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VITAMIN K AND PROTHROMBIN

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CHAPTER I

INTRODUCTION

The nutrition and metabolism of vitamin K have been investigated extensively in the last twenty-five years, and the major emphasis of most of these investigations has been with the vitamin K requirement for haemostasis. The original observations relating vitamin K and blood clotting (1) came from studies of chicks raised on a fat free diet which developed superficial and internal bleeding. The search for a lipid substance in the diet capable of reversing these hemorrhagic symptoms led to the isolation and characterization of the naturally occurring forms of vitamin K. When the original observations were made there was no knowledge of the type of alteration in blood clotting responsible for the observed hemorrhagic tendencies. The view persisted for some time (2) that an inhibitor of the clotting process was present in the plasma of vitamin K deficient animals.

A definitive experiment to distinguish between lowered levels of clotting factors and the presence of inhibitors of clotting (3) demonstrated that plasma from deficient animals clotted normally when a prothrombin preparation was added, but active prothrombin could not be obtained from plasma of deficient animals. This evidence supported the

idea that a dietary deficiency of vitamin K lowered the levels of clotting factors in plasma.

Hemorrhagic tendencies correlated with obstructive jaundice and experimental bilary fistulas were recognized to result from impairment of absorption of the vitamin (4) and symptoms disappeared when vitamin K was given by injection. Since these clotting changes in birds and mammals were readily corrected by administration of vitamin K it was proposed (5) that the lack of prothrombin is the only immediate result of vitamin K deficiency and hence the sole cause of bleeding in the deficient state.

With the advent of the coumarol series of vitamin K antagonists, and, as knowledge of the process of blood clotting increased, it became apparent that vitamin K is involved in factors affecting prothombin activation as well as in prothrombin formation (6). Tests for vitamin K dependency of new blood clotting factors have been applied as these new factors have been characterized (7) and the so called "prothrombin group" of factors derive their name from a common sensitivity to vitamin K antagonists. Thus, according to the system of nomenclature adopted by the International Committee for Haemostasis and Thrombosis, factors II, X, IX and VII are all dependent on adequate amounts of dietary vitamin K.

Of these four factors only factor II (prothrombin) has been purified to the extent of satisfying physicochemical criteria of homogeneity (8). Some controversy exists in the literature as to the independent existence of these factors (9), or their common origin as intermediates produced during, and mediating the autocatalytic activation of

prothrombin (10). However, the enigma of vitamin K action remains whether the factors affected be one, or four, in number.

One of the earlier hypotheses to arise as to the effect of vitamin K on clotting activity (11) proposed that a possible function could include the vitamin as a prosthetic group on the prothrombin molecule. The more recent evidence for vitamin K mediated control of the levels of four independent clotting factors makes this hypothesis seem less probable. Also it is known that a thorough investigation of prothrombin using both chemical and biological assays (12) fails to reveal any evidence for the presence of vitamin K.

A more recent idea put forth to explain the action of vitamin K (13) proposes an involvement of napthoquinones in coupling electron transport and oxidative phosphorylation. This theory proposes that the "prothrombin group" of clotting factors have a very short half-life in vivo, and the first manifestations of a decrease in the efficiency of energy production could be seen as a lowered level of these factors. Inherent in this model are the following premises: (1) in vitro studies would be expected to show decreased P/O ratios in mitochondrial preparations from deficient animals, (2) protein synthesis in these animals would be expected to be proceeding at a decreased rate as compared to controls.

Martius reported a decrease of one-third in P/O ratios of mitochondria isolated from the liver of chicks deficient in vitamin K. Subsequent investigators (14) were not able to detect this decrease in P/O ratio, and it is generally accepted now that some vitamin K antagonists do exist which have no effect on mitochrondrial respiration, and a lack

of vitamin K does not alter this respiration. Failure to demonstrate any effect of vitamin K deficiency on *in vivo* incorporation of labelled ^{14}C amino acids into protein (15) or the dietary induction of tryptophan pyrrolase indicates a lack of any general effect of vitamin K on protein metabolism.

Since all the information seems to indicate that vitamin K specifically affects only the prothrombin group of proteins, attention has been focused on the individual steps in protein synthesis where control mechanisms could be expected to function. The proposal of the "Operon Hypothesis" (16) suggested new ways in which the vitamin could regulate the synthesis of one or several specific protein molecules by a derepressor-like function. The availability of antibiotics which specifically block individual steps in protein synthesis provided new ways of studying the synthesis of prothrombin which occurs when vitamin K is given to deficient or dicoumarol treated animals. The first experiments to be reported using these new methods (17) showed that it was possible to give vitamin K deficient chicks a large enough dose of actinomycin D to prevent the normal recovery of prothrombin levels observed after menadione administration, when measurements of prothrombin level were made 6 hours after the administration of actinomycin D.

It is readily apparent that information from control animals administered equivalent doses of actinomycin D must be obtained in order to interpret these results. The biological half-life of the individual proteins, the half-life of the message RNA coding for the synthesis of the proteins, and the particular clotting factors measured in the assay procedure could all influence the final result.

At the time this experiment was carried out there was no information available on the half-life of prothrombin in the chick. Data on the prothrombin group obtained from human subjects treated with the vitamin K antagonist, 4-hydroxy coumarin (7) indicate half-lives of 60, 5, 30 and 45 hours for factors II, VII, IX and X respectively. It is known, however, that coumarol derivatives and vitamin K seem to compete with each other, and certainly it is not practical to give human subjects large enough doses of vitamin K antagonists to completely block prothrombin synthesis even if such a treatment is possible. Probably a better means of estimating the half-life of clotting factors (18) involves measuring the disappearance of prothrombin administered to hypoprothrombinemic patients. In these conditions a rapid turnover corresponding to a nine hour half-life was observed.

When rats were given doses of actinomycin D sufficient to block prothrombin synthesis in vivo (19) a rapid drop in blood prothrombin level was observed, and the half-life appears to lie in the range of five to eight hours. Interpretation of these data is rendered difficult due to the necessity for considering the half-life of the message RNA involved - therefore, the estimates probably represent a maximum half-life.

If both prothrombin and its message RNA turn over quite rapidly any synthesis of prothrombin occurring in the presence of actinomycin D must be measured before pre-existing message decays. Data obtained from this type of experiment indicate that vitamin K-dependent induction of prothrombin synthesis does occur in actinomycin D treated animals. The absence of any in vitro effects of vitamin K on the clotting ability of

plasma obtained from vitamin K-deficient animals, and other lines of evidence (20) indicate that vitamin K-dependent clotting factor activity requires de novo clotting factor synthesis. This view has been substantiated by experiments using puromycin (21) which show insignificant amounts of prothrombin synthesis after giving vitamin K to deficient, puromycin-treated animals. In vitro studies on synthesis of another member of the prothrombin group using liver slice techniques (22) support the in vivo findings.

It seems fairly clear that protein synthesis is required in order to obtain a response to vitamin K. Now the question must be asked, "Is vitamin K required for the specific de novo incorporation of amino acids into a polypeptide ultimately destined to become a member of the prothrombin group?" We must have an answer to this question if we are to produce a precise operational model for the control of synthesis of specific proteins. It should be noted that the requirement for protein synthesis in the production of prothrombin activity cannot be taken as evidence for a direct role of vitamin K in controlling the synthesis of the prothrombin group. Several other explanations are possible, and a few of the more plausible ones are considered below.

All members of the prothrombin group are probably glycoproteins. Vitamin K could have a role in the synthesis or attachment of a carbohydrate moiety required for enzymic and immunological activity. There is little evidence in the literature to support such a view. Although purified prothrombin is reported to contain 5 to 6% neuraminic acid by weight (23), complete removal of the neuraminic acid by treatment with clostridial neuraminidase was accompanied by only a 30% decrease in

prothrombin activity. It was proposed that this inhibition could be due to residual proteolytic activity known to exist in the neuraminidase preparation. These data indicate that enzyme activity which depends on carbohydrate moieties would have to depend on sugar residues other than, and independent of attachment to neuraminic acid.

Vitamin K could act in joining subunits together such that the proper quaternary structure required for enzymic activity is produced. It is known that dogs treated with clinical doses of dicoumarol (24) do not possess significant amounts of cross-reacting material to prothrombin antibody. If prothrombin were composed of immunologically unreactive subunits, then these could be present in plasma lacking prothrombin (25), and their proper assembly could involve alignment requiring vitamin K, or an enzyme containing vitamin K as a prosthetic group. Prothrombin preparations have been reported (26) with a mean of sixteen disulfide bonds per molecule. The degree of complexity present in such a system could necessitate an enzyme which catalyzes correct pairing. Vitamin K is known to complex with free sulfhydryl groups (27). The most powerful objection to this hypothesis is the failure of vitamin K to produce prothrombin activity *in vitro* in plasma from deficient animals which lacks this activity. Data now in press (28) reinforce the idea that neither free vitamin K nor any protein bound form of the vitamin occurring *in situ* demonstrate this type of activity.

The evidence does seem to indicate a functional form of vitamin K which is modified in some manner from that of the free vitamin.

Estradiol can replace vitamin K requirements for haemostasis (29) in rats, and the enzymic binding of estradiol and some of its

structural analogues to proteins has been documented (30). Thus it is possible that a similar type of binding could be occurring between vitamin K and an apoenzyme to produce a species required for clotting factor synthesis. If this binding were of a specific nature, then it should be possible to detect an enzyme containing vitamin K as a prosthetic group by investigating the distribution of radioactively labelled vitamin K administered to deficient animals.

Protein binding of estradiol appears to be mediated through free radical production via the NADPH microsomal oxidation pathway (30). Unfortunately this type of reaction is neither completely enzymic nor very specific and binding produced will be quite random. If vitamin K is bound as a prosthetic group on an enzyme it might not be detectable using radioactive tracers due to non-specific binding to many species of cell protein.

With an awareness of these limitations there may still be some value in checking the intracellular distribution of vitamin K, especially of the naturally occurring dietary vitamin K_1.

The object of this study was to conduct a series of experiments which could give evidence to support a hypothesis of vitamin K action consonant with the known facts about this system, yet avoiding the difficulties encountered in previous models.

CHAPTER II

MATERIALS AND METHODS

Materials

Reagents

Urea, imidazole, tris (hydroxymethyl) amino methane, reduced glutathione, bovine pancreatic ribonuclease A, and dextran with molecular weight of 60,000 to 80,000 were purchased from the Sigma Chemical Company, St. Louis, Missouri.

The Aldrich Chemical Co. Inc. were suppliers of 2-mercaptoethanol, 40% aqueous methylamine, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB).

Dithiothreitol (Cleland's Reagent) was purchased from Calbiochem.

Sodium warfarin and dehydroascorbic acid were bought from K and K Laboratories, Inc., Plainview, N. Y.

Commercially available materials used routinely in the clotting assay include: fibrinogen (Cohn fraction I) citrated, obtained as a dry powder containing 60% protein with about 67% of the protein clottable from Sigma Chemical Corp., a lyophilyzed human plasma (Diagnostic Plasma) obtained from General Diagnostics Division of Warner-Chilcott, Morris Plains, N. J., and bovine thrombin preparations of standard con-

centration sold as Thrombin Topical by Parke, Davis Co., Detroit, Mich.

Bovine serum which has been kept with the clot for thirty hours at 24° C, and then 18 hours at 4° C was used as a source of factors V, VII and X. This material was stored at -15°C, and quick-thawed before use.

Different forms of Sephadex (a registered trademark of the Pharmacia Co., Uppsala, Sweden) used for dialysis and ion exchange procedures, including Sephadex G-25, carboxymethyl (CM)-Sephadex and diethylaminoethyl (DEAE)-Sephadex, have been purchased from the Sigma Chemical Corp. .

Ultrafiltration

A Sartorius Membranfilter has been used for ultrafiltration. The membrane in this apparatus is a collodion bag with pore size of less than 50 millimicrons. A product of West Germany, it is marketed in this country by Carl Schleicher and Schuell, Keene, N. H.. The approximate molecular exclusion size of the 50 millimicron pores corresponds to a molecular weight of about 30,000.

Ultrafiltration has also been carried out using membranes which are hydrous gels of the complex interaction product of polyanions and polycations and are marketed under the name Diaplex by the Amicon Corp., Cambridge, Mass.. Solvent flow through these membranes is said to occur by a process of true molecular diffusion (31) as opposed to hydraulic pore flow observed in conventional ultrafilters. Various upstream surface formulations are available to allow screening of solute molecules of different molecular weight or charge.

Rats

Male albino rats weighing 60-80 grams were purchased from Sprague-Dawley of Madison, Wisconsin. These rats were maintained on purified diets containing adequate levels of all nutrients known to be required, with the exception, where indicated, of vitamin K.

Experimental Diets

The diet used is known to be inadequate (32) with regard to vitamin K content. When coprophagy is prevented, male rats maintained on this diet develop severe hemorrhagic symptoms, and usually die within 5-10 days of the beginning of coprophagy restriction. For composition of the diet refer to Table 1. Methionine was purchased from the Sigma Chemical Co., sucrose was a commercial product obtained locally, and the remainder of the constituents were purchased from the Nutritional Biochemicals Corporation, Cleveland, Ohio. Control rats were maintained on the above diet supplemented with 1.0 grams of menadione per 100 grams of vitamin premix or on a commercial pellet diet obtained from Rockland Laboratories, Teckland Inc., Monmouth, Illinois.

Coprophagy Prevention

When vitamin K deficient rats were required, animals with a weight range of 160 to 180 grams which had been maintained on the vitamin K deficient diet were selected. These animals were placed in tubular coprophagy-preventing cages (33) and left for four days. Then the whole blood clotting time was measured by the single stage method as an index (34) of vitamin K depletion. Varying degrees of clotting activity reaching as low as ten percent of normal have been present in rats used

TABLE 1

COMPOSITION OF THE VITAMIN K DEFICIENT DIET

Constituent	Weight Percentage of the Total Diet
Sucrose	66.9
Soy Protein (solvent extracted)	20.0
Methionine (D,L)	0.5
Glyceryl trioleate	3.0
Methyl linoleate	0.6
Vitamin premix (in cerelose)*	5.0
Salt mixture #446 (Nutritional Biochemicals)	4.0
	100.0

*VITAMIN PREMIX

Constituent	Amount Present in 100 gm of Cerelose-Premix
Thiamin hydrochloride	4.0 mg
Riboflavin	4.0 mg
Calcium pantothenate	20.0 mg
Pyridoxine	3.0 mg
Nicotinic acid	40.0 mg
Biotin	0.04 mg
Folic acid	0.4 mg
Vitamin B_{12} (0.1% in mannitol)	20.0 mg
Choline chloride	200.0 mg
Vitamin A	4000 I.U.
Vitamin D	400 I.U.
Vitamin E (α-tocopherol succinate)	60.0 mg

in this study. Daily measurement of clotting activity was usually sufficient for obtaining deficient animals before death occurs by internal hemorrhage.

Methods

Krebs-Ringer Buffer Preparation

Krebs-Ringer buffer was prepared from the following components:

1)	0.9% NaCl	100 parts
2)	1.15% KCl	4 parts
3)	2.11% KH_2PO_4	1 part
4)	3.82% $MgSO_4 \cdot 7H_2O$	1 part
5)	1.30% $NaHCO_3$ (gassed with CO_2)	21 parts

This buffer was made up freshly each day before use, and gassed with 5% CO_2, 95% O_2 for ten minutes to increase the level of dissolved carbon dioxide.

Prothrombin Assay

The two-stage assay procedure (35) for the measurement of prothrombin in defibrinated plasma has been slightly modified for the assay of isolated prothrombin. Reagent solutions used in the assay are listed below:

1) A 0.9% solution of NaCl in distilled water.

2) Imidazole buffer pH 7.2, prepared by dissolving 1.75 grams of dry imidazole in 90 ml of 0.1 N HCl and adjusting the volume to 100 ml with distilled water.

3) A 14% w/v solution of dextran in 0.9% NaCl.

4) Thromboplastin and calcium solution which was purchased in the lyoph-

ilized form as "Simplastin", and dissolved in distilled water as specified.

5) "Accelerator globulin" solution which was prepared from the specially treated serum listed under materials by diluting 8 parts of serum with 0.9% NaCl to a total of 1000 parts.

6) Fibrinogen solution was prepared by dissolving the commercial product in a mixture of 9 volumes 0.9% NaCl and 1 volume of imidazole buffer, to obtain a final concentration of 1% on the basis of clottable protein.

The prothrombin preparation to be assayed was diluted in the "accelerator globulin" solution, and the assay procedure carried out as follows:

Mixture A

14% dextran in physiological saline	2 parts
imidazole buffer pH 7.2	1 part
physiological saline	1 part

Mixture B

mixture A (above)	1 part
thromboplastin-calcium solution	1 part

Mixture C

mixture B (above)	2 parts
diluted prothrombin	1 part

Prothrombin was activated to thrombin in this mixture. After a seven minute incubation at 37°C fibrinogen was added to the solution.

Mixture D

mixture C (above)	3 parts
fibrinogen solution	1 part

The interval of time elapsing between the addition of a sample of mixture C to fibrinogen, and the formation of a measureable clot, was recorded as an index of prothrombin concentration. Clot formation has been measured mechanically on a "Fibrometer" manufactured and marketed by the Baltimore Biological Laboratory Division of Becton, Dickinson and Company, Baltimore, Maryland. Measurement was based on the increase in viscosity of the reaction mixture required to operate a microswitch stoping a digital readout clock whose cycle was initiated when mixture D is made.

Prothrombin time is then related to prothrombin concentration by comparing with clotting times observed for known dilutions of bovine thrombin of known standard concentration. All the reagents are stored at $<4^{o}C$ in either plastic or siliconized glass containers before the assay.

Prothrombin Preparation

Prothrombin was isolated according to the procedure of Moore et al. (8) with only a few deviations from their published protocol. The following is an outline of the procedure including some minor modifications.

Fresh bovine blood was obtained at the time of slaughter from the Canadian Valley Packing Company of Oklahoma City and diluted 1:8 with 2.85% aqueous trisodium citrate. Cooling was started on the trip from the packing house to laboratory by packing the containers in ice. Plasma was prepared from six liter batches of blood, and either used immediately in the preparation, or stored at $-20^{o}C$. Approximately three liters of plasma were used in an individual preparation, and all subse-

quent steps were carried out at 4°C using plastic or siliconized glass containers.

Barium citrate adsorption. A barium citrate precipitate was formed by slowly adding 1.0 M $BaCl_2$ (10 ml per 125 ml of plasma) to the citrated plasma with gentle stirring. Stirring was continued for 10 minutes, and the precipitate was collected by centrifuging at 3600 x g for 25 minutes in 300 ml polycarbonate bottles. The supernate was discarded, and the precipitate stirred with a small volume of a 1:10 dilution of stock citrate-saline (9.0% NaCl and 0.2 M $Na_3C_6H_5O_7 \cdot 2H_2O$ in distilled water) until a uniform suspension was made up to one liter. The suspension was stirred for 10 minutes and then allowed to equilibrate for one hour. Barium chloride addition was repeated as before. Stirring was continued for ten minutes after the addition had been completed and then the mixture was allowed to stand undisturbed overnight. In the morning the barium citrate was collected again by centrifuging for 25 minutes at 3600 x g. Again the supernate was discarded, the precipitate resuspended in sodium chloride-citrate, and $BaCl_2$ addition repeated.

Elution and dialysis. After one hour the barium citrate was collected for the third time, the supernate discarded, and about 30 ml of 0.2 M Na EDTA pH 7.40 were added to each of the four centrifuge jars. When the material had been uniformly suspended it was distributed among ten 14 inch sections of Visking dialysis tubing pretreated with boiling EDTA, and stored at 4°C in distilled water prior to use. The centrifuge jars were washed out with EDTA solution, and the wash was added to the dialysis bags to give a total volume of 400 ml (40 per bag).

The material was dialysed for 30 minutes against 360 ml of

0.2 M EDTA, 360 ml of stock citrate-saline and 2880 ml of distilled water. Subsequent dialysis was carried out against 3.6 liters of 1:10 30 minute intervals. Dialysis was continued overnight in the last change.

Ammonium sulfate precipitation. Ammonium sulfate was added as the saturated solution adjusted to pH 7.0 with concentrated NH_4OH. Dropwise addition of one volume of saturated solution was carried out while the solution was being stirred. Stirring was continued five minutes after addition was completed, and the precipitate was removed by centrifuging for 30 minutes at 3600 x g. A second volume of saturated ammonium sulfate was added to the supernatant slowly with constant stirring. The solution was allowed to stand for 30 minutes to allow complete salting out, and then it was centrifuged as before to collect the precipitated prothrombin. This precipitate was washed with 15 ml of 67% saturated $(NH_4)_2SO_4$ solution and again collected by centrifuging for 30 minutes at 3600 x g. The combined precipitate from all the centrifuge tubes was dissolved in 45 ml of distilled water, placed in a section of dialysis tubing and dialysed overnight against 4 liters of deionized water with 3-4 changes of dialysate. The pH of the water should lie between 6.0 and 8.0, as the buffering capacity of the preparation was low at this stage.

Isoelectric precipitation. After testing to make sure all sulfate ion had been removed, the pH of the solution was adjusted to 5.40 with 0.25% (v/v) HCl. The precipitated protein was removed by centrifuging for 30 minutes at 3600 x g and the pH of the supernate adjusted to 4.60. After sitting for fifteen minutes the suspension was centrifuged for 10 minutes at 1000 x g to collect the prothrombin precipitate.

Dialysis against phosphate buffer. The precipitate from the above step was dissolved in five ml of phosphate buffer (0.05 M in 0.1 M NaCl, pH 6.86 ± 0.02, I 0.2), transferred to a dialysis bag with a 5 ml rinse, and dialysed against the same buffer for six hours. The 4 liter dialysate volume was changed twice, after the second and fourth hours.

Adsorption with kaolin and bentonite. After dialysis the protein concentration was adjusted to 15 mg per ml by diluting with phosphate buffer. Kaolin was added to the solution at a concentration of 125 mg per ml, and the suspension was shaken in an iced Doubnoff incubator for 15 minutes. The kaolin was removed by centrifuging at 3600 x g for 15 minutes, and then bentonite was added to the supernate at a concentration of 10 mg per ml and the adsorption procedure repeated. After removal of the bentonite the material was placed in a plastic container and stored at -20°C.

Determination of protein concentrations. Two different methods were used for the determination of protein concentration, depending on the material to be analysed. If the presence of suspended non-soluble protein was suspected, as in a crude cell homogenate, then the method of Lowry et al. (36) was used. Following purification procedures which remove most non-protein substances, concentrations were estimated by ultraviolet adsorption (37).

Disulfide reduction in prothrombin. Again two separate methods were used in reducing the disulfide linkages in prothrombin. The major method follows that of Givol et al. (38). Sufficient urea to make an eight molar solution was added slowly with stirring to a 1% solution of

prothrombin in 0.05 M $KHPO_4^-$, 0.1 M NaCl, pH 6.86 ± 0.02. As the procedure was normally carried out this involves adding about 0.48 g of urea to 0.630 ml of 1% prothrombin solution. Then 4 µl of 2-mercaptoethanol and one drop of 5% aqueous methylamine were added, the tube was gassed with nitrogen, stoppered and left for 12 hours at 24°C. After sitting, the solution was acidified with a drop of glacial acetic acid, and placed on a 1 x 25 cm column of G-25 Sephadex previously equilibrated with 0.1 N acetic acid. The column was eluted with the equilibrating acetic acid, and the effluent monitored on a Beckman-Spinco Model 135 Spectromonitor. Ultraviolet absorption at 280 and 260 mµ was recorded as the effluent passed through the instrument. It was also possible to measure ultraviolet absorption at 240 mµ as a sensitive test for the presence of 2-mercaptoethanol.

The protein emerging in the void volume of the G-25 Sephadex was usually collected in two or three samples of 60 drop size. Collection was always stopped before the appearance of 2-mercaptoethanol, as measured by 240 mµ absorption.

Free sulfhydryl groups present in the fractions of the column effluent were measured in a colorimetric reaction with 5,5'-dithiobis-2-nitrobenzoic acid (39). DTNB was used as a 2.0 mM solution in 0.05 M $KHPO_4^-$ pH 6.86, and 0.20 ml of the reagent was added to 5.0 ml of sample or standard diluted with 0.05 M $KHPO_4^-$ buffer. The optical density was read at 412 mµ on a Beckman DB spectrophotometer after the reaction had gone to completion in fifteen minutes at 24°C. As developed here the assay was sensitive to as low as 10^{-2} µmoles of sulfhydryl residue.

Protein concentration in the individual tubes was measured

from the ultraviolet absorbance at 260 and 280 mμ in a Beckman DB spectrophotometer, and the moles of sulfhydryl group per milligram protein were recorded.

The alternate method for reduction of disulfide bonds involved the use of dithiothreitol (40). A 1% solution of prothrombin was treated with an approximate sixty-fold molar excess of dithiothreitol (1.0 mg per ml) for 48 hours at 24°C. The flocculent precipitate formed was dissolved by adding urea, guanidine, or diluting with nine volumes of 0.1 N acetic acid pH 2.8.

Storage of the reduced material was carried out by maintaining it at a concentration of 1 mg per ml in 0.1 N acetic acid under a nitrogen atmosphere at 4°C. No attempts have been made to store such material for a period longer than two weeks.

Reactivation of Reduced Prothrombin

Reactivation experiments were carried out in Krebs-Ringer buffer modified by the omission of Ca^{++} ions which interfere with the clotting assay. This limitation prevented large modifications of the pH or ionic strength of the reaction mixture. Krebs-Ringer buffer contained:

0.9 % NaCl	100 parts
1.15% KCl	4 parts
3.82% $MgSO_4 \cdot 7H_2O$	1 part
2.11% KH_2PO_4	1 part
1.30% $NaHCO_3$	21 parts

Stock solutions were stored at 4°C to minimize bacterial growth. The buffer was made up freshly before use, and gassed for ten minutes with

a mixture of 95% O_2 5% CO_2. The pH was 7.0 after the freshly made buffer was saturated with carbon dioxide and held at a temperature below 4°C. The total reaction volume in the reactivation system was 2.10 ml unless otherwise noted. All additions to the system such as enzyme fractions and cofactors were diluted in 2.0 ml of the Krebs-Ringer buffer, and 0.10 ml of the reduced prothrombin preparation was added to obtain the final volume. Control tubes contained the reduced prothrombin, but no enzyme. These initial steps were carried out with all fractions in an ice bath.

Zero time values were obtained by removing 0.5 ml from each reaction mixture before incubation, and assaying for prothrombin. For incubation, the entire reaction mixture remaining was placed in a Doubnoff shaking incubator maintained at 37°C under an atmosphere of 95% O_2 and 5% CO_2. After the prescribed incubation period the tubes were removed from the water bath and placed on ice until aliquots could be removed for analysis.

Assay for Reactivation

Half ml aliquots of the reaction mixture were removed before and after the incubation. These aliquots were then added to a source of factors V, VII and X prepared by diluting bovine serum (prepared as described earlier) fifty times in physiological saline. One half ml of the serum derivative was combined with the samples to be assayed for prothrombin, and again all the operations were carried out in an ice bath. The clot assay is carried out at 37°C and since the only effect desired in the preincubation is the conversion of prothrombin to thrombin all pre-warming is kept to a minimum. The sequence of addition of reagents to the 37° clotting assay was as follows:

0 minus 30 seconds	put dextran-imidazole on heat
0 minus 15 seconds	add sample to be assayed
0 time	Ca^{++} - thromboplastin added
0 plus 7 minutes	add fibrinogen solution and time the development of the clot.

All assays were carried out in triplicate as a standard practice. Experimental values have been corrected for changes which appear during incubation of the control to obtain a true estimate of enzymic reactivation. Enzyme specific activity is expressed in units of prothrombin produced per minute per milligram of enzyme protein in the incubation system.

Enzyme Purification

Livers were removed from rats immediately after decapitation and placed on ice. The livers were weighed and then homogenized in nine volumes of 0.25 M sucrose, 0.005 M $MgCl_2$, 0.05 M KCl and 0.025 M tris pH 7.6. A glass and teflon Potter-Elvehjem homogenizer was motor driven at 500 rpm, and three complete passes of the pestle in and out of the glass were used for complete homogenization. Particulate cell fractions were removed by centrifugation for one hour at 105,000 g or for two hours at 78,000 g in a preparative ultracentifuge. An aliquot of the supernate was removed to measure protein concentration, and it was then dialysed against 0.01 M $KHPO_4^-$ pH 6.86 and assayed for enzyme activity.

Ammonium sulfate precipitation. One volume of a 4°C saturated solution of $(NH_4)_2SO_4$ adjusted to pH 7.0 with NH_4OH was added dropwise with stirring to the remaining microsomal superantant. All operations at this point were carried out at 4°C. When ammonium sulfate addition was completed the solution was allowed to stand for ten minutes and then

centrifuged for 20 minutes at 3000 g to remove the precipitate.

The supernate from this first precipitate was removed, and a second volume of saturated ammonium sulfate was added dropwise with stirring. The precipitate formed was collected by centrifuging and dissolved in a minimal volume of deionized water. It was then applied to a 4 x 30 cm column of Sephadex G-25 and the column developed with 0.01 M $KHPO_4^-$ pH 6.86. The column effluent was monitored for ultraviolet absorption at 260 and 280 mμ, and the conductivity was also measured. The protein fraction freed of ammonium sulfate was now carried on to the next step.

CM-Sephadex fractionation. The desalted 33 to 66% $(NH_4)_2SO_4$ precipitate was placed on a 4 x 40 cm column of CM-Sephadex equilibrated with 0.01 M $KHPO_4^-$ at pH 6.86 and the flow rate adjusted to 40 ml per hour. After 200 ml of effluent had been collected the molarity of the eluting buffer was increased to 0.05 M, and after a further 200 ml were collected a second increase was made to 0.1 M buffer. The column was run at this molarity until all protein was eluted. The protein peak emerging with the front of the 0.05 M buffer was carried on to the next step.

Ultrafiltration and DEAE ion exchange. The material described above was placed inside a Sartorius Membranfilter with a pore size <5 mμ, and a negative pressure of 50 cm of mercury was applied to aid filtration. The filtrate which passed through the pores of the ultrafilter was collected, and concentrated using a UM-3 membrane in an Aminco Diaflow cell. This membrane has a molecular exclusion size of approximately 300 Daltons. After this concentration step the salt content of the material was reduced by washing in the Diaflow cell with deionized water.

The remaining material was placed on a 1 x 15 cm DEAE-Sephadex column equilibrated with 0.01 M $KHPO_4^-$ pH 8.0 and protein eluted with an increasing gradient of KCl. All fractions were collected and assayed for enzyme activity.

Intracellular Distribution of ^{14}C (U) Vitamin K_1

^{14}C (U) vitamin K_1 obtained from Hoffman LaRoche had a specific activity of 13.7 μc per mg. Vitamin K deficient rats were given ten to twenty μc of the vitamin K_1 by intraperitoneal injection, and were sacrificed from six to twelve hours after receiving the isotope.

Livers were homogenized in 9 volumes of 0.25 M sucrose, 0.005 M KCl and 0.025 M tris, pH 7.6. Nuclei and cell debris were removed by centrifuging for ten minutes at 2000 g. With all operations carried out at 4°C, the nuclear fraction was resuspended in the same medium and collected once more at 2000 g for ten minutes.

Mitochondria were pelleted by spinning the nuclear supernate and wash for fifteen minutes at 10,000 g and again the pellet was washed once.

Microsomes were collected by centrifuging the mitochondrial supernate for two hours at 105,000 g. Microsomal pellets were combined and resuspended in 0.001 M $MgCl_2$ and 0.05 M tris pH 7.6 so that microsomes from one gram of liver were suspended in 3.2 ml. At this point the microsomal suspension was divided in half, with one half being treated as a wash, while the other half was made up to 0.5% in sodium desoxycholate. The washed microsomes and the ribosomal fraction were collected by spinning for 90 minutes at 105,000 g.

All the pellets were resuspended in 0.001 M $MgCl_2$ 0.05 M tris,

pH 7.6 and protein concentrations measured by the method of Lowry et al. (36). Half ml aliquots of the suspensions were removed and diluted to 1.0 ml volume with 0.2 N NaOH. After storing at 37°C for 24 hours the samples were quantitatively transferred to scintillation vials containing 15 ml of aqueous scintillation medium (41) and counted in a Packard Tri-Carb liquid scintillation counter. Efficiencies were calculated in individual samples by adding an external standard of known activity, and from this data dpm per mg protein was calculated for the different fractions.

Folch Extraction

A chloroform-methanol extraction (42) of some fractions has been carried out after lyophilization to measure the distribution of ^{14}C activity between lipid soluble and lipid insoluble fractions. Radioactivity in the lipid soluble material was measured in the liquid scintillation counter and compared with the total radioactivity in the sample before extraction.

Molecular Weight Determination

The analytical ultracentrifuge was used to study physical properties of the reduced prothrombin molecule.

Sedimentation coefficient. The apparent sedimentation coefficient ($s_{app.}$) was measured at 1.0 and 0.25% protein concentration. A linear dependence of s on concentration was assumed in extrapolating to a value of s_{app} at zero concentration. This value was converted to standard conditions of water at 20°C by the equation

$$s_{20,w} = s_{app.} \left[\frac{\eta_t}{\eta_{20}}\right] \left[\frac{\eta}{\eta_0}\right] \left[\frac{1 - \bar{v}\rho_{20,w}}{1 - \bar{v}\rho_t}\right]$$

where $\eta t/\eta$ 20 corresponds to the viscosity of water at the experimental temperature t, relative to that at 20°, η/η_o is the relative viscosity of the solvent to that of water. $\rho_{20,w}$ and ρ_t are the densities of water at 20° and the solvent at t degrees respectively, and $\bar{V}$ is the partial specific volume of the protein.

Diffusion coefficient. A value for the apparent diffusion coefficient, $D_{app.}$, was obtained from the following formula

$$\sigma^2 = \frac{1}{2\pi} \left[\frac{A}{H_{max}}\right]^2$$

Values of the second moment σ^2 were plotted against time after boundary formation in a synthetic boundary cell. The slope of the plot is equal to 2D. The value of D_{app} was converted to standard conditions as follows

$$D_{20,w} = D_{app} \left[\frac{293}{273 + t}\right] \left[\frac{\eta_t}{\eta_{20}}\right] \left[\frac{\eta}{\eta_0}\right]$$

Molecular weight is obtained from the formula

$$M = \frac{RTs}{D\,(1 - \bar{V})}$$

Where R is the gas constant, 8.314 ergs/moles/degree, and T is the absolute temperature.

CHAPTER III

RESULTS

Hepatic Intracellular Distribution of ^{14}C (U) Vitamin K_1

The most notable observation about the distribution of ^{14}C labelled vitamin K_1 in the liver intracellular regions is a complete randomness of binding to all sub-cellular fractions. Not only was the distribution quite random, it also varied widely between the liver sub-cellular fractions from animal to animal. The total counts recovered in each sub-cellular fraction (Table 2) for ten individual rats failed to indicate any binding preference.

The recovery of administered ^{14}C in the livers after sacrifice was also quite variable, ranging from 50% down to less than 1% of the dose. These differences probably reflect different rates of transport from the injection site and/or different rates of excretion in the bile. The relatively low specific activity of the vitamin K preparation assures that the amount of vitamin K in these livers was adequate for normal physiological function. At 13.7μc/mg the animals were receiving at least 0.7 mg of vitamin K_1. This dose is almost 100 times the amount required to cure deficiency symptoms when given to rats of 200 gm body weight.

Further assurance that adequate amounts of vitamin K have reached the liver in the interval between injection and sacrifice comes

TABLE 2

TOTAL RADIOACTIVITY RECOVERED IN LIVER SUBCELLULAR FRACTIONS OF RATS INJECTED WITH 10 μc ^{14}C (U) VITAMIN K_1

Subcellular Fraction	Rat Number									
(total dpm)	1	2	3	4	5	6	7	8	9	10
Microsomal Supernate	5510	53100	17220	28600	7040	5160000	2540000	4050	30000	770
Nuclei and Cell Debris	87200	40800	30830	60000	8830	69000	1960000	10900	286000	786
Mitochondria	8080	13760	9915	16050	6750	4420000	2890000	1800	138600	89
DOC Soluble Microsomal Membrane	3840	47200	5000	18060	6510	1476000	1347000	920	56700	520
Ribosomes	4500	39880	3560	4000	778	105300	63000	0	3420	0
Total	109000	194700	66500	126700	29900	11230000	8800000	17680	514200	2165

from single stage measurements of blood prothrombin. In this series of experiments all the animals except one (see Table 3) had been maintained on the purified diet without added vitamin K, and these animals showed decreased prothrombin levels in comparison to rats on a normal ration. In all nine cases the prothrombin level showed an increase after vitamin K injection which was indicative of physiological dose levels. The single normal control animal also showed the random labelling pattern among all sub-cellular fractions. This was taken as evidence that no particular cell fraction has sufficient vitamin K bound to tie up all the sites available for binding the vitamin, even when the animal is on a vitamin K adequate diet.

General trends include a larger amount of radioactivity in the fractions containing most of the cell mass. The lipid content of the particular cell fraction has no obvious effect on the amount of vitamin K bound. The supernatant fraction contains as much of the total radioactivity as the nuclear and cell debris fraction, or the lipid rich microsomal fraction.

Since the distribution of radioactivity within the cell corresponded closely to the distribution of mass in the different fractions, the data have been expressed in terms of dpm per unit amount of protein in each of the five fractions. This has been done by dividing the percentage distribution of counts in each fraction by the averaged protein content of each fraction on a dry matter basis. A completely random binding to protein should give a ratio of 1.0 for this calculated value. In Table 4 a look at the standard deviations from the averaged percentage distribution of radioactivity shows that the calculated ratios do not

TABLE 3

BLOOD PROTHROMBIN LEVELS OF RATS
RECEIVING ^{14}C (U) VITAMIN K_1

Parameter	Rat Number									
	1	2	3	4	5	6	7	8	9	10
Initial Prothrombin Level (Percent of Normal)	8	100	7	<2	8	7	10	<2	5	7
Hours Elapsed Between Vitamin K_1 (^{14}C) Administration and Sacrifice	5	11	6	6	11	10	10	3	3	4
Final Prothrombin Level (Percent of Normal)	100	100	100	100	100	100	100	3	28	15
Lipid Soluble Radioactivity in DOC Soluble Microsomal Membrane (% of Total)		56				59			95	98

TABLE 4

DISTRIBUTION OF INTRACELLULAR PROTEIN AND ^{14}C (U) VITAMIN K_1

Subcellular Fraction	Radioactivity Distribution (Percent)											Protein Distribution $\bar{x}$ Percent	Ratio of Radioactivity to Protein
	Rat Number												
	1	2	3	4	5	6	7	8	9	10	$\bar{\bar{x}}$		
Micro. Supt.	5	27	26	23	24	46	29	23	6	35	24 ± 12	22	1.1
Nuclei and Debris	80	21	46	47	30	1	22	62	56	36	40 ± 22	56	0.7
Mitochon-dria	7	7	15	13	23	39	33	10	27	4	18 ± 11	13	1.4
Membrane	4	24	8	14	22	13	15	5	11	24	14 ± 7	7	2.0
Ribosomes	4	21	5	3	2	1	1	0	1	0	4 ± 6	1	4.0

differ significantly from a value of 1.0.

The generally non-polar characteristics of vitamin K_1 make it an illogical candidate for complex formation with cell proteins. The small amount of radioactivity recovered in the cell sub-fractions could be interpreted as a direct association with hydrophobic regions of protein, and lipoprotein components present in the sub-fractions. As vitamin K is a natural component of the diet one is probably safe in assuming some metabolic alterations of the vitamin to occur during its stay in the body. Water soluble metabolites of the vitamin could account for binding of radioactivity to proteins. There are three components which have some bearing on this problem, and are easily investigated. Lipid soluble material resembling vitamin K_1, lipid soluble material with differing physical properties, and protein bound metabolites of vitamin K_1 are all easy to quantitate.

Chloroform-methanol extraction (42) of some sub-cellular fractions from individual rats was used to assess the partition of recovered radioactivity into the three fractions mentioned above. The chloroform was removed under a stream of nitrogen, and the lipid residue redissolved in petroleum ether. After removal of an aliquot for liquid scintillation counting, the remaining material was spotted on alumina impregnated filter paper (Schleicher and Schuell, #288) and developed in cyclohexane-benzene 7:3 (v/v). Vitamin K_1 had an Rf of $\tilde{0.9}$ in this system, and could be readily separated from benzo and napthoquinone compounds lacking the isoprenoyl side chain, or having more than one unsaturated isoprene unit in the side chain. These latter compounds were slightly more polar than vitamin K_1, and move relatively slowly in this solvent system.

Nuclear, mitochondrial, and sodium desoxycholate solubilized microsomal material from rats 9 and 10 were treated in the above fashion. A scan of the radiochromatograms shown in Figure 1 indicates that most of the extracted radioactivity has an Rf corresponding to vitamin K_1. Only in the microsomal membrane fraction is there evidence for the presence of a lipid soluble metabolite with an Rf corresponding to the 2-methyl-1,4-napthoquinone which could be structurally related to metabolic products of vitamin K_1.

The absence of any detectable in vitro effect of vitamin K, except in whole liver perfusion studies by Suttie (43), indicates that some metabolite of the vitamin is probably involved in control of prothrombin synthesis. Due to an ever present difficulty in overcoming the concept of vitamins acting in coenzyme form, the protein bound, lipid insoluble radioactivity has been compared to the total radioactivity in a few of these cell sub-fractions.

Results from microsomes of four separate rats are included in Table 3. Rats 2 and 6 which were given the ^{14}C vitamin K_1 11 and 10 hours respectively before sacrifice show 56% and 59% of the microsomal radioactivity to be in a lipid soluble form. Rat number 2 is a control rat with adequate dietary vitamin K, so the similarity of response is interesting. It should be noted that in ten hours rat 6 has completely returned to normal levels of blood prothrombin, so we are looking at a post-induction position of the label. The amount of time elapsed since the administration of the vitamin also suggests that any catabolic pathways for elimination of the vitamin will be fully active.

In the case of rats 9 and 10 the animals were killed during

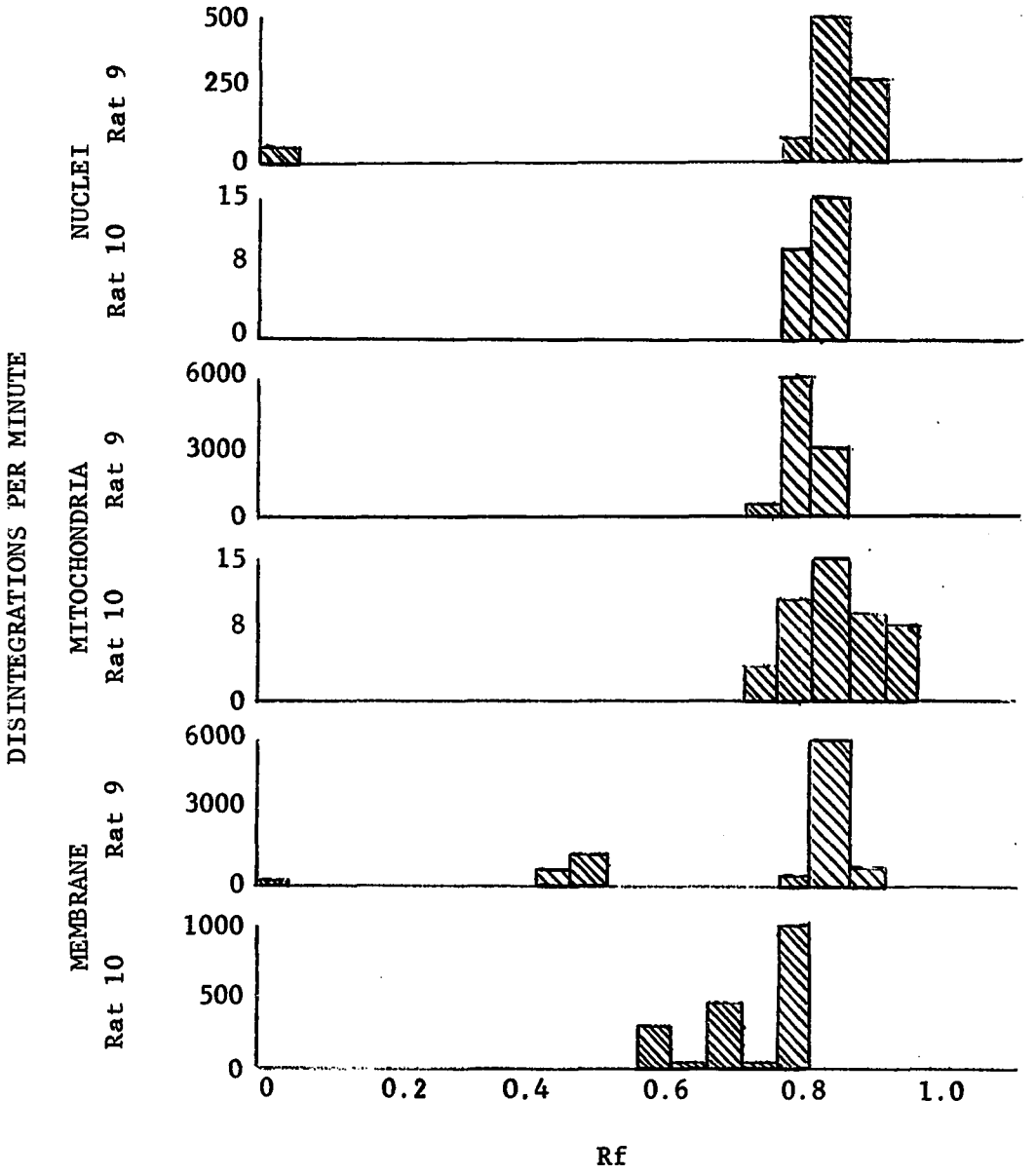

Figure 1. Radiochromatograms of subcellular lipid extracts. The solvent system is cyclohexane:benzene 7:3 and alumina impregnated paper was used in ascending chromatography. Chromatograms are cut in half inch strips and counted in a liquid scintillation counter.

the period of most rapid prothrombin synthesis, at three and four hours after administration of the ^{14}C vitamin K_1. At this time the results show more than 95% of the radioactivity in the microsomal membrane is lipid soluble. These results indicate that extensive catabolism of the vitamin, as seen in animals 2 and 6, is not required for prothrombin synthesis.

As indicated earlier, the finding of most of the microsomal, as well as the nuclear and mitochondrial lipid soluble radioactivity in a fraction of similar Rf to native vitamin K_1 suggests that this material may be present in considerable excess. Perhaps the excess available over physiological levels in accumulating in lipophylic regions of the cell unit it can be used or excreted.

Properties of Bovine Prothrombin

Bovine prothrombin prepared according to the method of Moore et al. (8) had specific activities ranging from 1500 to 3000 Iowa units per milligram of protein. Reasons for this variation in purity were not easily correlated with techniques used in the isolation procedure. It was possible to correlate specific activity of prothrombin preparations with indications of homogeneity obtained with the analytical ultracentrifuge. Sedimentation runs were carried out at 52,640 rpm at 25°C in a Spinco Model E ultracentrifuge. Prothrombin was in a solution of NaCl, potassium phosphate buffer, pH 6.86 at an ionic strength of 0.1 and the two preparations compared in Figure 2 were both at a 1% protein concentration.

Preparation #1 only assayed 1100 Iowa units per milligram of protein, and it showed the presence of a high molecular weight contami-

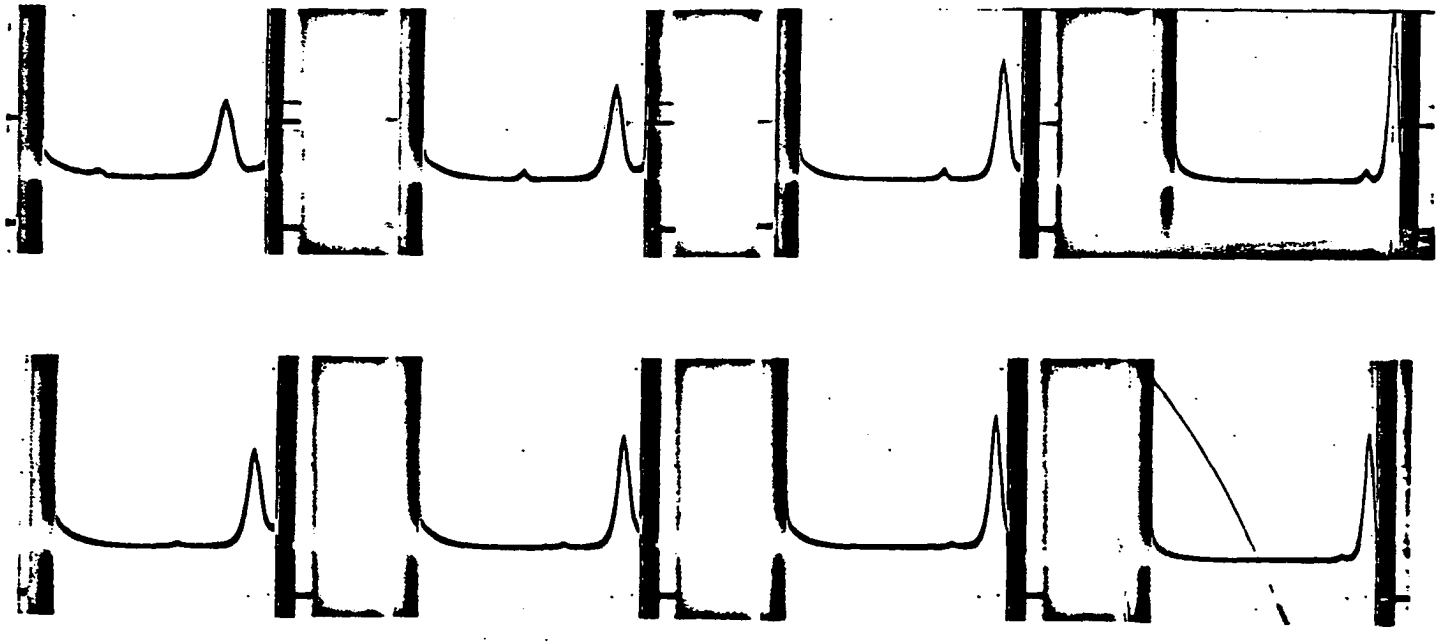

Figure 2. Comparison of Schlieren patterns and specific activity of two prothrombin preparations. The upper pattern shows preparation number 1, with a specific activity of 1100 units per mg. Exposures are at 8 minute intervals. Lower pattern is preparation number 4, assaying 2900 units per mg, exposures at 4 minute intervals.

nant. Preparation #4 assayed at 2900 Iowa units per milligram protein, and the contaminant species had almost completely disappeared. Cellulose acetate microelectrophoresis showed three or four bands staining for protein when preparation #1 was run for twenty minutes at 250 volts in 0.05 M pH 6.86 phosphate buffer. A comparison between preparation #1, and a second preparation assaying at about 2000 units per milligram protein is given in Figure 3.

No free sulfhydryl groups were detectable by the DTNB assay in native prothrombin. An estimate of the number of disulfide bonds present in the native molecule has been made by incubating prothrombin in 8 M urea with 10^{-3} M 2-mercaptoethanol at pH 8.5. Following acidification, and removal of urea and mercaptoethanol by Sephadex G-25 gel filtration, the reduced material was assayed for free SH-groups with DTNB. After measuring the concentration of protein and SH-groups in the same sample, the results were expressed as micromoles of SH-group per micromole of native protein (Table 5). The average of eight determinations gave a value of 15.3 SH-groups produced per molecule of native material, indicating a minimum of 8 disulfide linkages in the native prothrombin molecule.

Since the thrombin molecule is thought to have a molecular weight of 9000 to 12,000 Daltons, it is possible that two or more thrombin molecules could arise from the activation of a single prothrombin molecule. This could be most easily imagined if prothrombin contains two or more identical subunits. The structure of the reduced material was investigated for this reason.

Disulfide reduction in prothrombin at neutral pH without urea

Figure 3. Electrophoretic test of homogeneity. The two applications on the left are material from preparation 1. Four distinct species are present. In preparation 3, on the right, the material appears to be uniformly present at one location. Electrophoresis was run for 20 minutes at 250 volts in pH 6.86 buffer.

TABLE 5

DTNB REACTIVE SULFHYDRYL GROUPS PER MOLECULE OF REDUCED PROTHROMBIN

Prothrombin Preparation	SH Groups per Molecule of Reduced Material
Number 3	14.6
	14.7
Number 4	12.3
Number 5	13.9
	22.0
Number 6	17.6
	14.7
Number 9	12.9
Average	15.3

was accompanied by aggregation and precipitation of the protein. For this reason, no attempt was made to work with the material in the absence of urea. The sedimentation characteristics of prothrombin were studied in a solution of 8 M urea, 10^{-3} M 2-mercaptoethanol, and sufficient 5% aqueous methylamine to bring the pH to 8.5. The results of physical measurements made on this solution are given in Table 6.

Prothrombin Assay

The unusual relationship between concentration of prothrombin and clotting time of an assay system is commonly referred to in the literature (44, 45). Two aspects of the unusual relationship are: (1) Lack of reliability of dilution curves run on single samples for predicting dilution curves of other samples (2) Failure of the log - log plot of prothrombin concentration versus clotting time in seconds to reveal anything meaningful about the reaction being studied. The availability of purified prothrombin for study under conditions normally used for whole plasma was considered too great an opportunity to pass up.

Prothrombin assays referred to in the following section of this chapter have been performed on several different preparations of bovine prothrombin, of differing degrees of purity. The necessity for converting prothrombin times into units of prothrombin preparations be compared. Three preparations were compared for their behavior on dilution. Specific activities for the three preparations were 2500, 1600, and 1900 Iowa units per milligram of protein, for preparations 5, 9, and 6 respectively. If the impurities in preparations 9 and 6 represent substances which are inhibitory in the clotting assay, then dilution of these samples might have a different effect on clotting activity than

TABLE 6

MEASUREMENT OF PHYSICAL PARAMETERS OF
REDUCED PROTHROMBIN SOLUTION

Parameter		Experimental Value
ρ (gm/cc at 20°C)		1.1462
$\left[\frac{\eta}{\eta^o}\right]\left[\frac{\eta_t}{\eta_{20}}\right]$		1.5069
$\bar{V}$ (ml/gm)		0.714
$s_{20,w}$	1.0% solution	1.875
	0.25% solution	2.595
	0.0% solution[(a)]	2.80
D (cm^2/sec)		7.812×10^{-7}
M (Daltons)		48,052

(a) Value obtained by extrapolation to zero concentration.

diluting the purer preparation, number 5. Figure 4 shows a dilution curve prepared from averages of the three separate curves, with standard deviations from the average line being indicated at each point of measurement. The good fit of the data to one curve indicates the absence of inhibitors of the clotting reaction in protein contaminants in preparations 9 and 6.

These results raise the possibility that variations in dilution curves occurring between different plasma samples could result from different levels of activator or inhibitor substances present in plasma, but removed during the purification procedure. The averaged curve prepared from the three samples was then used as a standard curve for relating clotting time to prothrombin concentration in units. The increased scale drawing of the curve used in the assays in section four of this chapter is shown in Figure 5. Clotting time changes occurring during experimental procedures can be readily converted to units of prothrombin activity by using this graph.

Nothing has yet been said of the usefulness of this type of graph in determining chemical events occurring during the clotting process. Neither this linear plot of time versus prothrombin activity, nor Hemker's rectilinear log - log plot are very useful in predicting the type of reaction which is being observed. Since the value of kinetic data lies with its ability to give comparisons between model reaction schemes and individual situations under study it was considered useful to express the data in a more conventional form. The usual way of comparing enzyme concentration to activity involves plotting enzyme concentration against substrate consumed, or product produced per unit time at

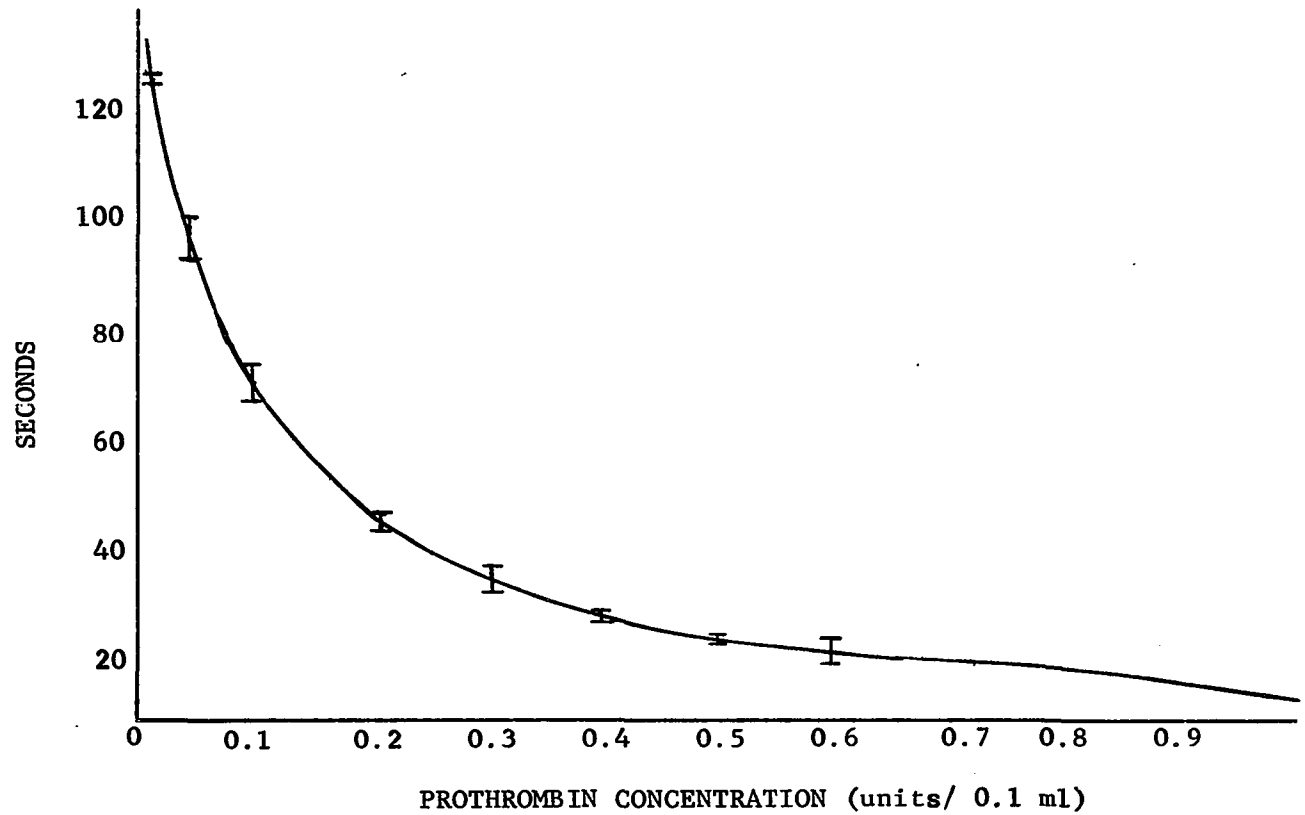

Figure 4. Comparison of clotting time with prothrombin concentration in purified preparations. Standard deviations are about the means of values obtained with preparations 5, 6 and 9.

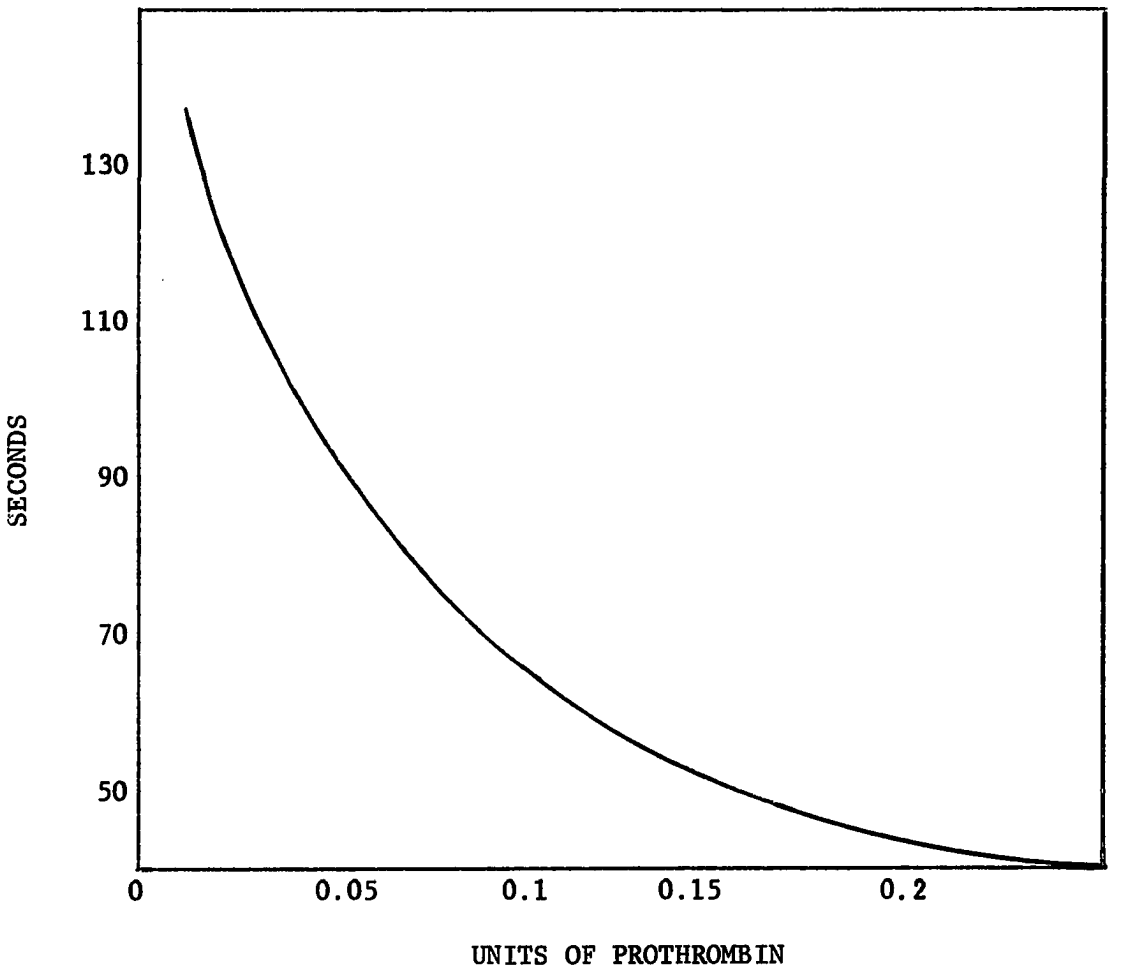

Figure 5. Prothrombin assay curve. The difference in clotting time between two samples is converted into units by referring to this graph.

a fixed substrate concentration.

As the two-stage assay is used it can only measure one level of product, i.e. the critical fibrin concentration required to trigger the polymerization reaction. However, it is still possible to think of amount of product formed per unit time. This can be done by considering the critical fibrin concentration as a unit amount of product. Then for a ten second clotting time one-tenth of the unit amount is produced per second, for a 100 second clotting time, one-hundredth of the unit amount is produced per second. Thus a plot of the reciprocal of clotting time versus prothrombin concentration should give results which are comparable to standard systems if the rate of fibrin production is linearly related to prothrombin concentration. This type of plot is given for dilutions of preparations 9 and 6 in Figure 6. This method of presentation can be supported mathematically, and the type of curve to be expected in a normal system is predicted by a look at Michaelis treatment of this situation. The standard form of Michaelis kinetics applicable here is

$$v = -\frac{dS}{dt} = \frac{kE_oS}{1 + K_s} \qquad (1)$$

Where the term - dS/dt refers to substrate disappearance per unit time, k is an empirical reaction constant, E_o represents total concentration of enzyme present in the system, S refers to concentration of substrate in the system and K_s is the equilibrium constant of the dissociation of ES into free enzyme and substrate.

If we integrate the differential form of the equation between the limits of the initial substrate concentration S_o and the final concentration remaining after a time t

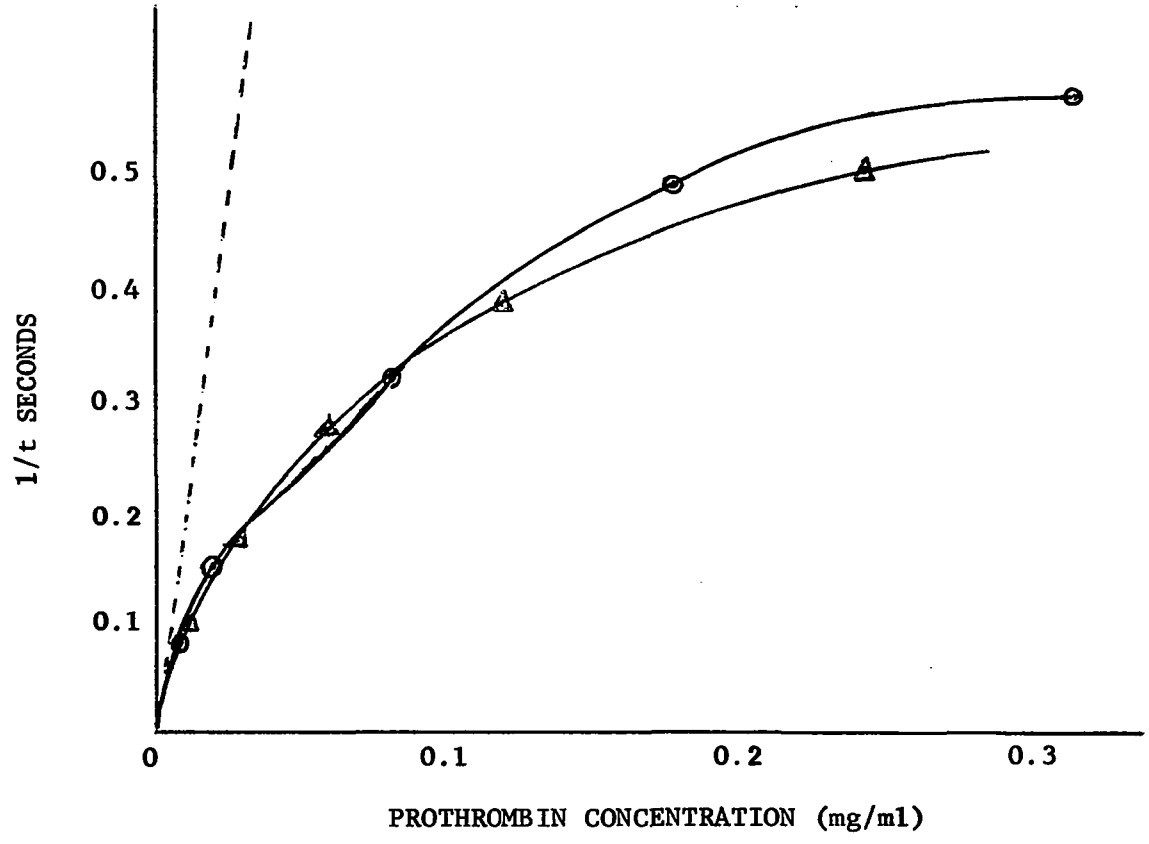

Figure 6. Plot of enzyme activity against concentration. The extrapolated initial rate is obtained by increasing the scale of the graph. Prothrombin preparations 6 Δ and 9 ⊙ are compared here.

$$\int_{S_o}^{S} -\frac{dS}{S_o} \quad (1 + K_s) \quad = k\, E_o \int_{0}^{t} dt \qquad (2)$$

Then integration gives

$$(1 + K_s) \quad (\ln S_o - \ln S) = k\, E_o \quad (t - o) \qquad (3)$$

and rearranging this equation for convenience shows that

$$\frac{(1 + K_s) \quad (\ln S_o/S)}{k\ t} \quad = \quad E_o \qquad (4)$$

Now we know that for the assay conditions used the term ln S_o/S should always be constant. This is based on the assumption that a constant amount of fibrinogen must be converted to fibrin to initiate the polymerization reaction actually serving as endpoint of the assay. Standardization of reagents for the assay provide a constant level of substrate in all situations. The we can let the constant terms from equation (4) be represented by $1/K^*$, and the equation reduces to

$$\frac{1}{t} \quad = \quad E_o \quad K^* \qquad (5)$$

Equation (5) is the description of a straight line having a slope of K^* and an intercept of zero when the reciprocal of clotting time is plotted against enzyme concentration.

A comparison between the predicted model and Figure 6 reveals that the data only fit the proposed model for very low enzyme concentrations. Curvilinearity appearing at higher enzyme concentrations is beginning to have an effect even at physiological levels of prothrombin concentration. For this reason it is interesting to propose models for explaining the data, and to test such models by comparison with kinetic parameters derived from this system.

The most obvious initial model which comes to mind is end-product inhibition of the thrombin mediated conversion of fibrinogen to fibrin by either fibrin or the fibrinopeptides. This model is based on the assumption that the initial rate of conversion of fibrinogen to fibrin is being measured only in the case of low prothrombin concentrations. Even if an initial linear rate of fibrin formation occurs at high prothrombin concentration, the requirement of the assay for a fixed fibrin concentration needed to initiate polymerization could be masking this initial rate by requiring the reaction to proceed to a point where product inhibition is important. A graphic portrayal of this idea is given in Figure 7.

The simplest kinetic treatment of this situation assumes simple competitive inhibition by product. The Michaelis expression for this situation is:

$$-\frac{dS}{dt} = \frac{k E_o}{1 + \frac{K_s}{S}\left[1 + \frac{I}{K_i}\right]} \qquad (6)$$

where I represents inhibitor concentration and K_i is the dissociation constant of inhibitor enzyme complex. Setting up for integration we have:

$$-\left(1 + \frac{K_s}{S}\right) \int_{S_o}^{S} \frac{K_s}{S} \cdot \frac{I}{K_i} \cdot \frac{1}{S} \, dS = kE_o \int_o^t dt \qquad (7)$$

The term $-(1 + K_s/S)$ from equation (7) will not vary under the assay conditions so is set equal to K'. Since the inhibitor in this system is hypothesized to be product, the value I can be expressed in terms of substrate concentration. When S_o is the initial substrate concentration

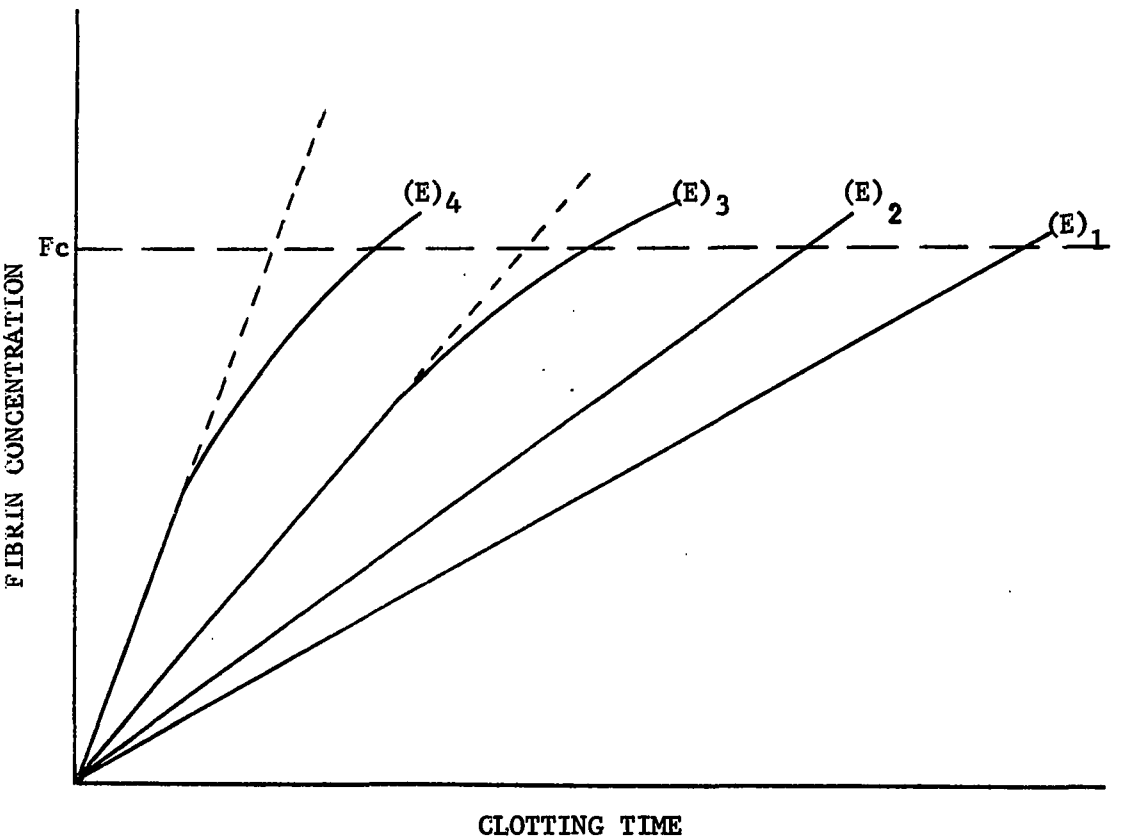

Figure 7. Hypothetical relationship between enzyme concentration and product inhibition. Assuming F_c to be the critical fibrin concentration required to initiate polymerization, then at high enzyme concentrations $(E)_3$ and $(E)_4$ the rate of fibrin production could be decreased before F_c is reached.

then

$$I = S_o - S \qquad (8)$$

Substituting equation (8) into (7) and rearranging gives:

$$K' \frac{K_s S_o}{K_i} \int_{S_o}^{S} \frac{dS}{S^2} - K' \frac{K_s}{K_i} \int_{S_o}^{S} \frac{dS}{S} = k E_o \int_0^t dt \qquad (9)$$

and integration yields:

$$\frac{K' K_s S_o}{K_i} (S - S_o) - \frac{K' K_s}{K_i} \ln \frac{S_o}{S} = k E_o t \qquad (10)$$

Again the absence of any variable entities in the left hand side of the equation under the conditions of the assay allow setting it equal to a constant K''. Now we see the familiar form:

$$\frac{1}{t} = \frac{k}{K''} E_o \quad \text{or} \quad \frac{1}{t} = K''' E_o \qquad (11)$$

This plot is obviously linear in nature, and does not fit the observed data. It appears that the peculiar conditions imposed by assaying to a fixed product concentration would mask effects of any competitive product inhibition. Thus the curvilinear nature of the enzyme activity versus concentration plot is not due to competitive product inhibition.

Another possible source of inhibitor in the clotting assay is the remaining portion of the prothrombin molecule which is not converted to thrombin. Bovine prothrombin is approximately six times as large as bovine thrombin on the basis of molecular weight. If an inhibitor of thrombin activity can arise during the activation of prothrombin the following situation should hold:

$$Z \longrightarrow E + I \rightleftharpoons EI \qquad (12)$$

where Z represents prothrombin, E represents thrombin, and I the hypothetical inhibitor. Then the total amount of enzyme produced on prothrombin activation, E_o, is equal to the amount of inhibitor, I produced. The concentration of enzyme actually assayed is (E) since

$$(E) = (E_o) - (EI) \tag{13}$$

The concentration of the EI complex is dependent upon its association constant K_a by the following definition:

$$(EI) = K_a (E) (I) = K_a (E)^2 \tag{14}$$

Now substituting equation (14) into (13) shows that

$$(E_o) = (E) + K_a (E)^2 \tag{15}$$

From this relationship it is apparent that at low levels of E_o, (E_o) should approximate (E), and for high concentrations of E_o, (E_o) will approach $(E)^2 K_a$. This observation predicts that a linear plot should be obtained by plotting enzyme activity against the square root of enzyme concentration at high enzyme concentrations.

Figure 8 shows that such a linear region is obtained if the general type of data presented in Figure 6 is plotted as the square root of enzyme concentration. Thus the data do fit the hypothesis of an inhibitor being produced from prothrombin as it is activated to thrombin.

The data available allow a calculation of the numerical value of the association constant of the enzyme-inhibitor complex. Such a calculation is based on the assumption that the linear region of the plot of enzyme activity versus concentration which is observed at very low enzyme concentration represents the situation where the inhibitor concentration has not become high enough to affect reaction rate. Extrapolation of this linear region of the curve then corresponds to a

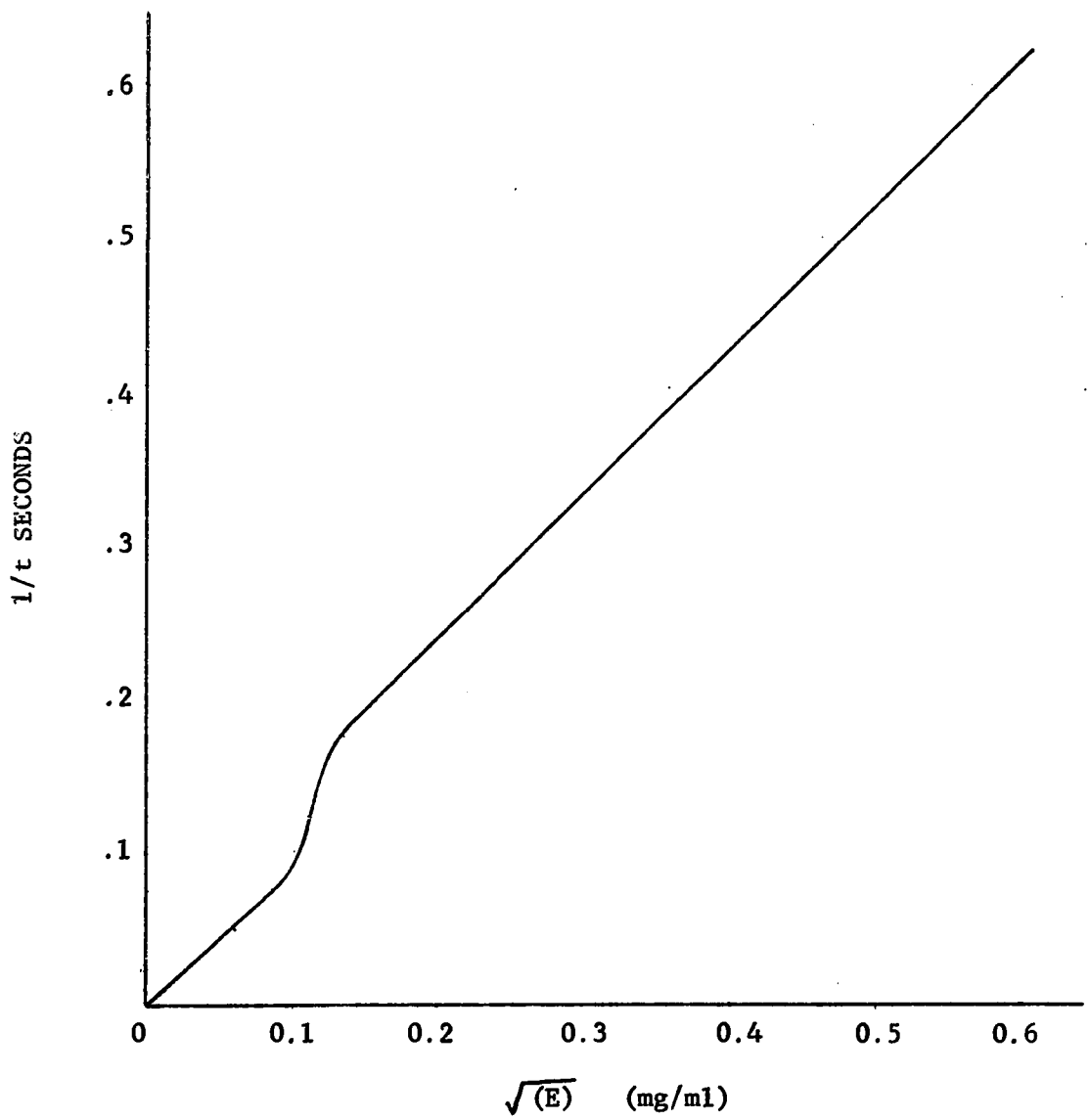

Figure 8. Plot of enzyme activity against the square root of enzyme concentration. Enzyme used for this experiment was prothrombin preparation number 6.

plot of activity against (E), while the actual curve represents (E_o) values (see Figure 6). From Figure 6 it is possible to correlate the assumed value of (E) with the known value (E_o) at any given level of enzyme activity. A value for K_a can then be obtained by substituting into equation (15). Empirical values for K_a calculated in this manner range from 140 to 160 liters grams $^{-1}$.

It is also possible to obtain an estimate of K_a from the relationships of the linear slopes of Figures 6 and 8. From (15) at high enzyme concentration the relationship

$$(E) = \frac{\sqrt{(E_o)}}{\sqrt{K_a}} \qquad (16)$$

holds true. The slope of the line in Figure 6 is given as K^* in equation (5) where it was assumed that (E_o) was equal to (E). Substituting equation (16) into equation (5) gives:

$$\frac{1}{t} = \frac{K^* \sqrt{(E_o)}}{\sqrt{K_a}} \qquad (17)$$

where the slope of the line in Figure 8 is now represented by the term $K^* / \sqrt{K_a}$. Now it is apparent that the slopes of the two lines are related and

$$\frac{\text{slope (Figure 6)}}{\text{slope (Figure 8)}} = \frac{K^*}{K^* / \sqrt{K_a}} = \sqrt{K_a} \qquad (18)$$

so K_a can be calculated from the following:

$$K_a = \left[\frac{\text{slope (Figure 6)}}{\text{slope (Figure 8)}}\right]^2 \qquad (19)$$

The value for K_a calculated from this equation is 225 liters grams $^{-1}$. Thus the estimations of dissociation of the enzyme-inhibitor complex, EI, give a K_i value of 0.85×10^{-7} M.

Prothrombin Reactivation

Simple proteins containing few disulfide bonds are known to be capable of regaining native characteristics after disulfide reduction (46). Appropriate conditions for slow reoxidation of sulfhydryl groups lead to some recovery of activity. This reactivation can occur most rapidly in the presence of a microsomal enzyme containing a free sulfhydryl group in the native form, and acting as a catalyst for disulfide exchange reactions.

This particular enzyme has no specificity for the type of protein molecule which is to be renatured. Since it is non-specific, the enzyme may be thought of as a free sulfhydryl donor, a sort of giant GSH molecule attached to the microsomal membrane. Unique properties of this enzyme must lie in the amino acid sequence around the reactive half cystine residue involved in disulfide interchange reactions (47).

Characterization of this type of activity does not seem to lead us any closer to an answer to the problem of assembling complex proteins containing inter as well as intra-molecular disulfide bonds. Although primary structure of proteins can dictate thermodynamic conditions which affect secondary, quaternary, and tertiary structure the existence of alternate mechanisms for specifying correct configuration cannot be completely ruled out. Moreover it becomes a weighty problem if one has to consider the logistics of correct pairing of half cystine residues in a protein containing more than five or six disulfide bonds.

Bovine prothrombin preparations reduced for 12 hours at 24°C in 8 M urea solution with 10^{-3} M 2-mercaptoethanol at pH 8.5 show no clotting activity after removal of denaturing agents. Since philosophi-

cal considerations require that the reduced form of the protein, or some mixed disulfide thereof be an intermediate between free amino acids and the active enzyme, the cell must be able to match sulfhydryl groups properly and oxidize them to give a configuration with enzyme activity. A search has been conducted for this type of activity in rat liver subcellular preparations.

A rational consideration of this type of activity suggests that it must reside at a location within the cell close to the protein synthesizing apparatus. Unfortunately microsomes are known to contain prothrombin activity which is released under appropriate incubation conditions. Thus a search for the type of activity of interest would be complicated by the problem of distinguishing between release of nascent prothrombin, and reactivation of reduced prothrombin in microsomes. For this reason reactivation activity has been investigated only in the microsomal supernate fraction.

Initial problems with the two-stage assay procedure indicated that potassium phosphate and tris buffers are both unsuitable for reactivation. Both these buffers cause problems in the assay technique. For simplicity a buffer was chosen for reactivation studies which would not interfere with subsequent assay techniques - Krebs-Ringer was the logical choice.

Results of the initial trial performed with Krebs-Ringer buffer are given in Table 7. The results given here are measurements of the clotting time of aliquots removed from the combination of 0.5 ml of incubation mixture and 0.5 ml of a 1:20 dilution of the bovine serum preparation in physiological saline. Incubation was carried out for 30

TABLE 7

EFFECT OF VARIOUS ADDITIONS ON THE REACTIVATION OF REDUCED PROTHROMBIN

Additions					
Reduced Prothrombin	GSH[a]	DHA[a]	Microsomal Supernate[b]	MDSES[c]	Clotting Time (Seconds)[d]
+					210
+		+			197
+		+	+		130
+		+		+	210
+		+	+	+	194
+	+				195
+	+		+		192
+	+			+	185
+	+		+	+	155

Incubation system consisted of 2.0 ml Krebs-Ringer buffer, pH 7.0, and 0.1 ml of reduced prothrombin (0.05 mg protein) in 0.1 M acetic acid for a final volume of 2.10 ml. GSH and DHA were dissolved in the incubation buffer. When rat liver enzyme was added, it replaced an equivalent volume of Krebs-Ringer buffer, so a constant incubation volume was maintained. Incubation was carried out at 37° for 30 minutes.

(a) GSH and DHA present at a final concentration of 1×10^{-3} M.
(b) microsomal supernate added as 0.5 mg of protein.
(c) microsomal disulfide interchange system added as 0.1 mg protein.
(d) see methods section for description of clotting assay.

minutes at 37°C in a shaking waterbath. Reduced glutathione (GSH) and dehydroascorbate were added to give a final concentration of 10^{-3} M. Activity in microsomal supernatant protein was investigated by adding 0.5 mg of dialysed microsomal supernatant protein prepared from rat liver. Microsomal disulfide exchange system was compared at the stage of purity obtained with ammonium sulfate fractionation of a microsomal acetone powder. One tenth of a milligram of protein was added, and this amount gives good reactivation of reduced bovine pancreatic ribonuclease (46).

The denatured prothrombin used as substrate in this assay contained 50 μmoles of free sulfhydryl group (SH) as measured by the DTNB reaction per μ mole prothrombin. Although it was not realized at the time this represents a considerable contamination of the sample with 2-mercaptoethanol. This may have a bearing on dehydroascorbate (DHA) requirement which appears uniquely in this experiment. This experiment was designed to test the ability of crude microsomal supernatant protein as compared with partially purified microsomal disulfide interchange enzyme in producing prothrombin activity.

The incubation systems containing dialysed crude microsomal supernatant show considerably more prothrombin activity than those with microsomal disulfide interchange system. No clear cofactor effects are observed here, although it is perhaps significant that controls lacking enzyme source, but containing GSH or DHA did not vary greatly from the control with no additions.

Rough testing indicated that most of the microsomal supernatant enzyme activity was present in protein precipitate from 33 to 66% saturation with $(NH_4)_2SO_4$. This preliminary evidence was verified in

the experiment presented in Table 8. Since the previous experiment showed the largest amount of prothrombin activity in the system containing dialysed crude microsomal supernatant and DHA, the effect of DHA was checked with the fractions of microsomal supernatant used this time. Ammonium sulfate fractionation of microsomal supernatant was carried out as described in the methods section, and desalted on Sephadex G-25. Again 0.5 mg of enzyme protein was added per incubation except for 0.1 mg of microsomal disulfide interchange preparation. Fresh substrate used in this assay showed a value of 18 moles SH per mole of prothrombin, and this value has subsequently proved much closer to an average than 50 moles SH per mole.

Without the excess SH present in substrate, DHA has no apparent effect on production of prothrombin activity. Microsomal disulfide interchange enzyme either alone, or in combination with other enzyme fractions shows no effect on the system. The largest amount of prothrombin activity appeared in the tubes containing the 33 to 66% ammonium sulfate cut of the microsomal supernatant. Again there was no effect of microsomal disulfide interchange enzyme, and heating the enzyme fraction for 2 minutes at 60°C prior to assay produced a marked reduction in activity. Due to an oversight the crude microsomal supernatant fraction was not dialysed before the assay, and failure to dialyse blocks all activity.

At this stage several questions arose as to the nature of the "prothrombin activity" appearing during the incubation of reduced prothrombin. Two of the major ones were: (1) is prothrombin activity appearing in the control tube during incubation? and (2) do all the

TABLE 8

ACTIVATING FACTOR (AF) ACTIVITY IN AMMONIUM SULFATE FRACTIONS OF MICROSOMAL SUPERNATE

Additions[a]						Clotting Time (Seconds)
$(NH_4)_2SO_4$ Fraction of Microsomal Supt.						
0-100	0-33	33-66	66-99	DHA	MDSES	
						120 ± 1.3
				+	+	125 ± 3.7
+				+	+	120 ± 3.7
	+			+	+	102 ± 3.3
		+		+	+	85 ± 4.2
			+	+	+	107 ± 0.8
		+				87 ± 2.6
		+[b]		+	+	115 ± 1.7

[a]System as described in Table 7. Enzyme was present as 0.5 mg protein where added.

[b]Enzyme fraction heated at 60°C for two minutes prior to addition to incubation tube.

incubation mixtures start out at zero time with the same amount of activity?

The first question is important in understanding whether enzyme is an absolute requirement for obtaining prothrombin activity in the incubation system. As the assay is being run presently there is no information available as to the effect of incubation in an air atmosphere at pH 7.0 upon production of prothrombin activity. These conditions result in rapid oxidation of half cystine residues to the sulfonic acid derivatives in the presence of heavy metal ions. Some disulfide formation may also be occurring. Is any of this oxidation restoring activity to the reduced material?

The second question is essentially asking if any prothrombin activity is being added with the enzyme protein. All other components of control and experimental system are identical. Results obtained so far are only valid if there is no prothrombin activity in the enzyme preparation. This has been assumed, since observations on the intracellular localization of prothrombin activity in subcellular liver preparations have shown the level of prothrombin to be below the threshold for detection in the soluble protein fraction.

In order to check on these two points all subsequent assays have been performed with non-incubated zero time controls for comparison with incubated values. All components were added to the incubation tubes while they were kept in an ice bath. When the total incubation volume of 2.10 ml is reached, 0.5 ml was withdrawn before incubation and added to the serum source of Factors V and VII. Zero time values were assayed while the remainder of the material was being incubated. When the pres-

cribed incubation period had been completed, the tubes were removed from the water bath, placed in an ice bath, and a second aliquot of 0.5 ml removed for analysis.

Initial results from this controlled form of the assay are presented in Table 9. It is apparent that the zero time values do not vary greatly among themselves. This observation has held up quite well over many assays, and the apparent uniformity of this value is taken as evidence that the enzyme fractions being added do not contain prothrombin activity. Variations which do occur are attributed to difficulty in reproducing the transfer of small volumes exactly.

This initial trial does show some activity developing in the control tube. Again this type of experiment has been repeated many times, and this observation has not been consistent or reproducible. There is no reason to suppose that air reoxidation of prothrombin cannot lead to some restoration of correct disulfide pairing and enzyme activity. The amount of such non-enzymic reactivation would be expected to show a dependence on the purity of the prothrombin preparation. Enzymic reactivation which must operate in vivo in an environment containing many heterologous protein species could show a lesser dependence on purity of substrate. The purity of the prothrombin preparations has varied slightly with no effect on ability to observe enzymic reactivation of reduced material. Therefore, the changes occurring in the non-enzymic control are routinely subtracted from gross activity in the experimental tubes to obtain a net enzymic reactivation.

The astute reader will note substitution of the term "reactivation" (renaturation) for the more general "appearance of prothrombin

TABLE 9

AF ACTIVITY IN CM-SEPHADEX FRACTIONS OF A 33-66% $(NH_4)_2SO_4$ CUT OF MICROSOMAL SUPERNATE

Enzyme Fraction (200 γ Protein)	Prothrombin Time (Seconds)				2 x Error Mean Square
	Non-incubated	45 Minute Incubation	Difference	Enzymic Difference	
None	156	147	9	0	4.8
Microsomal Supernate	158	148	8	0	7.9
33-66% $(NH_4)_2SO_4$	160	142	18	9[a]	0.0
CM-Sephadex Peak 1	158	135	23	14[a]	1.5
CM-Sephadex Peak 1a	156	131	25	16[a]	3.1
CM-Sephadex Peak 2	155	139	16	7[a]	3.6
CM-Sephadex Peak 3	162	165	-3	0	3.3

[a]Significant enzyme activity at the 0.05 level of probability.

Enzyme incubation system as in Table 7.

activity". It is felt that some justification has been provided for this semantic switch. If a temporary suspension of disbelief can be maintained further evidence to support this change is forthcoming.

Additional information about the system requires further purification of the enzyme. The results of initial purification procedures following the 33 to 66% ammonium sulfate cut were given in Table 9.

From 200 to 400 mg of dialysed 33 to 66% ammonium sulfate cut of the crude microsomal supernatant were placed on a 4 x 40 cm. column of carboxy-methyl Sephadex (CM-Sephadex) equilibrated with 0.01 M $KHPO_4^-$ pH 6.86 and maintained at 4°C. The column was developed by increasing the molarity of the eluting buffer. A typical elution pattern obtained by diverting the column effluent through a Beckman model 135 Spectromonitor, is presented in Figure 9. With a flow rate of 50 ml per hour the column performed best with about 300 ml volumes of each ionic strength of buffer in a stepwise gradient. The first peak to emerge, representing overload and unretained material, and coming off in 0.01 M buffer was called peak 1. Subsequently peaks 2 and 3 were eluted, with most of the protein emerging with the front of increasing conductivity. Materials eluted with 0.05 and 0.10 M buffer were called peaks 2 and 3 respectively. Since this material obviously did not represent any degree of homogeneity no attempt was made to check activity in individual fractions collected within a peak. Rather the entire material was combined and assayed. Results in the previous table show the greatest specific activity to be present in a peak which appears to be an adjunct of peak 2, which in this case emerged prematurely and was unfortunately called peak 1a. Again in this preparation the crude microsomal fraction was

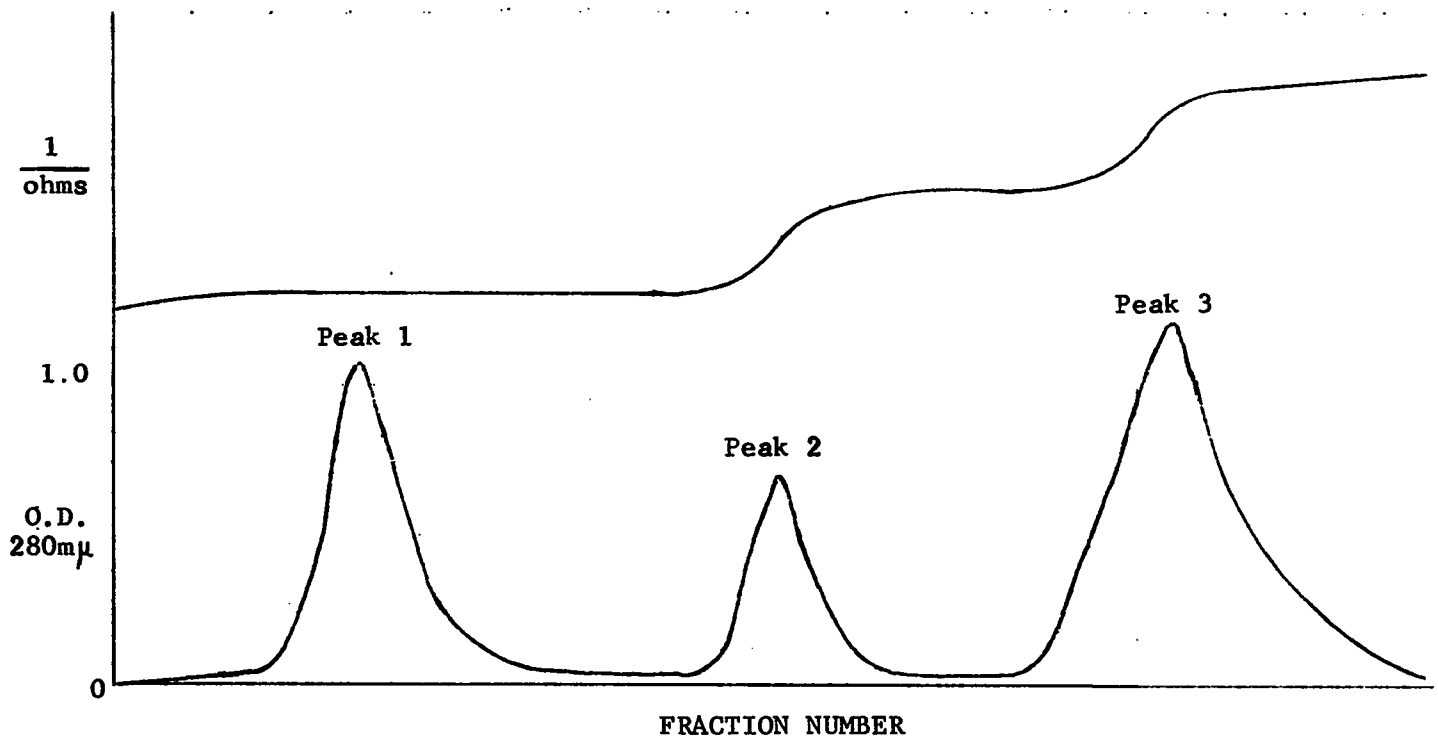

Figure 9. Protein elution pattern off CM-Sephadex. This is a typical example of the relationship between the conductivity of the eluting buffer, and the relative amounts of 280 mμ absorbing material eluted from a 33-66% ammonium sulfate cut of rat liver microsomal supernatant.

not dialysed, and shows no activity. Peak 3 appears to be slightly inhibitory in the clotting assay. Two tenths of a milligram of protein from the enzyme preparations was present in each tube.

One of the practical problems associated with column separation techniques, whether ion exchange or gel filtration is the dilution of sample which occurs during separation. The eluent collected off the CM-Sephadex column presented this problem of containing a small amount of protein in a large volume. Since concentration by lyophilization has the double disadvantage of possible loss of enzyme activity, as well as concentration of salt along with the protein, an ultrafiltration technique was attempted. The particular apparatus used consisted of a thick-walled collodion bag (Sartorius Membranfilter) with an opening for sample insertion and atmospheric equilibration, and a surrounding enclosed chamber. The chamber is evacuated to a pressure of 10 cm of mercury, and the pressure differential aids flow of liquid and small molecules through the pores in the collodion. Manufacturer's specifications give a pore diameter of <5 mμ. Although a defined pore size cannot specify exact pore exclusion size in terms of molecular weight, an approximate value for a 5 mμ pore would be a protein of 20,000 Daltons. With pore size <5 mμ one would expect a corresponding reduction in exclusion size.

In this fashion it was hoped to concentrate the activity sufficiently to attempt another purification step in a rapid manner which would not concentrate the buffering salts. Table 10 shows the unexpected results which were obtained on assaying the resulting materials. The activity present in peak 2 came through the ultrafilter membrane. Since the greater proportion of the molecules of <5000 Daltons has been left

TABLE 10

AF ACTIVITY IN CM-SEPHADEX ENZYME FRACTIONS

Enzyme Fraction[b]	Prothrombin Time (Seconds)				
	Non-incubated	20 Minute Incubation	Difference	Enzymic Difference	2 x EMS
Control (Expt 1)	234	212	22	0	22.8
Ultrafiltrate CM-Sephadex Peak 1, 4.0 γ	220	211	9		17.5
Non-filterable CM-Sephadex Peak 2, 125 γ	210	197	13		15.0
Ultrafiltrate CM-Sephadex Peak 2, 2.0 γ	217	192	25	3	7.3
Non-filterable CM-Sephadex Peak 3, 125 γ	186	191	-5		5.7
Ultrafiltrate CM-Sephadex Peak 3, 3.0 γ	172	174	-2		4.2
Control (Expt 2)	148	146	2	0	6.2
33-66% $(NH_4)_2SO_4$, 200 γ	154	131	23	21[a]	6.3
Non-filterable CM-Sephadex Peak 1, 100 γ	143	138	5	3	9.6
Ultrafiltrate CM-Sephadex Peak 2, 1.0 γ	147	129	18	16[a]	6.1

[a]Enzyme incubation system as in Table 7. Significant enzyme activity at the 0.05 level of probability.

[b]Treatment of a 33-66% $(NH_4)_2SO_4$ cut of rat liver microsomal supernate.

behind in desalting the ammonium sulfate cut on Sephadex G-25, the material coming through the filter only represents a small range of molecular size. Most of the protein was retained in the filter, and a good purification was obtained.

Some of the difficulties encountered with the assay technique are also revealed in this table. In the first part of the assay considerable variation occurred in results from the first control. This variation was almost large enough to mask enzymic effects occurring in the experimental tubes. Due to such problems, mean standard errors have been computed from standard deviations of the three zero time and incubated values. Differences between the two means must exceed twice the standard error of the means for statistical significance at the 0.05 level. This explains the presence of the doubled error mean square value in the tables.

At this point in history two sources of doubt about the validity of the assay technique were particularly worrisome.

Firstly, what is the source of the zero time, non-incubated prothrombin activity in the incubation system? Do these values represent residual prothrombin activity remaining after disulfide reduction?

The answer to this question is a very definite no. Two separate lines of experimental evidence supporting this negative conclusion are cited here. In the first place it is known that the bovine serum preparation used as a source of Factors V and VII contains residual prothrombin activity. The amount of this activity, and clear evidence of its origin, can be demonstrated easily with a different kind of control - one lacking reduced prothrombin. In this manner the knowledge

of an origin for this prothrombin activity, and the ability to quantitate it readily, can be used to provide definite evidence that no activity is present in the reduced prothrombin.

Table 11 shows results of an experiment designed to answer this question. It also answers the second major problem alluded to above. The uniformity of values obtained from the zero time assays has been taken as proof that no prothrombin activity is added to the incubation mixture with the enzyme. However, prothrombin activity could be produced from the AF enzyme during incubation.

Such a possibility has already been rendered unlikely by the estimate available for the approximate size of the molecule exhibiting AF activity. Bovine prothrombin is reported to have a molecular weight of 68,000, and the usual ideas of zymogen - active enzyme relationships make it difficult to conceive of a precursor molecule smaller than its native active form. As shown in Table 11 there is no appearance of prothrombin activity in incubation mixtures containing AF unless reduced prothrombin is also present.

Another important point is the effect of the AF preparation on activity of normal native prothrombin. This was investigated using purified prothrombin from bovine preparation number three corresponding to 0.05 units of prothrombin. AF activity was supplied in the form of 400 γ of protein from CM-Sephadex peak 2 level of purification. Table 12 shows that no significant differences in prothrombin activity appear between tubes incubated with and without enzyme. Native prothrombin does not seem to be affected by incubation with the AF enzyme.

The preceding results have been considered ample justification

TABLE 11

EFFECT OF TWO DIFFERENT FACTOR V AND VII PREPARATIONS ON THE POST-INCUBATION ASSAY

Additions				Prothrombin Time (Seconds)		
AF[a]	Reduced Prothrombin	Factor V & VII Prep #1	Prep #2	Zero Time	15 Minute Incubation	Difference
+		+		72	69	3
+			+	108	104	4
+	+	+		72	59	13
+	+		+	110	93	17

[a]Source of AF enzyme in this experiment is 100 γ of 33-66% ammonium sulfate precipitated microsomal supernatant protein from rat liver. Enzyme incubation system as in Table 7.

for the use of the term "reactivation" in describing the experimental observation, and calling the enzymic activity "Activating Factor".

A time curve of the reactivation process carried out with 1.0γ of AF protein at the level of purity obtained after ultrafiltration is shown in Figure 10. On the basis of this information, and an earlier time curve run with ammonium sulfate-precipitated protein, a fifteen minute incubation period has been used routinely. These data have now been converted from seconds of reactivation into units of prothrombin produced during the incubation. This conversion was essential since there is no absolute relationship between change in clotting time and units. The conversion has been made using values obtained by diluting two separate prothrombin preparations and plotting units of prothrombin versus clotting time (see section in this chapter dealing with assay methods).

One unit of AF activity is then defined as the activity required to renature one unit of prothrombin in one minute. Specific activity is then units of prothrombin renatured per minute per milligram of enzyme protein. Using these definitions, a purification table is presented (Table 13).

In this particular purification the starting material was liver from eight adult male rats averaging 250 gm body weight. Total wet liver weight was 65 gm. Enzyme activity in each fraction was estimated by using three different enzyme concentrations and a single, fifteen minute incubation period. Specific activities given in the table are taken from enzyme levels insufficient to consume all the substrate in the fifteen minute incubation time.

TABLE 12

EFFECT OF ACTIVATING FACTOR ON NATIVE PROTHROMBIN[a]

Enzyme Fraction	Prothrombin Time (Seconds) Zero time	15 Minute Incubation	Difference	Enzymatic Difference	2 x EMS
None	88	87	1	0	2.7
CM-Sephadex Peak 2, 200γ	85	83	2	1	3.4

[a]Enzyme incubation system as in Table 7 except that instead of reduced prothrombin, the native protein has been used as substrate in this assay. Bovine preparation number three is used here at a final dilution of 1:40,000.

TABLE 13

ENZYME PURIFICATION

Fraction	Total Activity (Units)	Yield (Percent)	Specific Activity Units per mg Protein	Purification
Crude Microsomal Supernate	17.82	100	0.0033	1
33-66% Ammonium Sulfate Fraction	2.71	15	0.0040	1.2
CM-Sephadex Peak 2	2.81	15	0.027	8.1
Membranfilter Filtrate	1.25	7	1.60	485

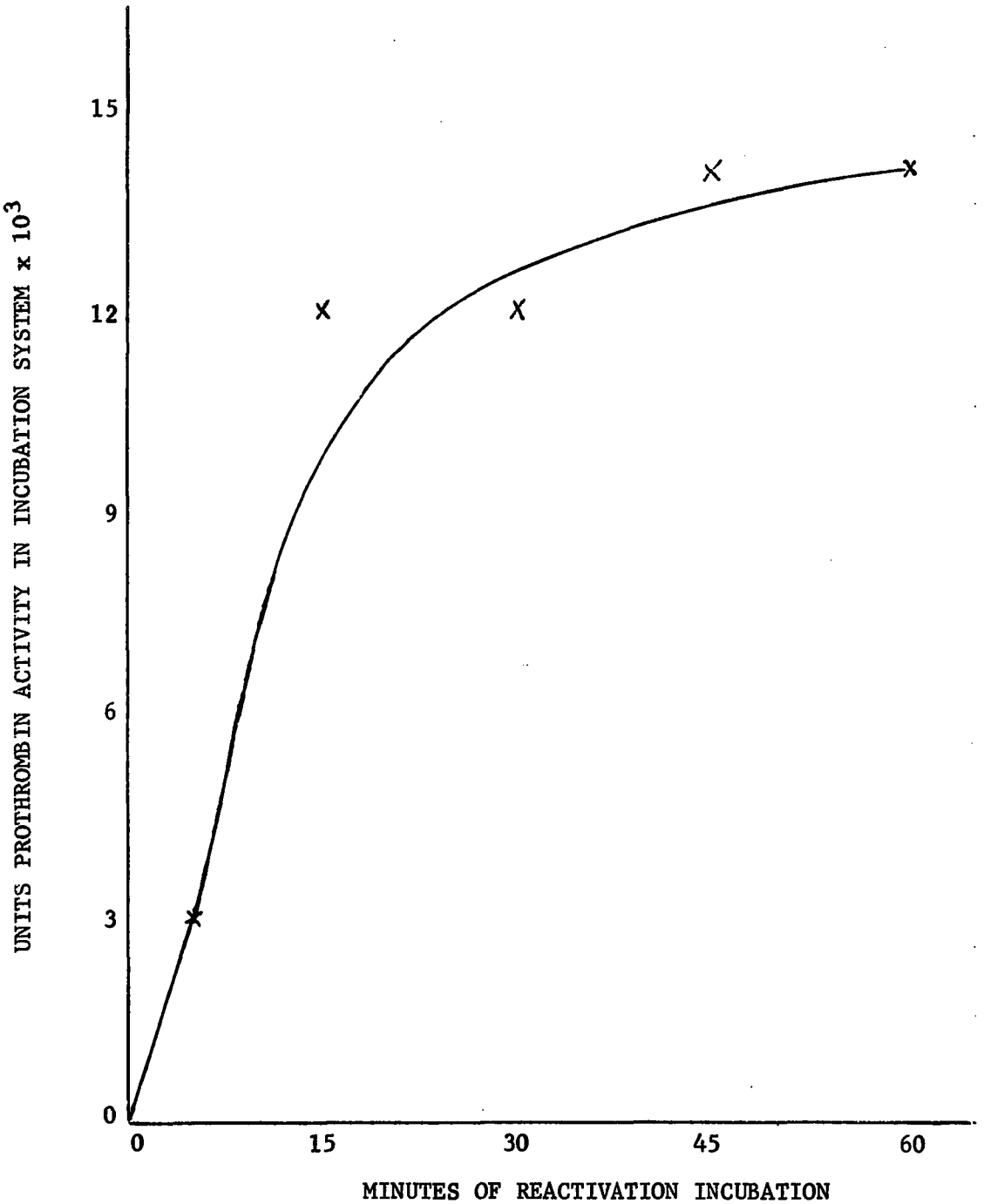

Figure 10. Effect of incubation time at 37° on amount of prothrombin activity produced. In this experiment, reduced prothrombin was present at a concentration of 45 γ per ml and enzyme purified to the level of S&S ultrafiltrate was used at a concentration of 0.5 γ per ml in the reactivation system. Values represent differences between control tubes lacking enzyme, and simultaneously incubated tubes containing enzyme.

It should be noted that low yield at the ammonium sulfate precipitation step is mainly a result of mechanical difficulties. Using G-25 Sephadex for desalting large amounts of protein with a fixed column size leads to overlapping between the protein and salt peaks. Monitoring of the column effluent for conductivity can be used to discontinue collection before appreciable ammonium sulfate has come through. Thus the overlapping protein is sacrificed. Incidentally, the emergence of AF activity in the G-25 void volume at this step is taken as evidence of a molecular weight greater than 5000.

At the level of purity reached after ultrafiltration the ultrafiltrate has occasionally been concentrated by lyophilization. When lyophilization is used instead of ultrafiltration with a membrane of an exclusion size of less than 1000 molecular weight, then a good part of the dry product obtained is buffering salt. On one occasion the salt accumulated during lyophilization was removed by another passage through G-25 Sephadex. It was of considerable interest to observe two separate peaks of ultraviolet absorbing material in the column effluent. The peak emerging from the void volume had a specific activity of 1.2 AF units per milligram protein. The smaller peak representing retained protein had a specific activity of only 0.067 AF units per milligram of protein.

No mention has been made yet of the amount of reduced prothrombin protein added as substrate in the incubation system. Exact control of amount of substrate between different experiments is not required, since meaningful comparisons are made on data within, not between experiments. Protein concentration in the different prothrombin preparations varies from 6 to 15 mg per milliliter. The concentration is diluted on

addition of urea, 2-mercaptoethanol and methylamine. Gel filtration techniques used for removal of reducing agents further dilutes the protein concentration down to a region of 1 mg per ml.

How much of the original activity is represented by activity produced during enzymatic reactivation? A wide range of substrate concentrations has been investigated, and the amount of reactivation obtained remains constant until substrate becomes the limiting factor. The theoretical yield of activity is only 0.5% at a concentration of 20.8 γ per ml of reoxidation system. As the substrate concentration is lowered to 6.0 γ per ml theoretical recovery is 1.2% and at 1.25 γ per ml recovery has increased to 4.2% of the activity added. When the substrate concentration is at 0.6 γ per ml the largest percentage of reactivation is obtained. At 0.1 micrograms per ml the limits of the assay system have been reached, and no reactivation can be measured.

The maximum recovery of added activity observed to date with the standard reactivation system is 12% when substrate is present at 0.5 micrograms per ml. A summary of results obtained from experiments in this area is presented in Table 14.

At this stage it is pertinent to ask about the type of function such an enzyme could have in vivo. This problem was attacked by investigating enzyme levels in vitamin K deficient rats. If any changes in the level of AF activity can be observed in vitamin K deficiency then we have presumptive evidence for the involvement of this enzyme in in vivo prothrombin synthesis.

Initial investigations were made by pooling livers of several vitamin K-deficient rats. Two separate pools were made of four rat livers

TABLE 14

EFFECT OF SUBSTRATE CONCENTRATION ON THEORETICAL YIELD OF ACTIVITY DURING REOXIDATION

Substrate Concentration Micrograms per Milliliter	Units Recovered x 10^2	Percent Reactivation
20.8	2.2	0.5
10.4	2.5	1.1
6.0	1.4	1.2
5.2	2.6	2.3
1.2	1.0	4.2
0.6	1.4	12.0
0.1	0	0
0.0	0	-

Enzyme incubation system as in Table 7, except the concentration of substrate has been varied.

each. Microsomal supernate was prepared in standard fashion, and AF activity was assayed at the 33 to 66% saturated ammonium sulfate-precipitable fraction level of purification. Fifty micrograms of enzyme protein was used per assay, and normal rats were used as a source of control enzyme. The first group of four had whole blood single stage prothrombin times of 27, 41, 71 and 24 seconds, for an average prothrombin level of 20% of normal. The second group of four had clotting times of 30, 78, 33 and 53 seconds and averaged 14% of normal prothrombin level. Table 15 gives the results of an assay comparing these two enzyme preparations with enzyme from normal animals. No statistical significance appears between the amount of reactivation produced in the normal preparation as compared with the preparation from vitamin K deficient animals. A trend was observed toward lower activity in enzyme from the group of rats having 14% of normal prothrombin levels as compared with the group having 20%.

Five more rats were investigated, but this time the 33 to 66% ammonium sulfate cut of the crude microsomal supernatant was prepared and assayed for activity independently for each rat. This group of rats was allowed to become more severely depleted of vitamin K than the previous group. Results from activity assays of this group of enzyme preparations shows statistically significant depression of activity in all five preparations as compared to the normal. Activity present in the AF preparations from the deficient animals ranged from zero to thirty-six percent of normal control enzyme. Thus it seems that a severe vitamin K deficiency is required to completely eliminate AF activity.

TABLE 15

CORRELATION OF IN VIVO PROTHROMBIN LEVEL WITH IN VITRO AF ACTIVITY IN VITAMIN K DEFICIENT RATS

Rat Number	Prothrombin Level (Percent of Normal)	AF Activity (Percent of Normal)
14	25	
18	12.5	
19	3	
22	39	
$\bar{x}$	20	125
16	20	
25	1	
26	17	
$\bar{x}$	14	70
15	6	0
20	6	9
21	3	19
23	5	35
24	6	0

Enzyme incubation system as in Table 7.

CHAPTER IV

DISCUSSION

Kinetic analyses has limitations, but a different kinetic approach to familiar data can give unexpected results. No attempts are being made to attack the utility of accepted methods of plotting clotting time in seconds against prothrombin concentration in units. This method is as good as any available for the estimation of unknown concentrations of prothrombin.

The disadvantages of this type of plot become apparent if any attempts are made to understand a sequence of enzymic events which occur during clot formation. When information about the relationship between enzyme concentration and rate of enzyme action is desired, it is provided best in this situation by plotting 1/t (seconds) against enzyme concentration. Justification for this approach is provided through manipulations of the Michaelis rate equation. When data obtained with dilutions of either citrated plasma or purified prothrombin are plotted against physiological ranges of enzyme concentration, the line obtained approximates linearity. A slight departure from the linear condition is consistently observed in the higher concentration ranges which approximate normal plasma prothrombin levels.

The assay conditions which seem to measure initial enzyme activity at low prothrombin concentration begin to give decreasing

increments of enzyme activity with unit increases in enzyme concentration. This situation becomes more pronounced as prothrombin concentration is raised to 2 and 3 times normal physiological concentrations. In the type of plot shown it is possible to propose the presence of an inhibitor of the clotting reaction.

If this hypothetical inhibitor were assumed to arise from fibrinogen during its conversion to fibrin, then it is always possible to express inhibitor concentration in terms of initial and final substrate concentration. Integrating the rate equation for competitive inhibition between the limits of initial and final substrate concentration shows that inhibition in this system would not be detectable as deviation from the expected linear relationship. The same holds true for noncompetitive and uncompetitive inhibition as long as the inhibitor concentration can be expressed in terms of the constant initial and final substrate concentrations. Integration of both these rate equations would give the same form as observed for the competitive case. Product inhibition occurring in a system which has only one measureable product concentration will not be detected by the assay techniques used here.

In the situation where inhibitor arises from proenzyme along with enzyme, the results are different. Here the kinetic assumptions fit the observed data. A calculated K_i for the enzyme-inhibitor complex is 0.85×10^{-7} M, indicating a strong affinity between enzyme and hypothetical inhibitor. Superficially, the process of developing inhibitor action along with enzyme from the same proenzyme seems inefficient and illogical. In this case, however, there may be some justification for such a mechanism. The production of too much thrombin activity in

response to a bruise or cut can be just as fatal as the production of none. Thrombus formation in the circulatory system is as likely to be terminal as unregulated hemorrhage. Internal regulation of thrombin activity could be an important aspect of homeostasis.

Wide intracellular distribution of ^{14}C (U) vitamin K_1 probably represents a combination of hydrophobic interactions and charge-transfer complex formation with proteins. Removal of most of the radioactivity by chloroform-methanol extraction provides evidence that this material is probably not active in prothrombin synthesis. This evidence is based on the lack of *in vitro* activity of vitamin K_1, except in liver slice (43) or perfused liver (22) experiments.

The presence of radioactive compounds in microsomes with an altered Rf value compared to vitamin K_1 indicates that some breakdown of the vitamin may be occurring in the microsomal membrane. The major metabolite, with an Rf approximating 2-methyl-1,4-napthoquinone, could be an intermediate in conversion of vitamin K to some active prosthetic group.

Lack of *in vitro* activity of vitamin K_1, itself, in cell-free systems could be due to several factors. It could be due to the form in which the vitamin is supplied, or possibly to failure to provide conditions adequate for a *de novo* protein-synthesizing system. Some essential cofactor for vitamin K action could be lacking in the *in vitro* system.

In considering these factors which could be preventing *in vitro* activity of vitamin K_1, it should be said that if vitamin K acts in prothrombin synthesis after having been degraded itself, then no information is available about such a metabolite. It is possible that reactions

which degrade vitamin K are closely linked to reactions which bind the vitamin to proteins, and the free metabolite is never detected. Without the stereospecificity available in a protein bound complex it is difficult to see how vitamin K could specifically affect the synthesis of only one group of proteins. Bioassayable amounts of vitamin K have been found covalently bound to rat liver subcellular fractions (48). According to Hecker - Mueller (30), the types of covalent linkages existing between protein and vitamin K may be fairly varied. The 5,6,7,8-tetralin p-quinol is a structural analogue of the A and B rings of estradiol. It also bears a strong resemblance to the napthoquinone nucleus of the vitamin K series. a fact which is not pointed out by the authors. There is a doubly interesting involvement here, since female rats, or male rats fed sufficient estradiol are quite resistant to the symptoms of vitamin K deficiency (29).

The tetralin p-quinol is a product of the action of an NADPH-dependent microsomal mixed function oxidase on 5,6,7,8-tetrahydro-2-napthol. It is proposed that the mixed function oxidase produces oxygen free radicals which attack the tetrahydro-2-napthol, giving a free radical form which reacts with acceptor molecules. Acceptor molecules may be small molecules in the reaction environment, or protein of the system. Ability of p-chloromercuribenzoate and N-ethyl maleimide to inhibit the enzymic system suggest a requirement for SH groups in the radical generating or accepting system. It seems to be more than coincidence that metabolites of estradiol and vitamin K are both bound to microsomal protein by an NADPH-dependent microsomal mixed function oxidase, are structurally related to a close degree and are both able to aid in prothrombin synthesis.

The time dependent appearance of protein bound radioactivity could be thought of as support for a covalently bound functional form of the vitamin. Although a considerable amount of time is required to obtain a large proportion of the label in the lipid insoluble fraction, it is possible that most of this non-extractable material is really non-specifically bound. Thus there is no evidence for a physiological role in binding which occurs after the deficient animal has synthesized enough prothrombin to restore the normal level of the blood. In its present form this data is not very helpful with regard to understanding vitamin K function.

At this point, discretion was considered to be the better part of valor, and a completely new approach was attempted. If the first approach has been based upon assumptions that intracellular location of the vitamin should help to reveal its mode of action, then the second attack assumes that something peculiar exists in the protein molecule affected by this vitamin.

The procedure used here for the preparation of prothrombin gives a product of good purity. The purity does not remain completely constant between preparations, as measured by specific enzyme activity, but this could be due to a slight degree of hemolysis in some of the plasma samples.

Calculation of an $s_{obs.}$ value for the purest preparations obtained gives a figure which is lower than the $s_{20,w}$ values presented in the literature. Literature values range from 5.3 according to Seegers (49), down through 4.9 obtained by Lamy and Waugh (50), to a low of 4.6 (51). Moore *et al.* (8) use the analytical ultracentrifuge as a tool to

provide evidence for the homogeneity of their preparation, but no mention is made of an s value which could be obtained from their sedimentation runs.

It is possible that the difference in purity of different preparations may have some effect on the s values. Seegers gives his values for specific activity in terms of Iowa units per mg tyrosine, and his best values correspond to 2200 Iowa units per mg protein. The preparation used in this preparation had a specific activity corresponding to 2900 units per mg protein. Thus the data indicate that bovine prothrombin may have a molecular weight in the region of 45,000 to 55,000, rather than 68,000.

The number of disulfide bonds present in the native molecule gives evidence for a complex structural situation. Evidence also exists for a complete dependence of enzyme activity upon the integrity of these disulfide bonds. Incubation with dithiothreitol at pH 7.0 and 0°C for 24 hours gives destruction of half the clotting activity when dithiothreitol is present is present in a 0.5 fold stoichiometry compared with disulfide bonds. If incubation is carried out at 24°C then all clotting activity is destroyed in 24 hours while at 37°C 99% of the activity is lost after two hours. Purified prothrombin can be incubated at 37°C for 24 hours with no change in the clotting activity present.

Since the aim of this study lies more in elucidation of control mechanisms than in repeating the structural studies of others, consideration has been given to the question of how the disulfide bonds pair correctly during the synthetic processes. Shapiro *et al.* (52) have proposed a mechanism for subunit assembly in gamma globulin. Using mouse

plasma cell tumours, they found L chains to be synthesized more rapidly and in excess of H chains. Free L chains appeared in the cytoplasm of the tumour cells, but no free H chains were observed. The evidence indicates that free L chains attach to H chains before the release of H's from the polyribosome complex. Thus correct configurational alignment and disulfide pairing are occurring during the translation process.

If the prothrombin molecule were composed of subunits, it could be proposed that vitamin K functions in the assembly of these subunits into native protein. The finding of a molecular weight of 48,000 for the species in 8 M urea and 1 x 10^{-3} M 2-mercaptoethanol rules out the possibility of any subunit structure in the native prothrombin molecule. A precise molecular weight determination on the reduced prothrombin molecule. A precise molecular weight determination on the reduced prothrombin molecule requires a more complete study of concentration dependence of the $s_{20,w}$ value, but the data conclusively show that native prothrombin does not contain two or more identical thrombin precursor subunits. If vitamin K cannot be involved in subunit assembly could it be involved with tertiary structure formation? Two logical alternatives exist: (1) primary amino acid sequence thermodynamically dictates correct disulfide pairing during or immediately after message translation, or, (2) some stereo-specific enzyme activity performs this function during or after polypeptide synthesis. If the formation of disulfide bonds occurs as in postulate (1), then this is positive evidence that vitamin K is not involved in this step of prothrombin synthesis.

A precedent already exists for determining the effect of primary amino acid sequence on disulfide pairing. Rearrangement of randomly

paired disulfide bonds in bovine pancreatic ribonuclease (46) can restore enzyme activity. Free sulfhydryl compounds, or a microsomal disulfide interchange system catalyse this rearrangement.

Observations made in this thesis suggest that systems which restore enzymic activity to randomly reoxidize ribonuclease are without effect on reduced prothrombin. The microsomal disulfide interchange system shows no activity, either with or without added dehydroascorbate. The effects of several oxidizing agents, including NAD, NADP, GSSG and DHA, are all negative. Reoxidation at neutral pH by exposure to air gives no restoration of prothrombin activity. All this negative evidence suggests that prothrombin does not have sufficient thermodynamic information present in its primary structure to allow resumption of native conformation after disulfide reduction. What then of the possibility of a specific enzyme carrying out this function only in the prothrombin group of proteins? The final section of the preceding chapter deals with the characterization of such an activity in rat liver microsomal supernate.

Several difficulties inherent in assaying for this type of activity were apparent before the experiments were performed. Among these was the question of species specificity. Practical considerations ruled out the use of combinations other than rat liver enzyme and bovine prothrombin. A lack of rat blood available as a prothrombin source has discouraged any thoughts of preparing rat prothrombin. Bovine prothrombin is only present at a concentration of 5 mg percent, and recovery procedures are generally not more than 50% efficient. Bovine liver was not used as an enzyme source due to the difficulty in manipulating rumin-

ant nutritional status through dietary changes. It may be hoped that the lack of species specificity observed for this enzyme is relevant to the large number of species which require vitamin K for prothrombin synthesis.

Another predictable difficulty in this system is the effect of concentration of reduced prothrombin on the theoretical yield obtained in the reoxidation reaction. If the RNase story is followed chronologically, it appears that Anfinsen started at a concentration of 2.0 mg of reduced ribonuclease per ml of reoxidation system in 1961 (46). In this paper there is no mention about the relative amount of reactivation observed. In 1963 (53) he has used a standard concentration of 0.018 mg per ml of reoxidation system to get a 70% theoretical recovery of activity. By 1964 (54) the concentration of reduced material has dropped to as low as 0.009 mg per ml in systems which give complete theoretical recovery. Apparently protein concentration, or, perhaps more accurately reduced protein-SH concentration is quite critical in any reoxidation system. Recovery of native activity can be measureable, but meaningless, if it represents an insignificant proportion of the theoretical activity present.

The conditions required for a good theoretical recovery of activity are subject to limitations imposed by the minimum assayable enzyme concentration. However, with prothrombin the random formation of intermolecular disulfide bonds may not be the only problem. Decreasing the protein concentration will only prevent incorrect intermolecular bond formation, not the improper intramolecular bonds which may be formed almost irreversibly in this case. It is possible that regulation of the

oxidation potential of the system could give higher values for the maximum obtainable amount of enzymic reactivation.

The minimum assayable concentration of prothrombin is about 0.1 to 0.2 γ per ml. The maximum recovery obtained on enzymic oxidation has been 12% at a concentration of 2.4 γ per ml of reoxidation system. Before assay this is diluted 1:1 to give only 1.2 γ of protein per ml in the assay. It seems that the limitations of the dilution process are imminent.

A critical assessment of the activation factor, of the product of its reaction with prothrombin, and of the reaction conditions strongly indicate that prothrombin activity is arising from oxidation of reduced prothrombin.

Preliminary observations relating activity of the AF enzyme with vitamin K nutritional status allow some speculation as to the regulatory function of vitamin K at the molecular level. Based on conclusive evidence that the primary site of regulation of prothrombin synthesis is really translation rather than transcription, the following model accounts for the facts known about the action of vitamin K.

Reading of prothrombin message RNA into prothrombin polypeptide requires that correct disulfide bond formation occur during the translation process. Without correct pairing, either enzymically inactive and immunologically inactive material is formed and released, or else the translation process just stops with incomplete polypeptide attached to the ribosome. Correct pairing requires an enzyme to align the SH groups properly, and the enzyme also functions as an electron acceptor in the oxidation of SH to disulfide bonds. This enzyme is required for correct

formation of two or more of the disulfide linkages occurring in the native prothrombin molecule. In its absence, either synthesis of prothrombin is halted before proceeding very far, or else the translation process occurs, but the product is inactive, and cannot be converted to native prothrombin even in the presence of enzyme. Vitamin K, or some metabolite of the vitamin is a cofactor in this enzyme, probably being reduced to the hydroquinone form by cysteine sulfhydryl groups, and then being reoxidized to the quinone form by oxygen.

The value of this model, like any other, lies in the types of creative thinking which it can produce, rather than in any absolute truth or untruth contained within. It is of necessity rather narrow and introspective. The specificity of the system under study demands this. Nevertheless, its principles reflect a logical continuity with the ideas of "molecular biology", and they may have implications for those interested in the regulation of protein biosynthesis.

CHAPTER V

SUMMARY

Vitamin K, given in large doses, is distributed uniformly among the liver subcellular fractions of both vitamin K deficient, and normal rats. In the short time interval between vitamin K administration to deficient animals, and initiation of prothrombin formation, most of the administered vitamin K is extractable with lipid solvents. Covalent binding of vitamin K_1 radioactivity to protein accounts for a considerable proportion of the recovered radioactivity only at a time period after the prothrombin level has returned to normal. The rather low specific activity of the vitamin K_1 used in the experiment does not allow a clear choice between requirements for covalent, or hydrophobic binding of the vitamin in the production of prothrombin.

Bovine prothrombin contains a minimum of eight disulfide bonds per molecule. DTNB provides an extremely sensitive assay method for the low concentration of sulfhydryl groups present after reduction of dilute protein solutions. Ionic environment about the free sulfhydryl groups may affect reactivity with DTNB, so the values obtained only represent a minimal number. Attempts to demonstrate the presence of subunits in the bovine prothrombin molecule have been unsuccessful.

Prothrombin which has undergone complete disulfide reduction

does not regain any enzymic activity on atmospheric reoxidation. Common biological oxidants such as dehydroascorbic acid, oxidized glutathione NAD and NADP are ineffective in restoring enzyme activity when present in the reoxidation system. Incubation with a heat labile, non-dialysable fraction from rat liver microsomal supernatant can restore up to 10 percent of the original activity of reduced bovine prothrombin. Initial purification of this activating factor has been carried out in an attempt to characterize its action. Rats which are severely depleted of vitamin K do not possess assayable amounts of activating factor.

It is proposed that vitamin K, or some metabolite of the vitamin, may be a cofactor of the AF enzyme which is required in pairing and oxidizing sulfhydryl groups during prothrombin synthesis.

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