578</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>13.895</td>
<td>4.9733</td>
</tr>
<tr>
<td>(6) March, 4</td>
<td>1</td>
<td>0.5</td>
<td>14.590</td>
<td>4.7912</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>14.112</td>
<td>4.9535</td>
</tr>
<tr>
<td>(7) March, 11</td>
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<td>0.5</td>
<td>14.025</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>13.917</td>
<td>4.9923</td>
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<tr>
<td>(8) March, 14</td>
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<td>0.5</td>
<td>15.284</td>
<td>4.6023</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>15.415</td>
<td>4.5632</td>
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<td>(9) March, 16</td>
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<td>0.5</td>
<td>14.177</td>
<td>4.9308</td>
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<td></td>
<td></td>
<td>1.0</td>
<td>13.760</td>
<td>5.0802</td>
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<tr>
<td>(10) March, 17</td>
<td>1B</td>
<td>0.5</td>
<td>13.504</td>
<td>5.1460</td>
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<td></td>
<td></td>
<td>1.0</td>
<td>13.461</td>
<td>5.1625</td>
</tr>
<tr>
<td>(11) March, 31</td>
<td>1</td>
<td>0.5</td>
<td>13.765</td>
<td>4.9963</td>
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<td></td>
<td></td>
<td>1.0</td>
<td>14.112</td>
<td>4.8676</td>
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<tr>
<td>(12) April, 2</td>
<td>1</td>
<td>0.5</td>
<td>13.765</td>
<td>4.9306</td>
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<td></td>
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<td>1.0</td>
<td>13.678</td>
<td>4.9620</td>
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<tr>
<td>(13) April, 13</td>
<td>1</td>
<td>0.5</td>
<td>14.003</td>
<td>4.8452</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>13.548</td>
<td>5.0079</td>
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</table>
Table 2

<table>
<thead>
<tr>
<th>Date</th>
<th>Run No.</th>
<th>$\tau$ (µsec)</th>
<th>$\tau_c$ (µsec)</th>
<th>$D_{20,w}$ ($x 10^{-7}$ cm²/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>April, 20</td>
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<td>0.5</td>
<td>14,763</td>
<td>4.5970</td>
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<td>1.0</td>
<td>15,371</td>
<td>4.4151</td>
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<td>May, 2</td>
<td>1</td>
<td>0.5</td>
<td>15,999</td>
<td>4.2934</td>
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<td>1.0</td>
<td>15,675</td>
<td>4.3822</td>
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<td>May, 4</td>
<td>1</td>
<td>0.5</td>
<td>15,906</td>
<td>4.3186</td>
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<td></td>
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<td>1.0</td>
<td>15,284</td>
<td>4.4943</td>
</tr>
<tr>
<td>May, 7</td>
<td>1</td>
<td>0.5</td>
<td>14,915</td>
<td>4.6054</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>14,932</td>
<td>4.6003</td>
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<tr>
<td>May, 18</td>
<td>2</td>
<td>0.5</td>
<td>24,316</td>
<td>4.6603</td>
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<td></td>
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<td>1.0</td>
<td>24,577</td>
<td>4.6108</td>
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<td>May, 26</td>
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<td>0.5</td>
<td>20,442</td>
<td>4.6036</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>20,582</td>
<td>4.5723</td>
</tr>
<tr>
<td>June, 9</td>
<td>1</td>
<td>0.5</td>
<td>18,702</td>
<td>4.9439</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>19,543</td>
<td>4.7311</td>
</tr>
<tr>
<td>July, 7</td>
<td>1</td>
<td>0.5</td>
<td>17,553</td>
<td>4.6796</td>
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<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>17,994</td>
<td>4.5649</td>
</tr>
<tr>
<td>July, 21</td>
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<td>0.5</td>
<td>17,777</td>
<td>4.5724</td>
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<td></td>
<td></td>
<td>1.0</td>
<td>17,433</td>
<td>4.6626</td>
</tr>
</tbody>
</table>

$D_{20,w}$ average = $4.7241 \times 10^{-7}$ cm²/sec

Standard Deviation = ± $0.2328 \times 10^{-7}$ cm²/sec

$D_{20,w} = (4.72 \pm 0.23) \times 10^{-7}$ cm²/sec
The diffusion coefficient \((D_{20,w})\) for a sample of Lot #2 human prothrombin at 5 mg/ml was also determined. The diffusion coefficient \((D_{20,w})\) \(4.51 \times 10^{-7} \text{ cm}^2/\text{sec}\) at 5 mg/ml was close to the reported value \(4.60 \times 10^{-7} \text{ cm}^2/\text{sec}\)\(^{(22)}\). No appreciable increase in diffusion coefficient of Lot #1 human prothrombin was observed upon dilution (factor of 2). However, diffusion coefficient \((D_{20,w})\) values varying from \(5.6 \times 10^{-7} \text{ cm}^2/\text{sec}\) to \(6.2 \times 10^{-7} \text{ cm}^2/\text{sec}\) for bovine prothrombin at infinite dilution has been reported in the literature\(^{(22,33)}\). But, the solution in this technique could not be diluted lower than 1 mg/ml because the average counts in each channel of the autocorrelator became too low to measure the autocorrelation function with sufficient accuracy.

\[\text{(ii) Sedimentation Coefficients of Pure Human Prothrombin:}\]

The sedimentation velocity experiments on Lot #1 and Lot #2 human prothrombin prefILTERED through 0.22 \(\mu\) millipore filters were made by Dr. K. C. Aune of Baylor College of Medicine. Sedimentation studies were made at 20°C in 0.1M Tris-H\(_3\)PO\(_4\) (pH 6.0) and 0.1M Tris-HO\(_4\)A\(_5\)m Benzamidine - HCl (pH 6.0) buffer respectively. The sedimentation coefficients obtained were reduced to the viscosity of water at this temperature (see Appendix D). The average sedimentation coefficients \(S_{20,w}\) obtained for Lot #1 and Lot #2 human prothrombin were 5.37 S and 6.70 S, respectively. The measurements of diffusion coefficients and sedimentation coefficients can be combined\(^{(11)}\) to determine the molecular weight of prothrombin, if its partial specific volume \(\bar{v}_2\) is known. For the partial specific volume of 0.70 cc/gm\(^{(22,28)}\) molecular weights were calculated to be 92,000 and 120,000 for 2 mg/ml and 5 mg/ml concentration of human prothrombin. These results confirm the findings of Cox and Hanahan\(^{(22)}\) that molecular weight of prothrombin is
concentration dependent, which might be explained by partial aggregation of prothrombin at high concentration.

(iii) **Activation of Human Prothrombin with Taipan Snake Venom:**

For the first time the technique of light beating spectroscopy was used to study the conversion of prothrombin by monitoring the change of translational diffusion coefficient during its activation process. Since the intensity $I(t)$ of scattering per unit volume is proportional to the first power of the molecular weight ($M$) and particles concentration $c$, the reaction mixture components used for prothrombin activation had to be smaller in molecular size than the final product, thrombin. At counts per sample interval much less than 1 and at a clipping level of $k=0$, the single clipped autocorrelation function is approximately equal to $<I(t) I(t+\tau)>$. From this fact, it is immediately seen that the autocorrelation function is proportional to the second power of $M$ and $c$ and therefore very sensitive to the presence of big particles in the solution. To assure that the only component to the autocorrelation function was that arising from the conversion of prothrombin, all other reaction mixture components were verified by light scattering to give a negligible contribution to the autocorrelation function. To satisfy this requirement, nonphysiological activator taipan snake venom (oxyuranus scutellatus) and micellar phospholipid, Dihexanoyllecithin (DHL), were used. Both of them are several times smaller than thrombin in molecular size. Calcium chloride was used as a source of $Ca^{++}$ which completes the activation complex. In activation runs from 4 to 9 (see Table 3) optimum concentrations of
1:1800 diluted taipan snake venom and 100 μg/ml phospholipid were used as determined by Pirkle et al. The concentration of CaCl₂ was decided by the prothrombin precipitation threshold. The maximum concentration of CaCl₂ which could be used without prothrombin precipitation was 0.3 mM CaCl₂. All these concentrations are the final concentrations in the reaction mixture. In the first three runs (1 to 3) concentration of reaction mixture were not exact. All reaction mixture components were filtered through 0.22 μ and 0.1 μ before adding them to prothrombin sample. The runs were taken at different temperatures varying from approximately 9°C to 25°C. Temperatures greater than 25°C could not be used as aggregation was rapid.

Table 3 summarizes the diffusion coefficients of Lot #1 pure human prothrombin and its activated form with some pertinent remarks for nine different runs. For two temperatures T = 9°C and T = 15°C, rate of increase of diffusion coefficients were taken and plotted on linear scale (Fig. 8,10) and on log-log scale (Fig. 9,10). Increase in diffusion coefficient rate is related to the prothrombin activation rate. The criteria for complete conversion was taken to be no further change in the measured diffusion coefficient (Fig. 8). To verify that completely activated prothrombin product was formed, fibrinogen clotting test was performed as described later. The time given for the conversion in Table 3 is the approximate interval between the first and the last data taken prior to the reaction mixture becoming aggregated. The actual time for the complete conversion was observed to be between 3 and 5 days. The measured
FIG-8. $D_{20}, w$ vs $t$ for $T = 9\, ^\circ C$

SAMPLE OF MAY 19, '75
FIG-9. Log D vs Log t
SAMPLE OF MAY 19, '75

SLOPE = \frac{\log(0.74) - \log(0.72)}{\log(1.86) - \log(0.98)}

= \frac{0.011899}{0.278287}

= 4.276 \times 10^{-2}
FIG-10. $D_{2o,w}$ vs $t$ FOR $T = 15^\circ C$
SAMPLE OF MAY 26, '75
FIG-11. LOG(D_{20,w}) vs LOG(t) FOR T = 15°C
SAMPLE OF MAY 26, '75
Table 3

<table>
<thead>
<tr>
<th>Date</th>
<th>Diffusion Coefficients (Pure Human Prothrombin)</th>
<th>Diffusion Coefficients (Activated Human Prothrombin)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Feb. 17, 1975</td>
<td>4.8867</td>
<td>6.3541</td>
<td>Change took place in a week. Data taken @20°C and 25°C. Otherwise solution most of the time was in the refrigerator, @approx. 4°C.</td>
</tr>
<tr>
<td>(2) March, 17, 1975</td>
<td>5.1542</td>
<td>6.3705</td>
<td>Change took place in a week. Data taken @25°C. Otherwise most of the time solution was in the refrigerator, @approx. 4°C.</td>
</tr>
<tr>
<td>(3) May 4, 1975</td>
<td>4.4300</td>
<td>5.0292</td>
<td>Change in two days. Data taken @25°C. Solution aggregated when it was left @25°C overnight on May 6, 1975.</td>
</tr>
<tr>
<td>(4) May 13, 1975</td>
<td>4.9629</td>
<td>6.7680</td>
<td>Change took place in a week. Data taken @25°C and 9°C.</td>
</tr>
<tr>
<td>(5) May 19, 1975</td>
<td>5.1619</td>
<td>6.1235</td>
<td>Change took place in 8 days. Solution kept @9°C for eight days. Reaction rate data were taken on this sample.</td>
</tr>
<tr>
<td>(6) May 26, 1975</td>
<td>4.5244</td>
<td>5.8435</td>
<td>Change took place in 4 days. Solution kept @15°C for 4 days. Reaction rate data were taken.</td>
</tr>
<tr>
<td>(7) June 9, 1975</td>
<td>4.8964</td>
<td>5.7162</td>
<td>Change took place in 3 days. Solution was kept @15°C for 3 days.</td>
</tr>
<tr>
<td>(8) July 7, 1975</td>
<td>4.7215</td>
<td>5.3260</td>
<td>This change took place in a day. Data taken @20°C. Solution got aggregated when left @20°C.</td>
</tr>
<tr>
<td>(9) July 21, 1975</td>
<td>4.6175</td>
<td>4.9568</td>
<td>Change took place in a day. Solution aggregated when left @20°C.</td>
</tr>
</tbody>
</table>

Sample No. (1), (2), (4), (5) and (6) were completely activated prothrombin sample. These five data are used for standard deviation calculation.

(D_{20,w}) average = 6.2919 \times 10^{-7} \text{cm}^2/\text{sec}

Standard deviation = \pm 0.3414 \times 10^{-7} \text{cm}^2/\text{sec}

\therefore D_{20,w} = (6.29 \pm 0.34) \times 10^{-7} \text{cm}^2/\text{sec}
diffusion coefficient did not change for 2 to 4 days after that. Hence we can safely conclude that complete conversion of prothrombin had taken place. In the first four runs no attempt was made to determine the rate of prothrombin activation. The main concern at that time was whether data from this technique could be reproduced. When reproducible data for three different runs (1), (2) and (4) were obtained, an attempt was made to determine the activation rate of prothrombin. In two successful attempts (Runs 5 and 6) the rate of prothrombin activation was measured (Fig. 8 - 11) at 9°C and 15°C. The rate of prothrombin conversion was about 3.5 times faster at 15°C than at 9°C from the slope of log D vs log t plot. All attempts to determine conversion rate at higher temperature were futile, as prothrombin was very susceptible to aggregation.

The diffusion coefficient \(D_{20,w}\) of completely activated prothrombin from Table 3 was calculated to be \(6.29 \times 10^{-7} \text{ cm}^2/\text{sec}\) with the standard deviation of \(0.34 \times 10^{-7} \text{ cm}^2/\text{sec}\).

On a sample of Lot #2 human prothrombin the activation study was made using the same reaction mixture. It was found that diffusion coefficient \(D_{20,w}\) changed from \(4.51 \times 10^{-7} \text{ cm}^2/\text{sec}\) to \(5.30 \times 10^{-7} \text{ cm}^2/\text{sec}\) in less than an hour at 20°C. In less than 12 hours diffusion coefficient increased to \(5.41 \times 10^{-7} \text{ cm}^2/\text{sec}\). After that no further data could be taken on the solution on account of aggregation.

(iv) Fibrinogen Clotting Test: To detect thrombin activity a 3 mg/ml solution of fibrinogen was used as a substrate. The formation of the fibrin network was observed by a phase contrast microscope as follows.
On a clear slide were placed a drop of fibrinogen solution and a drop of the reaction mixture components and the time of formation of the fibrin network observed and recorded. Table 4 summarizes the result of a systematic study on four different slides. Taipan Snake Venom was found to be very slow activator by itself as reported by Pirkle et al.\(^{(31)}\). For all completely activated prothrombin samples the formation of a fibrin network was observed in less than 1 minute (limitation based on focusing of the slide).

Table 4

<table>
<thead>
<tr>
<th>Slide #</th>
<th>Fibrinogen Clotting Test</th>
<th>Fibrin Formation Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Fibrinogen + Prothrombin</td>
<td>No Clot</td>
<td></td>
</tr>
<tr>
<td>(b) Fibrinogen + Prothrombin + TSV + Ca(^{++})</td>
<td>Nothing seen in more than an hour.</td>
<td></td>
</tr>
<tr>
<td>(c) Fibrinogen + Prothrombin + TSV + Ca(^{++}) + Phospholipid (1 minute after mixing components)</td>
<td>Complete clot after 15-20 minutes.</td>
<td></td>
</tr>
<tr>
<td>(d) Fibrinogen + Completely Activated Prothrombin Sample (5-7 days later)</td>
<td>Immediate formation of network.</td>
<td></td>
</tr>
</tbody>
</table>

(v) **Molecular Weight From Sedimentation Equilibrium Method:** Two separate experiments were carried out by Dr. K. C. Aune of Baylor College of Medicine to measure directly the molecular weights of Lot #1 and Lot #2 human prothrombin. At sedimentation equilibrium, the logarithm of the fringe displacement, \(\ln f\), should be linearly related to the square of the
radial position $r^2$ for a homogeneous species according to the equation:

$$\ln f = \ln f_a + \frac{\bar{M}_w (1 - \bar{v}_2 \rho) \omega^2 (r^2 - r_a^2)}{2 \, RT}$$

where $\omega$ is the angular velocity, $R$ is the gas constant, $T$ is the temperature, and $\rho$ is the density of the solution. The slope of the linear plot leads to the determination of $\bar{M}_w$. For a sample of Lot #1 human prothrombin $\ln f$ vs $r^2$ plot was linear and the molecular weight from the slope was 69,000 ± 300. However, $\ln f$ vs $r^2$ plot was non-linear for a sample of Lot #2 human prothrombin indicating heterogeneity of the sample. Molecular weight for this sample was in the range of 70,000 to 140,000.
5. DISCUSSION

The results from all the various physical methods of characterizing human prothrombin that were employed in this study indicate that the prothrombin aggregates at high concentrations. The molecular weight of purified human prothrombin was reported by Kisiel et al.\(^{(20)}\) to be 72,000 ± 3,000. These measurements were made by analytical ultracentrifugation, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) and gel filtration on calibrated columns of Bio Gel P-200 on protein concentrations of 0.016 mg/ml to 0.80 mg/ml at pH 7.5. In a single sedimentation equilibrium experiment performed by Kisiel et al.\(^{(20)}\) to determine the influence of pH on the molecular weight, weight average molecular weight values of 115,000, 69,000 and 82,000 were obtained at pH 5, 7 and 9, respectively. The high molecular weight observed at pH 5 indicates considerable aggregation at this pH and most probably related to the increased sedimentation coefficient observed by Lanchantin et al.\(^{(23)}\) at low pH and low ionic strength. Molecular weights of our human prothrombin samples obtained from diffusion coefficient and sedimentation velocity experiments at pH 6.0 were 92,000 and 120,000 for 2 mg/ml and 5 mg/ml concentrations, respectively. Values obtained for the molecular weight were also determined directly from sedimentation equilibrium for each sample of Lot #1 and Lot #2 human prothrombin and values were found to be 69,000 and 70,000-140,000, respectively, at pH 6.0 and very low ionic strength.

Diffusion coefficient values from light beating spectroscopy are in agreement with the values reported by Cox and Hanahan\(^{(22)}\) and Lanchantin et al.\(^{(23)}\). But sedimentation coefficient values are higher than has been reported in the literature. Sedimentation coefficients
(S_{20,w}) determined in 0.1M NaCl, 0.001M trisodium citrate (pH 6.0) buffer by Cox et al.\(^{(22)}\) at 2 mg/ml and 5 mg/ml concentrations were 4.80 S and 5.20 S, respectively. Our values of sedimentation coefficient at the corresponding concentrations are 5.37 S and 6.70 S, respectively. However, sedimentation coefficients of human prothrombin measured by Lanchantin et al.\(^{(23)}\) at low ionic strength and pH range of 6.0 to 9.0 varied from 5.6 S to 10.0 S, which are comparable to our values.

An unusually long time of 3-5 days for the complete activation of Lot #1 human prothrombin via taipan snake venom, Ca\(^{++}\) and phospholipid has been observed in our study. In fibrinogen clotting test, an initial lag phase of 15-20 minutes was observed during which thrombin activity could not be detected (based on the absence of a fibrin network).

Somewhat similar behavior has been observed in Intermediate 1 activation with Factor Xa, Ca\(^{++}\) and phospholipid by Bajaj et al.\(^{(34)}\) and Kisiel\(^{(35)}\). Their reported initial lag phase times were 10-20 minutes. They also observed that the activation of Intermediate 1 by Factor Xa, Ca\(^{++}\) and phospholipid to form thrombin was an order of magnitude less than that of prothrombin activation for the same conditions. The same low reactivity of Intermediate 1 can, therefore, be expected when taipan snake venom is used as an activator. These findings suggest that prothrombin conversion to thrombogenic Intermediate 1 and inhibitory Fragment 1 (Intermediate 3) might have taken place because of some contaminating activated factor X (conc. \(\sim 10^{-5}\) ortho U/ml) and trace amount of thrombin in Lot #1 human prothrombin.

Other plausible reasons for such a low activation rate can be explored if our data are compared with that of Pirkle et al.\(^{(31)}\) who studied activation of human prothrombin via Taipan Snake Venom.
The optimum concentrations of the reaction mixture components for rapid conversion of prothrombin (< 1 hr.) determined by them were: 20 µg/ml prothrombin, at least 5 µg/ml Taipan Snake Venom, 25 to 250 µg/ml phospholipids (Asolectin, inositol and a mixture of the soya bean phosphatides of choline, ethanolamine) and at least 17.5 mM CaCl₂. The limiting effect of suboptimal amounts of CaCl₂ and phospholipid on the yield of thrombin was considerable. They found that if concentrations of CaCl₂ were as low as 0.3 mM used in our study (because of precipitation threshold of prothrombin) yields of thrombin after two hours of incubation with the reaction mixture at room temperature, were less than 30%. For a phospholipid concentration of 2.5 µg/ml, the yield of thrombin after two hours of incubation was less than 75%. Since all the reaction mixture components had to be smaller than the thrombin molecule, a micellar phospholipid, Dihexanoyllecithin had to be used in our study. The concentrations of the phospholipid were typically 100 µg/ml which fall within their optimal limits. The form of the phospholipid, however, could also affect the length of the activation time. The micellar phospholipid used has less surface area per unit mass compared to commonly used bilayer vescicles phospholipid (dia. ~ 250 Å), consequently less surface sites for activation\(^{(10)}\). The effect of suboptimal amount of Taipan Snake Venom on the final yield of thrombin was found to be negligible. However, the rate of activation is affected when TSV is present in an amount less than 5 µg/ml. In our study, the Taipan Snake Venom concentration in the final reaction mixture was approximately 2 µg/ml. The most interesting finding was the apparent inhibition of the activation rate by excess prothrombin (> 100 µg/ml). The prothrombin concentration of 1.33 mg/ml
(2/3 dilution because of reaction mixture) used will probably slow down the initial activation rate of prothrombin. Lastly, temperatures used in the activation study were much lower than physiological temperature (37°C) on account of prothrombin aggregation.

In Lot #2 human prothrombin, in contrast to Lot #1 human prothrombin, initial activation occurred rapidly. A change in diffusion coefficient of 18% was observed in less than an hour at 20°C. But complete conversion of prothrombin could not be studied as solution subsequently aggregated. This finding supports our hypothesis that trace amounts of thrombin (arising from Xa) in the prothrombin preparation will convert the prothrombin to Intermediate 1 and inhibitory Fragment 1, consequently slowing down the overall rate of prothrombin activation. Lot #2 human prothrombin preparation did not contain discernible levels of Factor Xa and thrombin.
6. CONCLUSIONS

The technique of light beating spectroscopy was successfully employed to measure the diffusion coefficient of human prothrombin and its activation via Taipan Snake Venom. The diffusion coefficient obtained from this technique had a relative error of ±5% as compared to an uncertainty of 10% from Sedimentation Boundary Curves\(^{(24,25)}\). The values of diffusion coefficient for purified human prothrombin measured from this technique at two concentrations were in agreement with the literature values\(^{(22)}\). Prothrombin aggregation at high concentrations was observed as seen by \(\Gamma \text{ vs } |\tilde{K}|^2\) plot and high molecular weights calculated from diffusion coefficients and sedimentation velocity experiments. Molecular weight dependence on concentration of prothrombin was in agreement with the observation of Cox and Hanahan\(^{(22)}\).

For the first time, the conversion of prothrombin in solution was observed by this technique. The diffusion coefficient values for completely activated prothrombin were reproducible. Because of limitations of our experiment very low activation rate of prothrombin was observed. The activated solution of prothrombin was also found to be highly aggregated. The diffusion coefficient values of thrombin observed at about 1.33 mg/ml was 30% less than the reported value of thrombin at infinite dilution \(D_{20,\text{w}} = 8.76 \times 10^{-7} \text{ cm}^2/\text{sec}\).
7. RECOMMENDATIONS FOR FUTURE WORK

The present study on human prothrombin characterization and its activation via taipan snake venom was performed at pH 6.0 and very low ionic strength. As a continuation of the work, effect of pH and ionic strength on aggregation behavior of human prothrombin can be studied in detail by light beating spectroscopy. Effect of pH and ionic strength on activation rate of human prothrombin will also be a useful information.

If this technique is used in conjunction with electrophoresis\(^{(36,37)}\), diffusion coefficients and electrophoretic mobilities of charged macromolecules can be measured simultaneously. Preliminary experiments on bovine serum albumin (BSA) performed by Ware and Flygare\(^{(37)}\) have determined both the electrophoretic mobility and diffusion coefficient within a standard deviation of 5%. The advantage of this technique is that it can resolve multicomponent system on spectrum analyzer, which was one of the shortcomings of our technique. It can also determine the chemical rate constants of dimerization\(^{(38)}\) and first order reactions of proteins. If it is applied to the prothrombin activation system, the proposed prothrombin activation mechanism can be verified. The main requirement of this technique is the suitable design of electrodes\(^{(39)}\) which will minimize heating, turbulence and bubbling near the electrodes.
REFERENCES


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APPENDIX A

Viscosity Measurements

The viscosity measurements were made on four different buffers to reduce diffusion coefficients and sedimentation coefficients to 'standard conditions', the 'standard' solvent being pure water at 20°C. Haake's falling ball viscometer was used to measure viscosity up to the uncertainty of ± 2%.

(1) 0.1M Tris-$H_3PO_4$ (pH 6.0) Buffer: First ball constant is determined using distilled water as a reference.

Falling time of the ball in distilled water at 24.95°C:

\[
\begin{align*}
 t_1 & : 92.2 \text{ secs} \\
 t_2 & : 91.8 \text{ secs} \\
 t_3 & : 92.0 \text{ secs} \\
 t_4 & : 92.2 \text{ secs} \\
 t_5 & : 92.2 \text{ secs} \\
\end{align*}
\]

\[ t_{av} = 92.08 \text{ secs} \]

Ball Constant = 0.006941

(i) Falling time of the ball in the buffer at 24.95°C:

\[
\begin{align*}
 t_1 & : 103.0 \text{ secs} \\
 t_2 & : 102.8 \text{ secs} \\
 t_3 & : 102.6 \text{ secs} \\
 t_4 & : 103.0 \text{ secs} \\
 t_5 & : 102.2 \text{ secs} \\
\end{align*}
\]
tav = 102.72 secs

Viscosity of the buffer at 24.95°C: 0.9745CP

(ii) Falling time of the ball in the buffer at 18.6°C:

\[ t_1 : 118.2 \text{ secs} \]
\[ t_2 : 118.8 \text{ secs} \]
\[ t_3 : 119.2 \text{ secs} \]
\[ t_4 : 119.0 \text{ secs} \]
\[ t_5 : 119.0 \text{ secs} \]

\[ \text{tav} = 118.84 \text{ secs} \]

Viscosity of the buffer at 18.6°C: 1.1275CP

(iii) Falling time of the ball in the buffer at 12.5°C:

\[ t_1 : 138.2 \text{ secs} \]
\[ t_2 : 137.6 \text{ secs} \]
\[ t_3 : 140.7 \text{ secs} \]
\[ t_4 : 138.8 \text{ secs} \]
\[ t_5 : 138.0 \text{ secs} \]

\[ \text{tav} = 138.66 \text{ secs} \]

Viscosity of the buffer at 12.5°C: 1.3155CP

(iv) Falling time of the ball in the buffer at 6.4°C:

\[ t_1 : 165.6 \text{ secs} \]
\[ t_2 : 165.8 \text{ secs} \]
\[ t_3 : 164.6 \text{ secs} \]
\[ t_4 : 165.8 \text{ secs} \]
\[ t_5 : 164.0 \text{ secs} \]
$t_{av} = 165.16 \text{ secs}$

Viscosity of the buffer at 6.4°C: 1.5669CP

Viscosity of 0.1M Tris-\(H_3PO_4\) (pH 6.0) buffer is plotted as a function of 1/T on semilog graph (see Fig. A-1).

(2) 0.1M Tris-HOAc/5mM Benzamidine-HCl (pH 6.0) Buffer: Viscosity calibration is first done using distilled water as a reference.

Falling time of the ball in distilled water at 6.3°C:

- $t_1 = 147.4 \text{ secs}$
- $t_2 = 147.6 \text{ secs}$
- $t_3 = 147.2 \text{ secs}$
- $t_4 = 147.8 \text{ secs}$
- $t_5 = 146.6 \text{ secs}$

$t_{av} = 147.32 \text{ secs}$

Ball Constant = 0.007112

(i) Falling time of the ball in the buffer at 6.2°C:

- $t_1 = 163.6 \text{ secs}$
- $t_2 = 163.8 \text{ secs}$
- $t_3 = 163.6 \text{ secs}$
- $t_4 = 164.8 \text{ secs}$
- $t_5 = 164.6 \text{ secs}$

$t_{av} = 164.08 \text{ secs}$

Viscosity of the buffer at 6.2°C: 1.6102CP
(ii) **Falling time of the ball in the buffer at 11.9°C:**

\[
\begin{align*}
t_1 & : 138.0 \text{ secs} \\
t_2 & : 137.8 \text{ secs} \\
t_3 & : 137.8 \text{ secs} \\
t_4 & : 137.4 \text{ secs} \\
t_5 & : 137.7 \text{ secs} \\
\text{tav} & = 137.74 \text{ secs}
\end{align*}
\]

Viscosity of the buffer at 11.9°C: 1.3559CP

(iii) **Falling time of the ball in the buffer at 18.4°C:**

\[
\begin{align*}
t_1 & : 115.0 \text{ secs} \\
t_2 & : 114.8 \text{ secs} \\
t_3 & : 115.2 \text{ secs} \\
t_4 & : 117.2 \text{ secs} \\
t_5 & : 115.2 \text{ secs} \\
\text{tav} & = 115.48 \text{ secs}
\end{align*}
\]

Viscosity of the buffer at 18.4°C: 1.1368CP

(iv) **Falling time of the ball in the buffer at 25.4°C:**

\[
\begin{align*}
t_1 & : 98.0 \text{ secs} \\
t_2 & : 98.4 \text{ secs} \\
t_3 & : 97.4 \text{ secs} \\
t_4 & : 97.2 \text{ secs} \\
t_5 & : 97.2 \text{ secs} \\
\text{tav} & = 97.64 \text{ secs}
\end{align*}
\]

Viscosity of the buffer at 25.4°C: 0.9612CP
Viscosity of this buffer is plotted as a function of 1/T on semilog graph paper (see Fig. A-2).

(3) **Buffer Mixture #1:** It is a mixture of 0.0025 M CaCl$_2$, 0.1 Tris-NaH$_2$PO$_4$ (pH 6.0) and 0.1M Tris-HOA/5mM Benzamidine-HCl (pH 6.0) in the ratios 1, 2 and 6, respectively.

**Falling time of the ball in distilled water at 6.3°C:**

\[
\begin{align*}
    t_1 & : 148.0 \text{ secs} \\
    t_2 & : 147.2 \text{ secs} \\
    t_3 & : 147.3 \text{ secs} \\
    t_4 & : 147.8 \text{ secs} \\
    t_5 & : 147.2 \text{ secs}
\end{align*}
\]

\[t_{av} = 147.5 \text{ secs}\]

**Ball Constant = 0.007104**

(1) **Falling time of the ball in the buffer mixture #1 at 6.25°C:**

\[
\begin{align*}
    t_1 & : 167.2 \text{ secs} \\
    t_2 & : 166.8 \text{ secs} \\
    t_3 & : 166.7 \text{ secs} \\
    t_4 & : 166.5 \text{ secs} \\
    t_5 & : 167.2 \text{ secs}
\end{align*}
\]

\[t_{av} = 166.88 \text{ secs}\]

**Viscosity of the buffer mixture #1 at 6.25°C: 1.6349CP**

(ii) **Falling time of the ball in the buffer mixture #1 at 12.7°C:**

\[
\begin{align*}
    t_1 & : 137.8 \text{ secs} \\
    t_2 & : 138.0 \text{ secs} \\
    t_3 & : 138.4 \text{ secs} \\
    t_4 & : 138.0 \text{ secs} \\
    t_5 & : 138.2 \text{ secs}
\end{align*}
\]
tav = 138.08 secs

Viscosity of the buffer mixture #1 at 12.7°C: 1.3528 CP

(iii) Falling time of the ball in the buffer mixture #1 at 19.1°C:

\[\begin{align*}
  t_1 & : 116.2 \text{ secs} \\
  t_2 & : 116.2 \text{ secs} \\
  t_3 & : 116.0 \text{ secs} \\
  t_4 & : 115.8 \text{ secs} \\
  t_5 & : 116.2 \text{ secs} \\
\end{align*}\]

\[tav = 116.08 \text{ secs}\]

Viscosity of the buffer mixture #1 at 19.1°C: 1.1372CP

(iv) Falling time of the ball in the buffer mixture #1 at 25.7°C:

\[\begin{align*}
  t_1 & : 99.2 \text{ secs} \\
  t_2 & : 98.4 \text{ secs} \\
  t_3 & : 98.8 \text{ secs} \\
  t_4 & : 98.6 \text{ secs} \\
  t_5 & : 98.7 \text{ secs} \\
\end{align*}\]

\[tav = 98.74 \text{ secs}\]

Viscosity of the buffer mixture #1 at 25.7°C: 0.9674CP

See Fig. A-3 for viscosity vs 1/T plot on semilog graph paper.

(4) Buffer Mixture #2: It is a mixture of 0.0025 M CaCl₂, 0.1 Tris-\(\text{NaH}_2\text{PO}_4\) (pH 6.0) and 0.1M Tris-H₃PO₄ (pH 6.0) in the ratios of 1, 2 and 6, respectively.

Falling time of the ball in distilled water at 6.5°C:

\[\begin{align*}
  t_1 & : 146.7 \text{ secs} \\
  t_2 & : 146.2 \text{ secs} \\
  t_3 & : 146.8 \text{ secs} \\
\end{align*}\]
t₄ : 146.8 secs  
\[ t₅ : 146.8 \text{ secs} \]
\[ \text{tav} = 146.52 \text{ secs} \]

Ball Constant = 0.007108

(i) **Falling time of the ball in buffer mixture #2 at 6.5°C:**

\[ t₁ : 162.3 \text{ secs} \]
\[ t₂ : 162.4 \text{ secs} \]
\[ t₃ : 162.0 \text{ secs} \]
\[ t₄ : 163.2 \text{ secs} \]
\[ t₅ : 162.6 \text{ secs} \]
\[ \text{tav} = 162.5 \text{ secs} \]

Viscosity of the buffer mixture #2 at 6.5°C: 1.5928CP

(ii) **Falling time of the ball in buffer mixture #2 at 12.4°C:**

\[ t₁ : 136.4 \text{ secs} \]
\[ t₂ : 136.6 \text{ secs} \]
\[ t₃ : 137.0 \text{ secs} \]
\[ t₄ : 137.0 \text{ secs} \]
\[ t₅ : 137.8 \text{ secs} \]
\[ \text{tav} = 136.9 \text{ secs} \]

Viscosity of the buffer mixture #2 at 12.4°C: 1.3425CP

(iii) **Falling time of the ball in buffer mixture #2 at 19.9°C:**

\[ t₁ : 111.6 \text{ secs} \]
\[ t₂ : 112.0 \text{ secs} \]
\[ t₃ : 111.6 \text{ secs} \]
\[ t_4 : 111.9 \text{ secs} \]
\[ t_5 : 112.0 \text{ secs} \]

\[ \text{tav} = 111.82 \text{ secs} \]

Viscosity of the buffer mixture \#2 at 19.9°C: 1.0961CP

Fig. A-4 is the plot of viscosity vs $1/T$ of this buffer mixture on semilog graph paper.
FIG-A1. VISCOSITY OF 0.1 M Tris-H₃PO₄ (pH 6.0) BUFFER (η) vs 1/T
**FIG-A2. VISCOITY OF 0.1M Tris-HOAc 5 mM BENZAMIDINE HCl (pH 6.0) BUFFER ($\eta$) vs $1/T$**
FIG - A3. VISCOSITY OF BUFFER MIXTURE #1 vs 1/T

BUFFER MIXTURE #1

10 cc. 0.0025 M CaCl₂
20 cc. 0.1M Tris - NaH₂PO₄ (pH 6.0)
60 cc. 0.1M Tris HOAc
5 mM Benzamidine HCl (pH 6.0)
BUFFER MIXTURE #2

10 cc. 0.0025 M Ca Cl$_2$

20 cc. 0.1 M Tris-Na$_2$H$_2$PO$_4$ (pH 6.0)

60 cc. 0.1 M Tris-H$_3$PO$_4$ (pH 6.0)

FIG-A4. VISCOSITY OF BUFFER MIXTURE #2 vs 1/T
Sample Calculations for Diffusion Coefficient $D_{20,w}$

A single clipped autocorrelation function ($<n(\tau) n_k(0)>$) for appropriate sample interval and duration was directly taken down from the autocorrelator on a data sheet. A sample of data for pure human prothrombin is shown in Table B-1. Background counts (also known as shot noise counts) were calculated by multiplication of average number of counts per sample interval, average number of clipped counts per sample interval and duration of the experiment. These background counts were corrected for the error in the digital correlator. The background corrections were made using white light bulb (see Appendix C for details). The corrected background counts ($<n(o)> < n_k(o)$) were substracted from clipped autocorrelation function. The resultant function ($<n(\tau) n_k(o) > - <n(o)> <n_k(o)> )$ was plotted which should decay exponentially (from eq. 38) for uniform particles solution as seen in Fig. B-1. Inverse of the slope gives coherence time $\tau_c$ which is related to translational diffusion coefficient $D$ and scattering vector $|\vec{K}|$ by the following equation:

$$\tau_c = \frac{1}{2D|\vec{K}|^2}$$

From equation (15)

$$|\vec{K}| = \frac{4\pi n}{\lambda} \sin \frac{\theta}{2}$$
For $\theta = 90^\circ$

$n = 1.333$ (for dilute solution of protein refractive index is the same as that of water)

$\lambda = 4579\text{Å}$

$|K| = 2.58676 \times 10^5 \text{ cm}^{-1}$

$|\overline{K}| = 6.691365 \times 10^{10} \text{ cm}^{-2}$

For data shown in Table B-1, the diffusion coefficient of human prothrombin at 26.1°C

\[
D = \frac{1}{2|\overline{K}|} \frac{1}{\tau_c}
\]

\[
= \frac{1}{2 \times 6.691365 \times 10^{10} \times 14.9370 \times 10^{-6}} \text{ cm}^2/\text{sec}
\]

\[
= 5.0042 \times 10^{-7} \text{ cm}^2/\text{sec}
\]

D represents the diffusion coefficient measured at temperature T in 0.1M Tris-H$_3$PO$_4$ (pH 6.0) buffer of viscosity $\eta$. Let $\eta_s$ be the viscosity of 'standard' solvent and $T_s$ be the 'standard' temperature, the 'standard' diffusion coefficient $D_s$ can be determined using Stokes-Einstein relation.

\[
D = \frac{kT}{3\pi\eta_d \eta}
\]

\[
D_s = \frac{k T_s}{3\pi\eta_s \eta_s}
\]
from these two relations,

\[
\frac{D_S}{D} = \frac{T_S}{T} \cdot \frac{\eta}{\eta_s}
\]

\[
\therefore D_S = \frac{T_S}{T} \cdot \frac{\eta}{\eta_s} \quad (40)
\]

Conventionally, 'standard' temperature \(T_s\) is 293.15 K and 'standard' solvent is water, therefore, \(\eta_s\) is 1.00203 CP.

From fig. A-1, \(\eta\) at \(T = 299.23\ K\) is 0.94 CP.

\[
\therefore D_{20, w} = \frac{293.13}{299.23} \times \frac{0.94}{1.00203} \times 5.0042 \times 10^{-7} \text{cm}^2/\text{sec}
\]

\[
= 4.6003 \times 10^{-7} \text{cm}^2/\text{sec}
\]
Run #1  

A sample of human prothrombin prepared by filtering through 0.22 μ and 0.10μ filter paper. Looked under microscope, it had about 3-4 tiny particles in the field of view, i.e. solution is good.

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<td>: 90°</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>65838</td>
<td>46</td>
<td>Wavelength</td>
<td>: 4579 Å</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>65049</td>
<td>47</td>
<td>Index of Refraction n</td>
<td>: 1.333</td>
<td></td>
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<tr>
<td>16</td>
<td>64048</td>
<td>48</td>
<td>Sample Temperature</td>
<td>: 1.044 mv</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>63008</td>
<td>49</td>
<td>Laser Power</td>
<td>: 180 mw</td>
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</tr>
<tr>
<td>18</td>
<td>62394</td>
<td>50</td>
<td>k = 0</td>
<td></td>
<td></td>
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<tr>
<td>19</td>
<td>62074</td>
<td>51</td>
<td>S/N at τ = 1.726</td>
<td></td>
<td></td>
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<tr>
<td>20</td>
<td>60742</td>
<td>52</td>
<td>Dark Current Counts</td>
<td>: 1.275 counts/sec</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>60518</td>
<td>53</td>
<td>Photo Current i</td>
<td>: 1.26 x 10^-8 amps</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>59805</td>
<td>54</td>
<td></td>
<td></td>
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<td>31</td>
<td>56511</td>
<td>63</td>
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</table>
SAMPLE INCREMENT ($\tau$) = 1 $\mu$Sec
COHERENCE TIME ($\tau_c$) = 14.937 $\mu$Sec
TEMPERATURE ($T$) = 26.1 °C

DATE: MAY 7, '75
RUN NO. # 1

FIG.-B.1. CLIPPED AUTO CORRELATION FUNCTION FOR SCATTERED LIGHT FROM A UNIFORM SAMPLE OF HUMAN PROTHROMBIN.
APPENDIX C

Background Correction

The autocorrelation of the photocurrent contains the desired autocorrelation plus a white shot noise background. The shot noise level is calculated from the total number of counts and clipped counts. Ideally, it should give the correct shot noise which is also known as background. But, in practice, for low sample time of 0.5 μsec and 1.0 μsec the background calculated this way was always less than the actual background. The correction in background was made using white light bulb (noise source). The intensity of the light bulb was adjusted to produce an average photocurrent \(<i>\) equal to that which was produced by the sample. The autocorrelation taken for white light bulb is essentially shot noise\(^{(13)}\), therefore, the light bulb effectively serves as a white noise generator. Background correction graph have been plotted as a function of average number of counts per sample interval for \(t_b\) sample interval of 0.5 μsec and 1.0 μsec. (Fig. C-1 and Fig. C-2).
FIG-C1. BACKGROUND CORRECTION FACTOR vs $\langle \eta(0) \rangle$
FOR SAMPLE INTERVAL OF 0.5 Sec
FIG-C.2. BACKGROUND CORRECTION FACTOR vs $\langle \eta(0) \rangle$

FOR SAMPLE INTERVAL OF 1 $\mu$Sec
APPENDIX D

Sedimentation Coefficient Correction for a Buffer

The sedimentation coefficients of protein, like diffusion coefficient, depend on the properties of the solvent and are normally reported as reduced to 'standard' conditions. For dilute protein solution sedimentation coefficient $s$, diffusion coefficient $D$ and molecular weight of protein are related by

$$ M = \frac{S \Omega T}{D(1 - \frac{\nu_2}{\nu_2})} $$

(41)

$\rho_0$ is the density of the solvent.

By using equation (40) and (41), 'standard' sedimentation coefficient $S_s$ can be computed,

$$ S_s = \frac{S\eta (1 - \frac{\nu_2}{\nu_2})}{\eta_s(1 - \frac{\nu_2}{\nu_2})} $$

(42)

where symbols without subscripts refer to the solvent in which measurements were made and the subscript $s$ refers to the standard solvent.

If the values $\eta_s$ and $(\rho_0)_s$ are those of water at 20°C the 'standard' sedimentation coefficient $S_{20,w}$ is obtained.

At 20°C for a sample of human prothrombin in 0.1M Tris-$\text{H}_3\text{PO}_4$ (pH 6.0) buffer $S = 4.73 \times 10^{-13}$ sec.
At 20°C $\eta = 1.09$ CP

$\bar{v}_2 = 0.700$ c.c./gm

$(\rho_o)_{s} = 0.9982$ gm/c.c.

$\eta_s = 1.00203$ CP

$\rho_o = 1.0078$ gm/c.c.

$S_{20,w} = \frac{4.73 \times 1.09 \times (1 - 0.7 \times 0.9982)}{1.00203 \times (1 - 0.7 \times 1.0078)} \times 10^{-13}$ sec

= $5.2626 \times 10^{-13}$ sec