STUDIES ON PROTHROMBIN

BY

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ABBREVIATIONS

Many of the abbreviations used are those recommended by The Biochemical Society as set out in "Policy of the Journal and Instructions to Authors", Biochem. J., 169, 1-27 (1978). Non-standard abbreviations are listed below.

Buffer A	: 0.25 M sucrose, 0.025 Imidazole, 0.08 M KCl, pH 7.5
CRM	: Cross-reacting material
DEP	: diethylpyrocarbonate
DMSO	: dimethyl sulphoxide
DTT	: dithiothreitol
EDTA	: ethylenediaminetetraacetic acid
fluorescamine	: 4-phenyl spiro furan-2(3H) 1'-phthalan-3, 3'dione
NADH	: nicotinamide adenine dinucleotide, reduced form
NP-40	: Nonidet P-40
PMS	: post mitochondrial supernatant
PMSF	: phenyl methyl sulphonyl fluoride
PPO	: 2, 5-diphenyloxazole
PP888	: benzyl-3, 5-dichlorol2, 6-difluoro-4-pyridyl ether
PP493	: 2,6-difluoro-3, 5-dichloro-pyridinol
PRM	: prothrombin-related material
SAC	: Staphylococcus aureus Cowan 1 strain cells
SDS	: sodium dodecyl sulphate
TEMED	: tetra methyl ethylene diamine
TLE buffer	: 0.2M Tris-HCl, pH9.0, 0.1M Licl, 22 mM EDTA, 1% SDS
V8	: Staphylococcal V8 protease

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CHAPTER 1

INTRODUCTION

1.1 Derivation of the Thesis

During routine toxicological testing, the potential herbicide PP888-benzyl-3, 5-dichloro-2, 6-difluoro-4-pyridyl ether (see Figure 1.7.1) was found to have anticoagulant activity which was overcome by the administration of vitamin K_1. In addition, it was noted that the effect on male rats was far more extreme than that on females, but sensitivity could be reduced by castration or administration of oestrogen to the male. It was felt that PP888 might be a useful tool for investigating the relationship between vitamin K_1 and the production of vitamin K-dependent coagulation factors in the liver.

1.2 Structure and Function of the Blood Coagulation Cascade

Blood remains fluid until it comes into contact with a foreign surface or until the blood vessels are damaged. In either case, physiological changes occur which result in the formation of a fibrin clot. The cascade model of coagulation, proposed independently by MacFarlane (1964, 1965) and Davie and Ratnoff (1964), was the culmination of over 50 years of clinical observation and investigation into clotting defects. In this model both the intrinsic pathway, initiated by contact with a foreign surface and the extrinsic pathway, activated through tissue damage, involve the sequential activation of a train of clotting factors. Each factor exists as an inert zymogen in the plasma until coagulation is triggered. On activation, each factor is transformed into an enzyme which activates the next member in the series. In this way, an

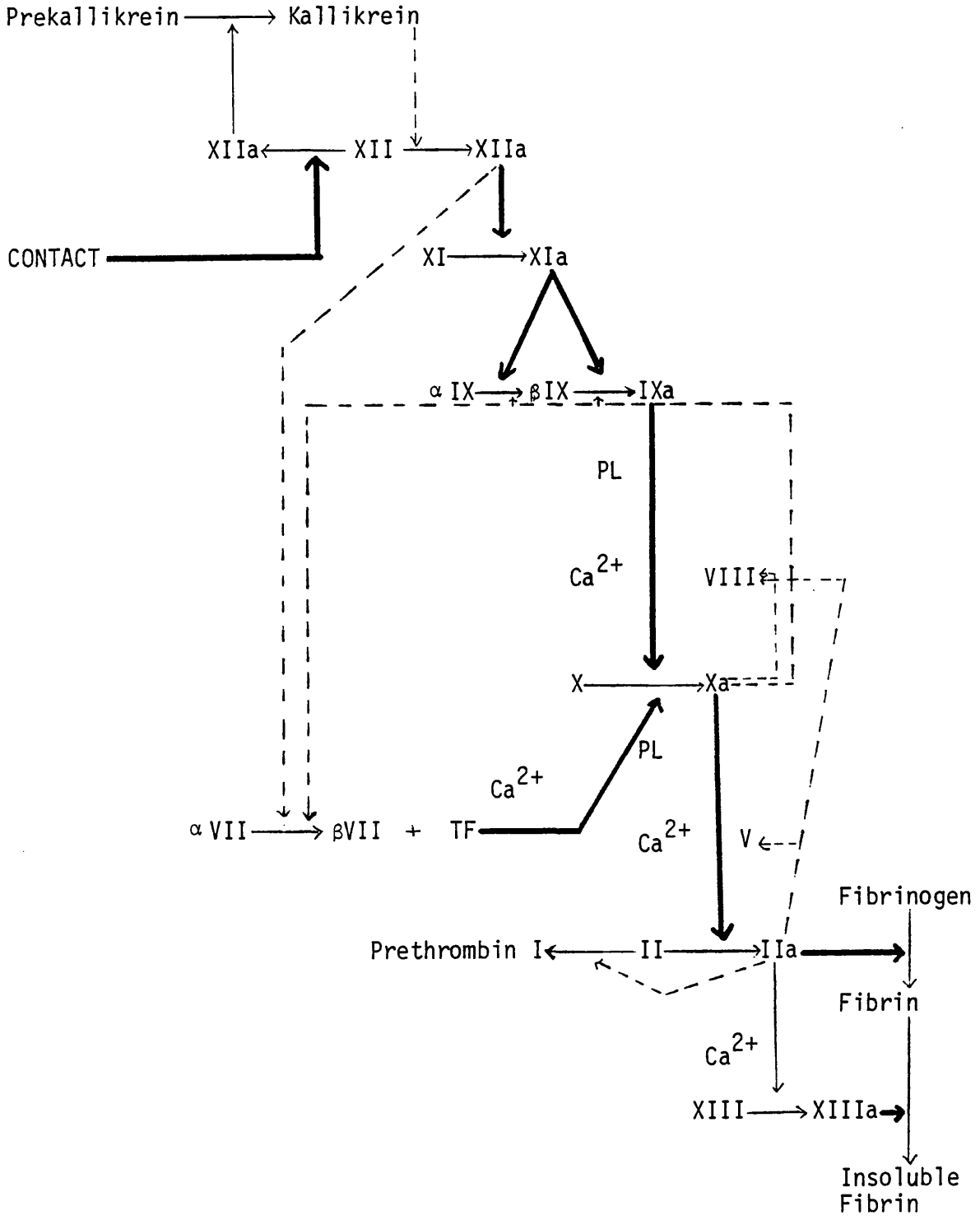


Figure 1.2.1 : A scheme for the interaction of the blood coagulation factors. Thick arrows denote the action of enzymes on their specific substrates. Positive and negative feedback loops are indicated by interrupted lines.

FACTOR	SYNONYM
I	Fibrinogen
II	Prothrombin
III	Thromboplastin; tissue extract
IV	Calcium
V	Accelerator globulin; proaccelerin
VII	Proconvertin
VIII	Antihaemophilic factor; antihaemophilic globulin
IX	Plasma thromboplastin component; Christmas factor
X	Stuart-Prower factor
XI	Plasma thromboplastin antecedent
XII	Hageman factor
XIII	Fibrin stabilising factor

Table 1.2.1 : The Roman Numerical nomenclature of blood clotting factors together with some common synonyms.

amplification of the initial stimulus is achieved and relatively large amounts of fibrin are produced at the site of damage.

The cascade model has been considerably modified to encompass the formation of multi-component complexes, to acknowledge the modifier role of several of the factors and to include the subtleties of feedback controls on and within the system. A far from complete blood coagulation scheme is given in Figure 1.2.1. The Roman Numerical nomenclature of blood clotting factors, together with some of their synonyms is given in Table 1.2.1.

The coagulation cascade does not occur in isolation, but is intimately linked with the kallikrein system, (involved in the inflammatory response), the complement cascade (involved in the immune response) and fibrinolysis (involved in clot lysis).

1.3 The Vitamin K-dependence of Several Clotting Factors

In 1935, Dam discovered that a haemorrhagic disease in the chick, associated with vitamin K-deficiency was due to the absence of prothrombin (factor II) in the plasma of these birds. Similarly, a haemorrhagic disease in cattle, associated with the consumption of clover containing dicoumarol was characterised by a decrease in blood prothrombin activity (Campbell and Link, 1941). Dicoumarol was found to act as an antagonist of vitamin K. The vitamin K-dependence of factors VII, IX and X was also recognised but the precise action of the vitamin in their production was obscure.

1.4 Evidence for the Existence of Inactive Precursors

Circumstantial evidence from coagulation studies on patients receiving anticoagulant therapy (Hemker et al, 1963) suggested the existence of a precursor protein involved in the formation of prothrombin. Support for the hypothesis came with the discovery of a biologically inactive form of prothrombin in the plasma of coumarin-treated cows (Stenflo, 1970). Using inhibitors of protein synthesis, Shah and Suttie (1971) provided evidence that vitamin K might be involved in the post-translational conversion of precursor protein to the biologically active prothrombin.

1.5 Identification of the Vitamin K-dependent Modification

Structural and functional comparison of prothrombin with its precursor led to the determination of the position and chemical nature of the vitamin K-dependent modification (Stenflo et al, 1974). The modification was found to occur near the NH_2-terminal of the precursor (Stenflo, 1973) where several specific glutamic acid residues are carboxylated to yield a new amino acid, ɣ-carboxy-glutamic acid (Stenflo et al, 1974; Nelsestuen et al, 1974; Magnusson et al, 1974). (See Figure 1.5.1.) Such modification confers biological activity on prothrombin by enabling it to bind calcium ions and hence take part in the protein-phospholipid interactions of the coagulation cascade (Nelsestuen et al, 1975; Esmon et al, 1975).

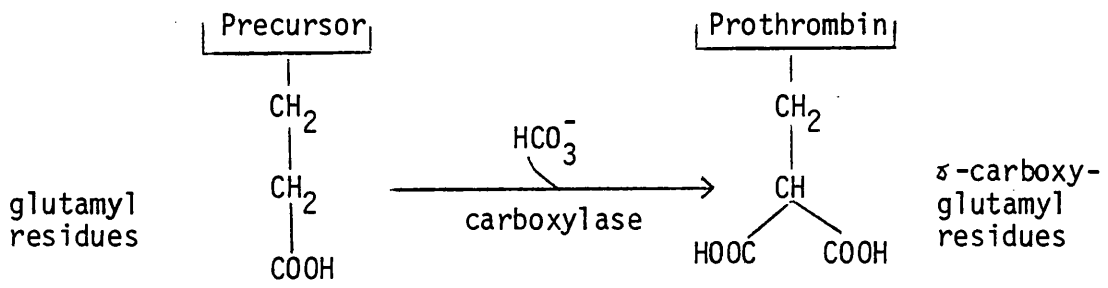


FIGURE 1.5.1 Vitamin K-dependent Carboxylation Reaction

The specific action of vitamin K in the biosynthesis of prothrombin is not known. It is generally assumed that the vitamin activates or transfers the HCO_3^- in the carboxylation reaction. It may also labilize the hydrogen at the γ-methylene group of the glutamyl residue so that it can accept the HCO_3^- (Suttie, 1977). The reaction has been shown to be biotin independent (Friedman and Shia, 1977) and recently Friedman et al, (1979) have found that the vitamin K-dependent γ-carbon-hydrogen bond cleavage can occur without concurrent carboxylation of the glutamyl residue.

1.6 The Site of Action of Vitamin K

The vitamin K-dependent proteins are synthesised in the liver. Suttie (1973) demonstrated a precursor pool in the liver of vitamin K-deficient rats and also rats treated with anticoagulants. He went on to isolate two different isoelectric forms of prothrombin precursor (Esmon et al, 1975a;Grant et al, 1976) from the microsomal fraction of the liver. Soon afterwards, Helgeland (1977) showed

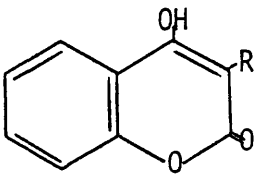
that the vitamin K-dependent carboxylation function is localised on the rough endoplasmic reticulum of microsomes.

1.7 Vitamin K Metabolism and the Vitamin K Antagonists

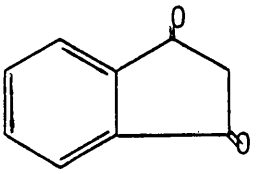
Antagonists of vitamin K - warfarin, phenylindanedione, 2-chloro K and tetrachloropyridinol (see Figure 1.7.1) - have been used to investigate the relationship between the various metabolites of vitamin K and carboxylation (see Figure 1.7.2). Results from these studies indicate that the anticoagulants can be divided into two groups (Ren et al, 1974). The first group, which includes warfarin and phenylindanedione, is thought to inhibit carboxylation through its action on the epoxide reductase activity (Ren et al, 1977). In warfarin-resistant rats, this enzyme is found to be much less sensitive to warfarin inhibition (e.g. Zimmerman and Matschiner, 1974). The second group, which includes 2-chloro K and tetrachloropyridinol, is thought to inhibit epoxidase (Willingham et al, 1976) and vitamin K reductase.

1.8 The Active Form of Vitamin K

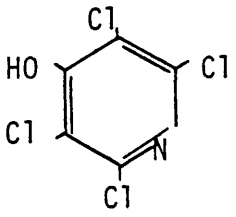
Willingham and Matschiner (1974) presented evidence for the involvement of a vitamin K - 2,3-epoxide cycle in the carboxylation reaction. Caldwell et al (1974) presented evidence against this whilst others (Sadowski et al, 1976; Girardot et al, 1976) demonstrated a requirement for the hyroquinone form of the vitamin. It seems that vitamin K- 2,3-epoxide, and hydroquinone are all intimately linked with the carboxylation reaction. A summary of vitamin K metabolism in liver microsomes is given in Figure 1.8.1.



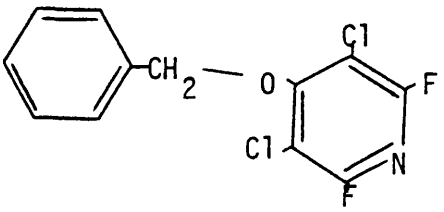
Coumarins



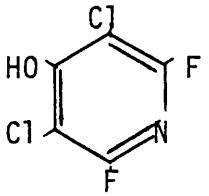
Indanedione



2,3,5,6-tetrachloropyridinol
(PP465)

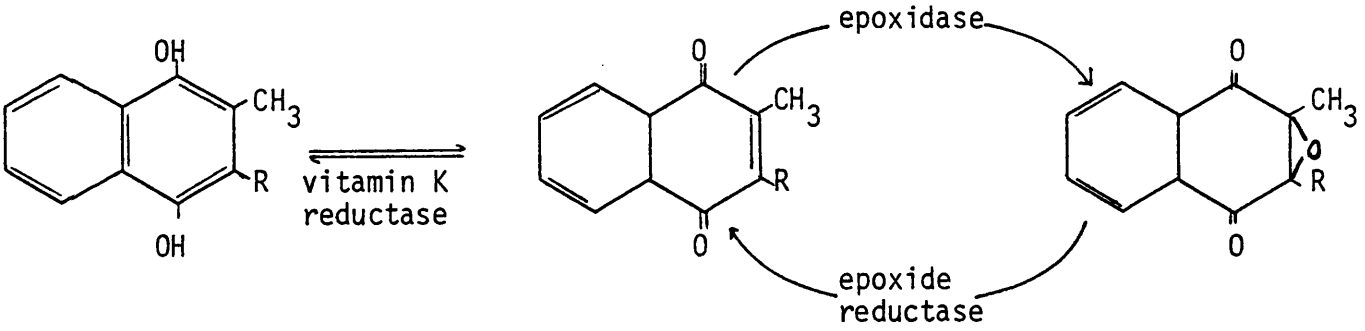


benzyl-3,5-dichloro-2, 6-difluoro-
4-pyridyl ether
(PP888)



2,6-difluoro-3,5-dichloro-pyridinol
(PP493)

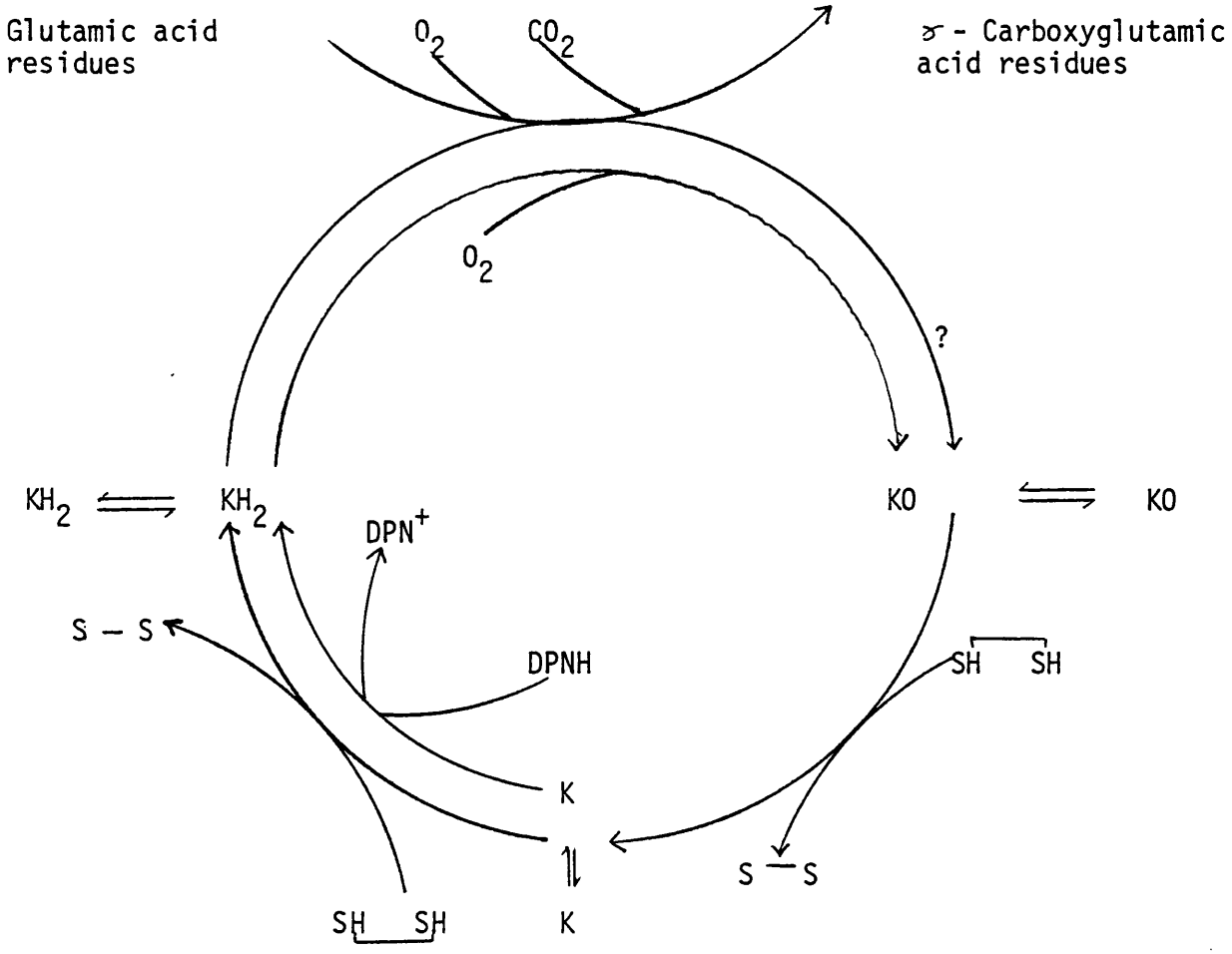
Figure 1.7.1 : Structure of vitamin K antagonists



HYDROQUINONE VITAMIN K 2,3-EPOXIDE

Figure 1.7.2 : Various metabolites of vitamin K which have been implicated in the vitamin K-dependent carboxylation reaction.

Figure 1.8.1 : Coupling of vitamin K-dependent carboxylation to vitamin K metabolism.



The ? in the figure indicates that intermediate forms of vitamin K may be involved in the carboxylation reaction. Esnouf et al, (1978) proposed that a superoxide intermediate is involved since they found that both carboxylation and epoxidation are inhibited by superoxide dismutase. On the other hand, Larson and Suttie (1978) have proposed that a hydroperoxide intermediate is formed during carboxylation since glutathione peroxidase, which reduces organic hydroperoxides, is found to inhibit both carboxylation and epoxidation.

1.9 The In Vitro Vitamin K-Dependent Carboxylation Assay

Much of the information regarding the requirements of the vitamin K-dependent carboxylation reaction has come from the in vitro system first developed by Shah and Suttie (1974). In this system, the microsomal fraction from vitamin K-deficient rats is found to respond to the addition of vitamin K by producing biologically active prothrombin. The carboxylation reaction requires HCO_3^-, O_2 and NAD(P)H though dependence on the latter can be removed if the hydroquinone form of the vitamin is used (Sadowski et al, 1976).

Studies on the carboxylation reaction have been greatly facilitated by the use of $H^{14}CO_3^-$ and the development of the pentapeptides, Phe-Leu-Glu-Glu-Val and Phe-Leu-Glu-Glu-Leu, which serve as synthetic substrates for the carboxylase reaction (Suttie et al, 1976). However, despite these innovations, the active metabolite of vitamin K and its precise mechanism of action remain unknown.

1.10 Post-Translational Processing of Prothrombin

Recently, using immunoadsorption techniques (Graves, Grabau and Munns, 1979), the precursor pool of prothrombin in H-35 hepatoma cells was investigated. Five different isoelectric forms of the precursor were identified having pI values of 7.2, 6.7, 6.2, 5.8 and 5.5. Graves et al, (1979) suggested that each form represented a stage in the processing of prothrombin and, from kinetic pulse data and subcellular fractionation data, they were able to order the processing events. (See Figure 1.10.1). They suggested that the 6.7 and 7.2 precursor species serve as the substrate and product, respectively, for the vitamin K-dependent carboxylase reaction. They also suggested that the precursors possessing pI values of 5.8 and 5.5 might represent asialo or partially sialated forms of prothrombin. It is clear from their work that the post-translational modification of prothrombin is very complex.

1.11 Aims and Achievements of the Present Study

Thrombin is the last active enzyme in the coagulation cascade and its zymogen, prothrombin, is synthesised in large quantities in the liver. It is therefore the most amenable of the vitamin K-dependent clotting factors to investigation. The conventional clotting assays for prothrombin measure only the biologically complete form. An assay for the determination of precursor prothrombin was therefore developed, utilising the venom of the carpet snake, Echis carinatus and the newly developed chromogenic reagent, Chromozym TH. The assay was used to investigate the distribution of the precursor in the rat liver fractions and to observe the effect of warfarin on this partition.

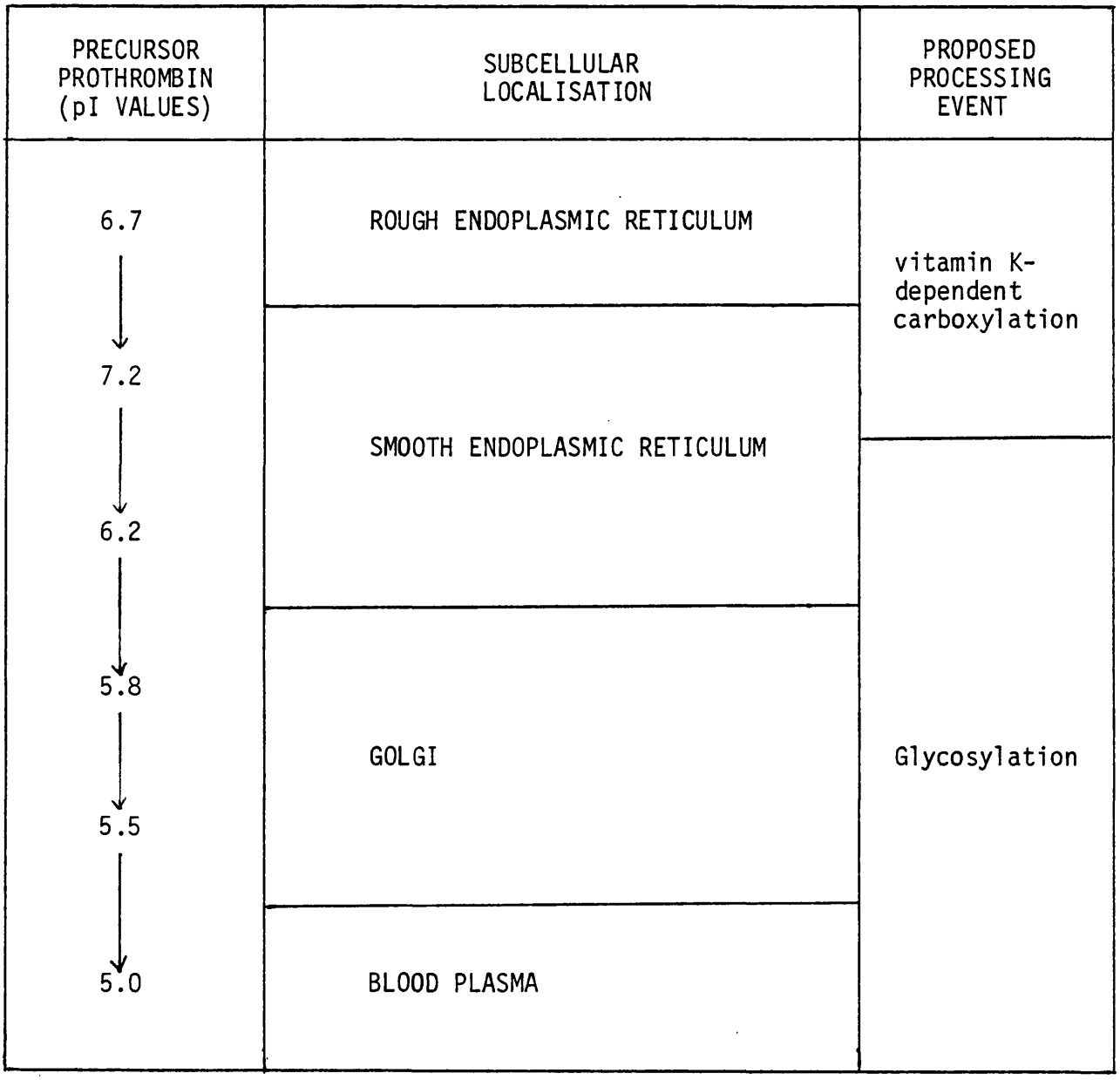


Figure 1.10.1 : Processing events associated with the maturation of precursor prothrombins (Graves, Grabau and Munns, 1979)

Post-translational modification of prothrombin was studied in an *in vitro* carboxylation system for precursor-prothrombin conversion. The action of the anticoagulant PP888 and the related compound PP493 (see Figure 1.7.1) on this system was compared with that of warfarin.

Because of the complex pattern of precursors in the rat liver, post-translational processing of prothrombin was investigated by isolating the primary, unmodified translation product. mRNA from rat liver was translated in the *in vitro* rabbit reticulocyte lysate translation system. The prothrombin-related translation product was recovered using specific antibody (raised against prothrombin which had been purified from rat plasma) and biochemically characterised. Its modification was then attempted using the *in vitro* carboxylation system.

CHAPTER 2 - MATERIALS

Chemicals used in the study are listed in alphabetical order. The source of each is given by a reference letter. The code to the lettering system together with addresses of commercial suppliers is given at the end of the list. All other reagents used were the finest grade available.

acrylamide	S	hirudin	S
amfix	MB	imidazole	BDH
ammonium persulphate	BDH	Instagel	P
antibodies	M	iodo(1-^{14}C) acetamide	RCA
ampholines	LKB	kaolin-cephalin reagent	DG
aprotinin	B	D-19 developer	K
barbitone	BDH	XR P5 film	K
barbitone (sodium)	BDH	^{35}S-methionine	RCA
benzamidine HCl	S	NN'bis methylene acrylamide	S
caesium chloride	BDH	β-mercaptoethanol	S
^{14}C-carbonate (sodium)	RCA	NADH	S
Chromozym TH	B	neomycin sulphate	S
chymotrypsin	BDH	Nobel agar	D
Coomassie blue R50	BR	Nonidet P-40	BDH
diethyl pyrocarbonate	S	oligo-dT cellulose	B
dimethyl sulphoxide	BDH	papain	S
dithiothreitol	S	PMSF	S
Echis carinatus venom	S	platelet substitute	DG
EDTA	BDH	Protein A-Sepharose CL-4B	PH
fibrinogen	KL	QAE-sephadex	PH
fluothane	ICI	rabbit reticulocyte lysate	RCA
Freund's complete adjuvant	D	sodium dodecyl sulphate	BDH
heparin	S	soya bean trypsin inhibitor	S

Staphyloccoccal V8 protease	S	triethylamine	PW
Taipan-$CaCl_2$	DG	Triton X-100	BDH
TEMED	S	trypsin	BDH
Thromboplastin	WH	vitamin K_1	S
Tricine	BR	Whatman filters	W

B : BCL Boehringer Corp., Lewes, Sussex

BDH : British Drug House Chemicals, Speke, Liverpool

BR : Bio-Rad Labs.Ltd., Watford, Herts.

D : Difco Lab. Ltd., East Molesey, Surrey

DG : Ortho Diagnostic, High Wycombe, Bucks.

K : Kodak Ltd. (Chemicals), Kirkby

KL : Koch Light Labs., Slough

LKB : LKB Instruments Ltd., South Croydon, Surrey

M : Miles Lab. Ltd., Stoke Poges, Slough

MB : May and Baker Ltd., Dagenham, Essex

P : Packard Instruments, Carversham, Bucks.

PH : Pharmacia (GB) Ltd., Hounslow, Middx.

PW : Pierce and Warriner (UK) Ltd., Chester, Cheshire

RCA : Radiochemical Centre, Amersham, Bucks.

S : Sigma London Chem. Co. Ltd., Poole, Dorset

WH : Withington Hospital, Withington, Manchester

W : Whatman, Maidstone, Kent

CHAPTER 2

METHODS

2.1 Animals Used

Alderley Park, Wistar-derived rats were housed in Wilmslow-type mobile rat units - 5 per cage. They were given free access to standard rat feed and water unless on special diet. Sprague Dawley rats used at Leicester University were housed in plastic cage units (North Kent Plastics Ltd.) with free access to standard rat feed and water.

2.2 Collection of Blood Samples

Rats were anaesthetised with fluothane, their thoracic cavities quickly opened and blood withdrawn by cardiac puncture using a 21g needle and plastic syringe. The blood was delivered into plastic tubes containing one-tenth volume of 0.013M trisodium citrate. The tubes were capped and inverted to ensure thorough mixing and to prevent the samples from clotting. The samples were placed in a Beckman Model TJ-6 centrifuge and spun at about 3000 rpm for 10 min. at $4^{o}C$. Finally, the citrated plasma was transferred to fresh plastic tubes for subsequent analysis.

2.3 Preparation of Plasma Pool

A normal plasma pool was constructed from the plasma of fifty male rats. Prior to pooling, the samples were screened using the kaolin-cephalin and prothrombin time tests (see below). Any samples showing abnormally short clotting times (indicating a degree of activation) or long clotting times (indicating factor deficiency) were discarded. The remainder were pooled and aliquots were stored in plastic tubes at $-20^{o}C$ until required.

2.4 Coagulation Screening Tests

2.4.1 Kaolin-Cephalin Time Test

Principle: The kaolin-cephalin mixture is used to assess the integrity of the intrinsic pathway. The test is sensitive to deficiencies in all clotting factors involved in the intrinsic pathway though sensitivity to factor I and II deficiency is limited.

Method: Kaolin-platelet substitute mixture was reconstituted with 10 ml water. 1 ml aliquots were placed in a series of glass tubes (50 x 10 mm) in a 37^{o}C water bath and the temperature was allowed to equilibrate. 0.1 ml of fresh citrated test plasma was added and allowed to incubate for 2 min. 0.1 ml of 0.05 M calcium chloride was added to trigger the reaction and the clotting time was measured. The test was carried out in duplicate and the mean clotting time for each sample was recorded.

2.4.2 Prothrombin Time Test

Principle: Thromboplastin is used to activate the extrinsic pathway. This test is sensitive to deficiencies in all the clotting factors known to be involved in the extrinsic pathway. However, its sensitivity to factor II deficiency is limited.

Method: Manchester Comparative reagent was diluted 1 in 10 with imidazole buffer (0.05 M imidazole, pH 7.4; 0.1 M sodium chloride) and an equal volume of 0.05 M calcium chloride was added. 0.2 ml of the calcium-thromboplastin mixture was placed in a series of glass tubes (50 x 10 mm) in a 37^{o}C water bath

and the temperature was allowed to equilibrate. 0.1 ml of test plasma was added, and the clotting time was measured. The test was carried out in duplicate and the mean clotting time for each sample was recorded.

The kaolin-cephalin time and prothrombin time determinations were routinely automated using the 'Coag-a-Pet' (General Diagnostic).

2.5 Specific Factor Assays

2.5.1 The Specific One-Stage Assay of Prothrombin (Denson et al, 1971)

Principle: Prothrombin is activated directly by the action of Taipan snake venom and the resulting thrombin acts on fibrinogen (supplied by adsorbed bovine plasma) to form a clot. (See Figure 2.5.1.)

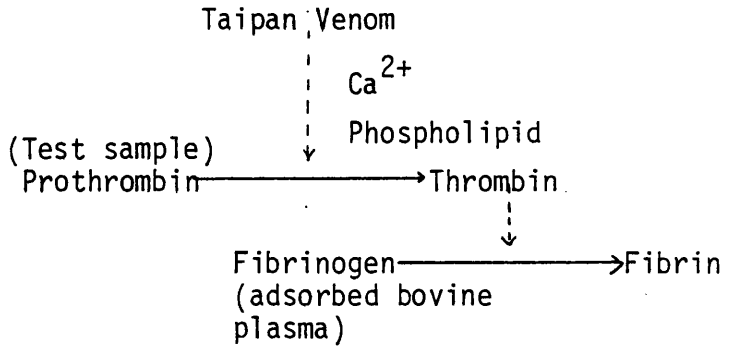


Figure 2.5.1 : The activation of prothrombin in the One-Stage Factor II assay using Taipan venom. (Factor II is provided by the sample under test.)

Method: 0.1 ml of adsorbed bovine plasma, 0.1 ml platelet substitute and 0.1 ml of control or test plasma dilution

(normal pooled plasma diluted 1 in 10, 1 in 20, 1 in 50 and 1 in 100 with imidazole buffer, test sample diluted 1 in 10) were placed in a series of glass tubes (50 x 10 mm) in a 37°C water bath, and the temperature was allowed to equilibrate. 0.2 ml of the Taipan/calcium chloride mixture was added and the clotting time measured. The test was carried out in duplicate and the mean clotting time for each control or test plasma sample was recorded.

Calculation: The reciprocal of the normal plasma concentration (1 in 10 dilution is arbitrarily assigned 100%) was plotted against clotting time using double log paper. (See Appendix 1) The level of prothrombin in test samples was expressed as a percentage of the normal plasma pool level by interpolation of the graph.

2.5.2 Echis carinatus Factor II Clotting Assay

Principle: Factor II is activated directly by Echis carinatus snake venom and the resulting thrombin acts on fibrinogen to form a clot. (See Figure 2.5.2)

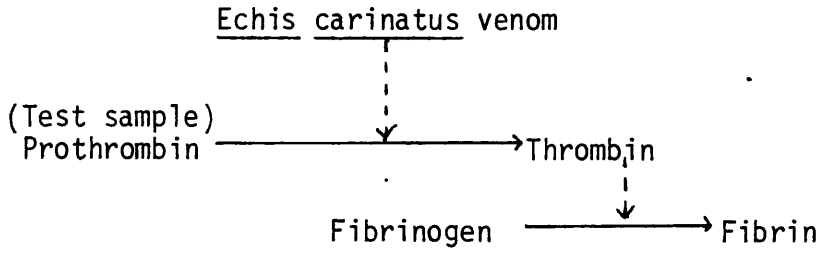


Figure 2.5.2 : The activation of prothrombin in the One-Stage Factor II assay using E.carinatus venom. (Factor II is provided by the sample under test).

Method: Normal pooled plasma was diluted to give a range of concentrations from 100% (1 in 10) to 5% (1 in 200) with imidazole buffer. 0.1 ml of the plasma dilution or test sample (diluted 1 in 10) was placed in a glass tube (50 x 10 mm) together with 0.1 ml of 1% clottable fibrinogen, in a 37°C water bath. To trigger the reaction, 0.1 ml of E. carinatus venom (1 mg/ml) was added and the clotting time measured. Each assay was repeated once and the mean clotting time was recorded.

Calculation: The reciprocal plasma concentration was plotted against clotting time on linear paper. A straight line relationship was found. (r=1) The level of prothrombin in test samples was expressed as a percentage of the normal plasma level by interpolation of the graph.

2.6 Chromogenic Assay for Prothrombin Determination

Principle: Prothrombin related material is activated by E. carinatus snake venom. The "thrombin" generated cleaves the synthetic substrate, Chromozym TH, to yield a coloured product, p-nitroaniline. The rate of appearance of the latter is measured at 405 nm.

Method: This is a modification of the methods of Latallo et al (1977) and the Boehringer test handbook.

2 ml of TRA-aprotinin buffer (0.1 M triethanolamine, 0.2 M NaCl, 0.5 mg/L aprotinin, pH 8.4) were placed in plastic cuvettes and prewarmed to 37°C. 0.1 ml of the suitably diluted sample was added. At time zero, 0.1 ml of E. carinatus snake venom (1 mg/ml) was

added and after thorough mixing, the cuvette was placed in a Perkin Elmer 356 spectrophotometer at 37°C. The mixture was incubated for 3 min. at which time 0.25 ml Chromozym TH (1.5 mM) was added with mixing. The rate of appearance of p-nitroaniline was recorded over the next 3 min. at 405 nm, and the enzyme activity of the sample calculated. All results were corrected for the control assay in which sample was replaced by buffer.

Calculation of Thrombin Activity:

$$\text{Enzyme activity} = \frac{Va \times 1000}{Vs \times \varepsilon \times d} \times \Delta \text{ A/min}$$

where Va = final volume of assay solution (2.45 ml)

Vs = sample volume (0.1 ml)

d = path length through cuvette (1 cm)

ε = molar extinction coefficient of p-nitroaniline

10,750 L/(mol x cm) = 10.75 cm/mol

(determined by standard dilution curve)

Hence thrombin activity = 2356 x ΔA_{405}/min (mU/ml)

2.7 Oral Dosing of the Rat

For intra-oral administration, a syringe fitted with a blunted hypodermic needle of sufficient length to pass completely into the stomach was used. The volume of the dose was calculated according to body weight.

2.8 Microsomal Fraction Preparation

Livers were fractionated according to the method of Sadowski, Esmon and Suttie (1976). The rats (150 - 200 g) were killed by dislocation of the neck. Their livers were quickly removed, washed, weighed and placed in 2 parts (w/v) of ice-cold homogenisation buffer (0.25 M sucrose, 0.025 M imidazole, pH 7.5). The livers were homogenised using 4 strokes of a teflon/glass homogeniser at approximately 1,000 rpm. The post-mitochondrial supernatant (PMS) was obtained by centrifugation of the homogenate at 10,000 rpm for 10 min. using a MSE Hi-spin 21 centrifuge. The PMS had an equivalent of 0.5 g of liver (wet weight)/ml supernatant . Microsomal fractions were prepared from the PMS by centrifugation at 105,000 g for 60 min. in a MSE Prep-spin 50. The supernatant (supernatant-2) was retained. The microsomal pellet was resuspended in buffer A (0.25 M sucrose, 0.025 M imidazole, 0.08 M KCl, pH 7.5) to give a final concentration of microsomes equivalent to 0.5 g of liver/ml and was either used fresh for vitamin K-dependent carboxylation, or was stored at -70^{o}C until required.

2.9 Vitamin K-Dependent Carboxylation

2.9.1 Unsolubilised System

This method is a modification of the methods of Esnouf (personal communication) and Bell (personal communication). (See Appendix 3) Microsomes were prepared as described, using rats treated with warfarin (10 mg/kg body weight). The incubation mix was as follows:

1.00 ml microsomal fraction

0.02 ml 3 mM DTT, NADH (50 mg/ml)

0.1 ml buffer A (containing inhibitors if required)

5×10^7 dpm of $Na_2{}^{14}CO_3$ (Amersham, 59 m Ci/mmole)

0.01 ml vitamin K_1 (10 mg/ml ethanol)

The reaction was started by the addition of the vitamin K. The tubes were sealed, shaken well and incubated in a covered bath at 27°C for 30 min. They were then placed in ice and 0.1 ml of 1 M $NaHCO_3$ was added. The incubation mix was centrifuged at 105,000 g for 1 hr. and the supernatant was discarded. The microsomes were surface washed with buffer and were then resuspended in 1 ml buffer with 0.03 ml of a 10% Triton X-100 solution (final concentration = 0.3%) and 0.1 ml of 1 M $NaHCO_3$. The mixture was frozen, thawed and vortexed. The suspension was centrifuged for 1 hr. at 105,000 g and the supernatant was decanted off into another tube. The pellet was surface washed with buffer which was added to the supernatant. 1 mg of albumin was added to the supernatant and the protein was precipitated with 10% TCA. The TCA precipitate was collected on a millipore filter, washed with 5% TCA, then dried and counted in 10 ml of Instagel scintillation fluid in a scintillation counter.

2.9.2 Solubilised System

The microsomes were resuspended in buffer A with 1% Triton X-100

to give a final concentration equivalent to 1 g of liver per ml. After standing at $4^{o}C$ for 1 hr, the mixture was centrifuged at 105,000 g for 60 min. Protein carboxylation assays were set up using the supernatant. After the reaction had been stopped by the addition of 0.1 ml of 1 M $NaHCO_3$, protein was TCA precipitated as previously described and the radioactivity determined.

2.10 Prothrombin Purification

Prothrombin was purified from rat plasma by the method of Grant and Suttie (1976a).

The blood from 35 rats, bled by cardiac puncture, was taken into one tenth volume of 0.001 M tri-sodium citrate. Following centrifugation at 5,500 rpm for 15 min. plasma was removed and frozen down at $-20^{o}C$. On thawing, the plasma samples were re-centrifuged at 8,500 rpm for 5 min. They were then pooled and heparin (7.3 mg/125 ml plasma) and soya bean trypsin inhibitor (2.5 mg/125 ml plasma) were added. The plasma pool was placed in a $4^{o}C$ cold room and 10 ml of 1 M barium chloride was added slowly whilst the mixture was stirred constantly. The cloudy mixture was stirred for a further 30 min. after which time the precipitate was collected by centrifugation at 3,700 rpm for 15 min. The precipitate was resuspended and washed 3 times in 10 ml aliquots of 0.005 M sodium citrate in 0.15 M sodium chloride. The final precipitate was dissolved in 10 ml of 0.2 M EDTA and was stored at $-70^{o}C$. On thawing, an equal volume of saturated ammonium sulphate was added and the mixture stirred for 15 min. The first ammonium sulphate cut was removed by centrifugation at 3,700 rpm for 15 min.

To the supernatant, a further volume of saturated ammonium sulphate was added and the mixture stirred for 15 min. The precipitated protein from the second cut was collected by centrifugation and then dissolved in 4 ml of 0.05M imidazole HCl (pH 7.8), 0.02 M sodium citrate, 0.001 M benzamidine HCl and 0.2 M ammonium chloride. This was dialysed against the same buffer for about 3 hr. and the dialysate was stored at -70^{o}C.

On thawing, the dialysate was passed through a QAE-Sephadex column, pre-equilibrated with 0.2 M ammonium chloride, using a salt gradient (0.2 - 0.45 M ammonium chloride) generated by a gradient mixer. The fractions collected from the column were assayed by chromogenic assay, and those showing the highest specific activity were pooled.

At each stage of the purification procedure a sample was retained for chromogenic assay and protein estimation so that specific activity could be monitored.

2.11 Antibody Production in the Rabbit

For antibody production, the prothrombin from the pooled fractions was precipitated by the addition of solid ammonium sulphate (to a final concentration of 66% saturation). The precipitate was redissolved in a small volume of saline (0.15 M) and dialysed against saline for several hours. The prothrombin concentrate was then ready for use.

6 ml of Freund's complete adjuvant was placed in a mortar and 3.5 ml of the prothrombin concentrate was added slowly, mixing thoroughly after each addition, to form an emulsion. The emulsion was forced several times through the nozzle of a syringe before it was ready for use.

An area on the side of the rabbit was shaved. Using a small bore needle 1 ml of the emulsion was injected intra-dermally into its side, at numerous sites. The antigen-adjuvant mixture was stored at $4^{o}C$. The rabbits were challenged in a similar manner 10 days later, and 7 days after this, 0.5 ml was injected sub-cutaneously in the scruff of the neck. After a further 7 days, 10 ml of blood was collected by ear bleed, and the anti-sera were characterised immunoelectrophoretically.

Ear Bleeding

Fur was shaved from the outer edge of the ear. Xylol was applied to the lower edge of the lobe to dilate the blood vessels, and vaseline was placed over the surface to stop the xylol evaporating. A small incision was made along the length of the marginal vein and about 40 ml of blood was collected, whilst pressure was applied proximal to the vein. To stop the bleeding, pressure was applied distal to the incision.

The blood samples were allowed to stand at room temperature until the clots had retracted. They were then spun at 3,000 rpm for 15 min. to collect the serum. The serum was treated with barium citrate - to remove endogenous prothrombin - prior to storage in aliquots at $-70^{o}C$.

2.12 Antibody Characterisation

2.12.1 Immunoelectrophoresis

Principle: This technique combines the immunological discrimination of double diffusion with zone electrophoresis.

Method: Immunoelectrophoresis tablets were dissolved in distilled water to yield a 1% agarose solution in 0.025 M Tricine buffer, pH 8.6. For 100 x 100 mm plates, 15 ml of the heated gel solution was applied to the agarose pre-coated plate. Using a template (see Figure 2.12.1), 7 wells were cut in the gel. Antigen sources were applied to the wells using narrow bore capillary tubes. Normal rat plasma (NRP) was treated with bromophenol blue which marked the front during electrophoresis. The plates were run at 280V (20A) for about 2.5 hr., using a 1 in 2 dilution of stock barbitone buffer (0.075 M sodium barbitone, 0.015M barbitone, pH 8.6) as electrophoresis buffer. When the front had moved about 60 mm the plates were removed and using a template, 6 troughs were cut between the wells. Antisera were applied to the troughs. The plates were placed in a moist chamber, and immunodiffusion occurred overnight. Precipitin lines could be seen in the gel between wells and troughs. The plates were washed in saline, then water, and then dried. The precipitin lines were stained using Ponceau S in trichloracetic acid, and the plates were finally destained with 5% acetic acid.

2.12.2 Crossed Immunoelectrophoresis (2-dimensional Laurell)

Principle: The combination of electrophoretic separation of proteins in agarose gel followed by electrophoresis

perpendicular to this in an antibody-containing gel produces 'rockets' where the antibody recognises antigen. The area enclosed by an individual precipitate is proportional to the antibody-antigen ratio of the system.

Method: This technique is the method of Laurell (1965). 1.5 g of agarose was dissolved in 100 ml distilled water with an equal volume of a 1 in 3 dilution of stock sodium barbitone buffer, (0.1 M sodium barbitone, 0.022 M barbitone, 3×10^{-4} M calcium lactate, pH 8.6). The agarose solution was heated above $56^{o}C$ and 18 ml were spread over each plate (100 x 100 mm) and allowed to cool at $4^{o}C$ in a moist chamber. When the gel had set, origin wells were cut using a 2 mm well cutter. (See Figure 2.12.2) A 4µl sample, labelled with bromophenol blue, was loaded into each well. The plates were placed in an electrophoresis tank with the origin towards the cathode and electrophoresis was carried out at 260 V (45A) using a 1 in 6 dilution of the stock buffer, until the front had moved about 60mm. Excess agarose was removed from around the first dimension tracks. 16 ml of antibody-containing agarose was then poured onto the remaining part of the plate and allowed to set. The plate was returned to the tank with the first dimension gel at the cathode (for investigation of anodically moving proteins). Electrophoresis was conducted at 140 V overnight. After the run, the gels were pressed, washed, dried, stained with Coomassie blue and destained.

2.12.3 Single Radial Immunodiffusion (Mancini, 1965)

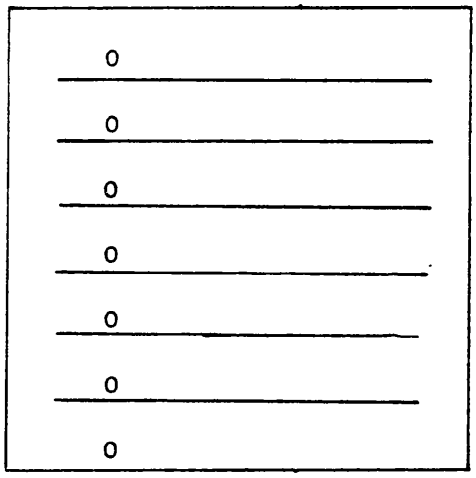
Principle: The antigen diffuses radially from a point application

into an antibody-containing gel, forming a circular precipitate at the equivalence point. With a constant antibody concentration and uniform gel thickness, the area encompassed by the precipitation ring is proportional to the antigen concentration. A plot of the diameter^2 against the antigen concentration yields a straight line.

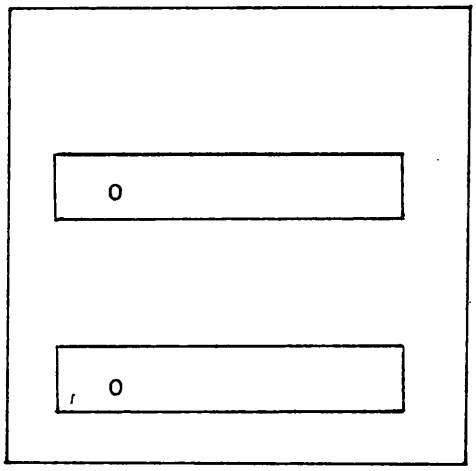
Method: Agarose immunodiffusion tablets were dissolved in distilled water to give a 1% agarose gel in Tris buffered normal saline (0.15 M at pH 7.2).

Between 10 and 40 µl of antiserum was added per ml of gel - the amount depending on the titre of the antiserum. Increasing the antibody concentration reduces the final ring diameter. The antiserum-agar mixture was spread evenly over clean 50 x 50 mm glass plates. After the gel had set, origin wells were cut in the gel according to the template. (See Figure 2.12.3.) Measured volumes of test and standard solutions were applied to the origin wells by micropipette. The loaded gels were placed in a moist chamber at room temperature, until completion of diffusion. Diffusion was deemed complete when the largest precipitin ring failed to increase in size as judged by daily measurement. For quantitation, the gels were placed in 1% tannic acid for 5 min to enhance the precipitin rings. The ring diameter was measured to the nearest 0.1 mm, using a graduated eyepiece. A standard curve was constructed by plotting d^2 versus standard concentration and the concentration of the test samples was determined using the curve.

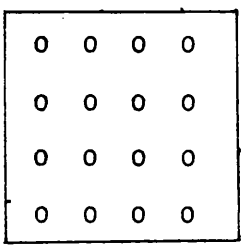
Figure 2.12 : Design of the plates used for standard immuno-electrophoretic techniques



i) Immunoelectrophoresis template



ii) Crossed immunoelectrophoresis template



iii) Mancini template

2.12.4 Immunoprecipitation and Chromogenic Assay

Principle: Protein A is a protein found on the surface of certain strains of Staph. aureus, which is known to bind non-specifically to antigen-antibody complexes. Following incubation of plasma with putative anti-rat prothrombin antibody, the antigen-antibody complexes are removed by adsorption to a protein A-Sephadex suspension. After centrifugation, the supernatant is assayed for thrombin activity using the chromogenic assay.

Method: Equal volumes of plasma and antiserum dilutions (1 in 100 and 1 in 1000) were mixed together and incubated at $37^{o}C$ for 1 hr. Following the incubation, 0.1 ml of a suspension of protein A-Sephadex in phosphate buffered saline was added and the mixture stirred gently for about 30 min to allow the protein A to bind the antigen-antibody complexes. The mixture was spun gently for about 2 min to pellet the sephadex beads. The supernatant was then tested by chromogenic assay.

2.13 mRNA Preparation

N.B. All glassware and reaction vessels were autoclaved; all solutions were sterilised or treated with diethyl pyrocarbonate (0.1%).

2.13.1 Direct Phenol Extraction (Noyes et al, 1979)

Rats were starved overnight prior to mRNA preparation. They were killed by dislocation of the neck, their livers quickly excised, washed and weighed. For every 10 g of tissue, 40 ml of phenol: chloroform:isoamylalcohol (50:48:2) and 40 ml of TLE buffer

(0.2 M Tris-HCl, pH 9.0, 0.1 M LiCl, 22 mM EDTA, 1% SDS) were added. The mixture was homogenised for 10 x 10 sec periods with 15 sec rests, on full speed in a Waring blender. The resulting homogenate was spun at 5,000 rpm for 15 min and the supernatant extracted with 20 ml of the phenol mixture. The aqueous phase was retained in a flask. The phenolic phase and the interphase were sequentially back extracted with two 15 ml volumes of TLE buffer, the aqueous phases being pooled after each centrifugation step. The interphase alone was extracted with a further 5 ml TLE buffer and again the aqueous phase was pooled. The pooled aqueous phases were shaken with 20 ml of the phenol mixture for 5 min and then spun at 7000 rpm for 10 min. The aqueous phase was used in all subsequent steps.

2.13.2 Centrifugation through a Caesium Chloride Cushion

The aqueous phase was layered over 0.2 volume of CsCl cushion (5.7 M CsCl, 0.1 M Na_2EDTA, pH 7.5) in cellulose nitrate centrifugation tubes. The samples were then centrifuged for 18 hr at 25,000 rpm and 25^{o}C in a Sorval AH650 centrifuge. DNA banded just above the CsCl cushion. Total RNA formed a clear gelatinous pellet at the bottom of the tube. After the supernatant had been removed, the pellet was taken up in 1 ml of sterile DEP-treated water (neutralised with Hepes buffer, pH 7.5). The total RNA samples were pooled. 0.1 volume of 2 M Na acetate, pH 5.0, and 2 volumes of ethanol were added and the RNA was precipitated overnight at -20^{o}C.

The precipitate collected by centrifugation at 18,000 rpm for 15 min was washed sequentially with a small volume of EtOH:H_2O: 2 M Na Ac. (20:9:1) and then ethanol. Finally it was taken up in 2 ml of sterile DEP-treated water and the optical density at 260 nm (OD_{260}) was determined in order to estimate the RNA concentration. (OD_{260} of 1.0 = 45.5μg RNA).

2.13.3 Oligo dT-cellulose Chromatography

Principle: Many mammalian mRNA's have a polyadenylate (poly (A)) rich region at their 3' end which allows them to bind to the oligo dT-cellulose column. Poly(A-) RNA (e.g. rRNA or tRNA) runs straight through the column.

Method: An oligo dT column was prepared by resuspending the oligo dT cellulose in binding buffer (0.4 M NaCl, 0.01 M Tris-HCl, pH 7.6, 0.02% SDS) and packing it in a column. The column was washed with 0.1 M NaOH and allowed to stand overnight. It was then washed with binding buffer until the pH was neutral. Solid NaCl and SDS were added to the total RNA sample to give a final concentration of 0.4 M and 0.02% respectively. It was then heated in a 60°C water bath for 2 min to completely denature the RNA and break up any double stranded RNA which might interfere with the separation on the column. The sample was applied to the column, which was then washed with between 10 and 20 ml of binding buffer. All the poly (A-) fraction was deemed to have come off the column when the OD_{260} had returned to the baseline level.

The column was then eluted with about 10 ml of elution buffer (0.001 M EDTA, 0.01 M Tris-HCl, pH 7.6, 0.02% SDS). The OD_{260}

of the fractions was determined and those containing RNA were pooled. NaCl was added to a final concentration of 0.4 M and the RNA was applied to a second oligo dT column which was washed and eluted as before. The poly (A+) fractions were pooled and ethanol precipitated overnight.

The mRNA was collected by centrifugation at 18000 rpm for 30 min, washed with ethanol and dried under vacuum. It was then taken up in 100µl of DEP-treated water and the OD_{260} of a small aliquot was determined. The concentration of the mRNA was adjusted to 1 mg/ml and the solution was then frozen down in aliquots at $-70^{o}C$.

2.14 In vitro Translation of Poly (A+) RNA

A commercially available rabbit reticulocyte lysate (prepared by a modification of the method of Pelham and Jackson, 1976) was used. It is nuclease-treated and therefore message-dependent. The routine assay was as follows:

80% (v/v) rabbit retic. lysate

20% (v/v) ^{35}S-methionine (1 µCi/ µl final concentration)

mRNA (not exceeding 10% by volume of the assay mix)

All reagents were kept on ice until required. The reaction was triggered by the addition of mRNA. Tubes were incubated at $30^{o}C$ for 90 min. after which time the reaction was complete.

2.15 Product Analysis

2.15.1 Counting of TCA-precipitable Protein

1 or 2 µl aliquots were withdrawn from the incubation mix,

placed on filter paper discs (Whatman No. 1) and allowed to dry. The discs were floated in 5% TCA with 2 mg/ml DL-methionine for 15 min. They were transferred to 5% TCA, boiled at 90°C for 5 min. and then rinsed in cold 5% TCA. The discs were dried by sequential rinsing in ethanol, ethanol-ether (3:1, v/v) and ether, and were counted by liquid scintillation in toluene with PPO and POPOP.

2.15.2 Immunoprecipitation

Principle: (Ivarie and Jones, 1979). Translation products are incubated with anti-prothrombin antibody. The immune complexes are then bound to Protein A on the cell wall of formalin-fixed, heat-inactivated Staphylococcus aureus Cowen Type 1 (SAC). The protein A binds rapidly and tightly to the Fc region of IgG molecules. The immune complexes bound to SAC can be readily isolated by centrifugation.

Method: For every 20 µl of translation mix, 5 µl of antiserum and 45 µl of immunoprecipitation buffer (50 mM Tris, 100 mM NaCl, pH 7.5, 0.1% NP40, 1 mM PMSF) were added. The mixture was allowed to stand overnight at 4°C. 20 µl of a 10% solution of washed SAC cells were added and the mixture incubated at room temperature for 30 min. The cells were isolated by centrifugation in a microfuge for 2 min. They were washed 3 times in immunoprecipitation buffer. After the final centrifugation step, the pellet was resuspended in 30 µl of Staph. A gel buffer (6 M urea, 4% SDS, 4% v/v β-mercapto ethanol, 10% v/v glycerol).

The suspension was boiled for 5 min. to release and denature the immune complexes. The cells were removed by centrifugation and the supernatant analysed by TCA counting or SDS gel electrophoresis for subsequent autoradiography or fluorography.

2.16 SDS Gel Electrophoresis (Laemmli, 1970)

Principle: Proteins treated with the detergent sodium dodecyl sulphate (SDS) completely unfold to form long, rodlike SDS-polypeptide complexes. The complexes contain a constant ratio of SDS to protein and differ only in mass. When subjected to electrophoresis in a molecular sieve gel, the rate of migration is determined by the mass of the complex. To calibrate the gel, markers of known molecular weight are run in parallel.

Method: The running gel was prepared from stock solutions A (3 M Tris-HCl, pH 8.9) and C (30% acrylamide, 0.75% NN' bis methylene acrylamide), water, 3% ammonium persulphate and 10% SDS. These were mixed together to give final concentrations of 0.375 M Tris-HCl, 0.1% SDS, 0.1% ammonium persulphate and an acrylamide concentration between 7.5 and 15%, depending upon the relative proportion of water to stock acrylamide solution. After degassing for 5 min. the gel was polymerised chemically by the addition of 0.03% by volume of tetra methyl ethylene diamine (TEMED).

The stacking gel was prepared from solutions B (0.5 M Tris-HCl, pH 6.7) and D (7.5%acrylamide, 0.25% NN' bis methylene acrylamide) instead of A and C to give final concentrations of 0.125 M Tris-HCl, 0.1% SDS and 3% acrylamide. The gel was polymerised as above. The electrode buffer (pH 8.3) contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS.

Samples were mixed with sample buffer to give final concentrations of 0.0625 M Tris-HCl (pH 6.7), 2% SDS, 10% glycerol, 5% mercapto-ethanol and 0.001% bromophenol blue as dye. Proteins were completely dissociated by boiling for 2 min. and were then applied to the tracks in the stacking gel. Electrophoresis was carried out at 100V constant voltage for about 3 hr. At the end of the run, the dye front and gel length were measured. Proteins were stained overnight with 0.2% Coomassie blue in 50% methanol, 10% acetic acid and destained with several changes of 25% methanol, 7% acetic acid. The gel was then dried down on a Biorad slab gel dryer for 1.5 hr under water vaccuum.

Mobility: Mobility was calculated by the method of Weber and Osborn (1969) using the following formula:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after staining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

When radioactively labelled samples were run, the gel was stained using a quick stain (0.1% coomassie blue in 50% methanol and 10% acetic acid) and destain (5% methanol, 10% acetic acid) by the method of Cleveland (1977).

2.16.1 For Autoradiography :

The gel was dried down onto filter paper. In the dark room, an X-ray film (Kodak, XRP5 film, 13 x 18 cm) was placed on top of the gel, which had been marked down one side with ^{14}C-labelled ink, and the two were sandwiched between metal sheets and wrapped up in silver foil. This was stored in a dark drawer under heavy lead weights until ready for development.

2.16.2 For Fluorography: (Bonner & Laskey, 1974)

The gel was soaked in 2 x 500 ml changes of dimethyl sulphoxide

(DMSO) for 2 hr to remove the acetic acid, methanol and water. It was then soaked for 3 hr in PPO/DMSO. After an hour of washing in several changes of water, the gel was dried down and an X-ray film exposed to it as described above. This was stored at -70°C under heavy weights until ready for development.

2.16.3 Development of X -ray Films

In the darkroom the film was taken out and placed in Kodak D19 Developer for 5 min. After a quick rinse in water, it was transferred to the fixer (Amfix, May and Baker) for a further 5 min. Finally the film was washed for 1 hr in running water and then dried.

2.17 Partial Peptide Digestion

Principle: Proteins isolated from SDS gels are cleaved by enzymatic proteolysis in the presence of SDS. Cleavage products are then analysed by polyacrylamide gel electrophoresis. The pattern of peptide fragments produced is characteristic of the protein substrate and the proteolytic enzyme and is highly reproducible. This is a modification (Macdonald, personal communication) of the method of Cleveland et al (1977).

Method: The protein to be digested, was run on a 10% SDS gel (0.042 mm thick). Protein bands were visualised by staining, and isolated by cutting the relevant bands from the destained gel. When radioactively labelled proteins were to be digested, they were visualised by cutting a narrow strip from the radioactive protein track and dissolving 2 mm slices in 100 µl of "10 volume" hydrogen peroxide at 60°C, overnight. 1 ml of Triton-toluene scintillation fluid (500 ml Triton X-100, 500 ml toluene, 8g PPO and 0.3 g POPOP) was then added and the

radioactivity determined. Once the peaks of radioactivity had been identified, the appropriate areas of the SDS gel were cut out. The bands were placed in 1 ml of SDS-PAGE V8 digest buffer, pH 6.8 (0.125 M Tris, 1mM EDTA, 0.1% SDS) and allowed to equilibrate, fresh buffer being added until the pH remained neutral. The gel slices were placed in the sample wells of a 12.5% SDS gel (1.5 mm thick, 80 mm deep) with a 10% stacking gel (50mm deep), and were overlayed with digest buffer containing bromophenol blue and 20% glycerol. The protease (1 µg/track) was then applied in the same buffer containing 10% glycerol. Electrophoresis was performed as usual until the dye front neared the bottom of the stacking gel. The power was then turned off and digestion allowed to take place for 1 hr. Electrophoresis was then completed and the gel stained, destained, dried down and autoradiographed or fluorographed as required.

2.18 Two-Dimensional Electrophoresis (O'Farrell, 1975)

Principle: Proteins are separated according to isoelectric point by isoelectric focusing in the first dimension and according to molecular weight in the second dimension, by SDS gel electrophoresis.

Method :

2.18.1 Sample Preparation

Following immunoprecipitation of the sample with anti-prothrombin antibody and SAC, the pellet was resuspended in lysis buffer (9.5 M urea, 2% NP-40, 2% Ampholines (comprising 1.6% pH range 5 - 7 and 0.4% pH range 3 - 10) and 5% β- mercaptoethanol). Following centrifugation, the sample was stored at -20^{o}C until required.

2.18.2 First Dimension

Isoelectric focusing gels were made in glass tubes (130 x 2.5 mm ID) presoaked in chromic acid and alcoholic KOH, rinsed and air-dried. The final constitution of the gel mixture was 9.2 M urea, 4% acrylamide, 2% NP-40, 2% Ampholines (pH range 3 - 10) and 0.01% ammonium persulphate. The tubes were filled to a depth of 120 mm and covered with gel overlay buffer (9 M urea, 1% Ampholines). After 1 to 2 hours this was replaced with 20 µl of lysis buffer and overlaid with water. After a further 2 hours, the gels were placed in a standard tube gel electrophoresis tank. Fresh lysis buffer was placed on the surface of the gel and overlaid with 0.02 M NaOH (the upper reservoir buffer which was degassed prior to use). The lower reservoir was filled with 0.01 M H_3PO_4 and connected to the positive terminal, the upper tank being connected to the negative terminal. The gels were pre-run as follows: 200 V for 15'; 300 V for 30'; 400 V for 30'. The power was turned off, the upper tank emptied and the samples loaded onto the surface of the gels. Overlay buffer and 0.02M NaOH were replaced and the chamber refilled. The gel was run overnight at 400 V and the voltage was increased to 500 V, 1 hr before it was turned off in order to sharpen the bands.

Gels were removed from the tubes under the pressure of water from a syringe and equilibrated in SDS sample buffer (10% glycerol, 5% β-mercaptoethanol, 2.3% SDS and 0.0625 M Tris HCl, pH 6.8) for about 30 min. with shaking. The buffer was then drained away and the gels stored at -20°C until required. One gel which had been run with lysis buffer on top instead

of a sample, was cut up into 5 mm slices on removal from the tube. Each slice was equilibrated with 2 ml of distilled water, and the pH was measured after about 30 min. In this way, a pH gradient was constructed.

2.18.3 Second Dimension

This was carried out using SDS gels which were prepared as previously described. Up to six gels could be run simultaneously After the SDS gels had been loaded onto the apparatus, the troughs were filled with a 1% solution of agar in SDS sample buffer and the isoelectric focusing gels were applied to the troughs before the agar set. The apparatus was sealed throughout with a 1% agar solution and the SDS running buffer was placed in the tanks. 100 μl of bromophenol blue tracker dye was put in the upper chamber, which was stirred continuously. The gels were run at a constant voltage of 100 V for about 6 hr until the dye fronts had reached the bottom of the gels. The gels were finally removed, quick stained, destained and fluorographed as required.

2.19 Amino Acid Analysis

Protein samples were hydrolysed for 72 hr in the presence of 6 M HCl containing 10 μM phenol at 105^{o}C in sealed evacuated tubes. After removal of the acid under vacuum, the hydrolysate was resuspended in 0.02 M HCl and aliquots were analysed using a single column Locarte amino acid analyser operating with sodium citrate buffers (Spackman et al, 1958). The chromatograph of the sample and standards (Hamilton, Type H) was integrated and the data normalised by the method of Ambler (1975).

2.20 Tryptic Digestion

The prothrombin-related translation product, isolated from 100µl incubation mixture, together with 2 mg of purified prothrombin was extensively dialysed against water and was then lyophilised. The lyophilised protein was redissolved in a minimum volume of 50 mM ammonium hydrogen carbonate. The mixture was agitated to form an even suspension, using an ultrasonic disintegrator at maximum output for 15 to 20 sec. Trypsin was added in an enzyme to substrate ratio of 1 - 2% (w/w) and digestion was carried out at 37°C for 4 hr, with thorough mixing at regular intervals. Digestion was arrested and the peptides recovered by lyophilisation. The tryptic peptides were rendered salt-free by repeated lyophilisation from 10% v/v triethylamine in water. They were then stored at -20°C until chromatography could be carried out.

2.21 High Voltage Paper Electrophoresis

Electrophoresis of peptides was carried out using Whatman No. 1 and 3 MM chromatography paper in water-cooled vertical tanks containing either toluene (pH 6.5 tank) or white spirit (pH 3.5 tank) as coolant. The electrode and wetting buffers were pyridine:acetic acid:water (0.5:10:89.5 for pH 6.5; 5:0.5:94.5 for pH 3.5).

The salt-free lyophilised sample was taken up in 200 µl of the pH 6.5 buffer and applied in a 1 x 1 cm box at the origin of the paper under a stream of warm air. Careful application of buffer to the paper on either side of the origin allowed focusing of the sample as the solvent fronts met at the origin. After removal of excess buffer, a marker was applied at the edge of the paper and it was loaded into the tank. Electrophoresis was carried out at 300 V (or 100 mA) for 50 min. The

paper was then removed from the tank and oven dried. Two-dimensional electrophoresis was achieved by excising the vertical protein track and stitching it horizontally to a second piece of Whatman No. 1 paper. The paper was wetted with pH 3.5 buffer to focus the peptides at the origin. Electrophoresis was carried out at 100 mA for 30 min using the pH 3.5 buffer. The paper was then removed and dried.

Radioactively labelled peptides were detected by autoradiography of the unstained chromatogram on Kodak Bluebrand 'Regulix' Film. Before staining the chromatogram, it was soaked in an acetone: triethylamine mixture (50:1) to remove acid groups, and was then oven-dried. The paper was then soaked in an acetone:pyridine mixture (50:1) containing a small amount of fluorescamine. Having dried the paper thoroughly, it was held under an ultraviolet light source and the flourescent spots were marked in pencil. The peptide map visualised on the paper was finally compared with that of the autoradiogram.

2.22 Iodo (1-^{14}C)-acetate labelling of proteins (Farrel et al, 1978)

Principle: Conditions are chosen which favour reaction between iodoacetamide and -SH groups, rather than imidazole or methionyl residues. (The latter are more reactive at lower pH.)

Method: A 10 µl sample of protein was mixed with 15 mg of urea, 2 µl 1.5 M Tris-HCl buffer, pH 8.8 and 6 µl of iodo(1-^{14}C)-acetamide. The tube was then gassed with nitrogen and allowed to stand at 20^{o}C for 3 hr. The labelled protein was then stored at - 20^{o}C until required.

2.23 Protein estimation using the Biorad Protein Assay Kit

Several dilutions of the Biorad protein standard (bovine gamma globulin) were prepared, containing from 0.2 to 1.4 mg/ml. 0.1 ml of the standards and appropriately diluted unknowns were placed in clean test tubes. 5.0 ml of diluted dye reagent were added and the tubes vortexed. After a period of 5 min, the OD_{595} was measured versus a reagent blank.

CHAPTER 3

PROTHROMBIN DETERMINATIONS IN BIOLOGICAL SAMPLES

3.1 Introduction

Prothrombin has no biological activity. To determine the prothrombin concentration of a sample, one converts it to thrombin and measures either the rate of thrombin formation or the total amount of thrombin formed. The conversion is accomplished using non-physiological activators, which do not require all the factors of the intrinsic or extrinsic pathway for their action. Several snake venoms have been discovered which activate prothrombin in the absence of any other clotting factors. The venoms of Echis carinatus and Oxyuranus scutellatus (Taipan) have been used in the present study.

The end point of conventional clotting assays comes with the formation of a fibrin clot. Since the clotting assays depend on visual observation of the clot formation they are subjective. With the development of synthetic substrates, more objective assays can be designed where the rate of formation of a coloured product is followed spectrophotometrically. Results can be evaluated as with the conventional assays, using a calibration curve, or alternatively activity can be expressed in international enzyme units.

The application of clotting assays and chromogenic assays to biological samples isolated from normal and warfarin-treated rats was investigated.

3.2 Application of Clotting Assays to Plasma and Liver Fractions

Denson et al (1971) developed a one-stage assay, specific for

prothrombin using Taipan snake venom (see Section 2.5.1). This snake venom is capable of converting prothrombin to thrombin in the absence of all other known clotting factors, though phospholipid and calcium greatly potentiate its action. The mechanism of activation of prothrombin by Taipan venom appears to parallel that of factor X_a (Owen and Jackson, 1973).

The prothrombin activating principle from E. carinatus venom was identified by Schiek et al (1972). This venom requires neither phospholipid nor calcium, and the mechanism of activation appears to be different from that of Taipan and factor X_a (Franza et al, 1975; Morita et al, 1976, 1978). A more important distinction is that the venom activates precursor forms of prothrombin (Nelsestuan and Suttie, 1972; Josso et al, 1968).

Using E. carinatus venom, a simple one-stage clotting assay was developed (see Section 2.5.2 and Appendix 1) which could be used in parallel with the one-stage Taipan assay.

The two assays were compared using plasma from control and warfarin-treated rats and were then applied to rat liver fractions.

3.2.1 Assay of Plasma Samples

Plasma samples were taken from rats which had been dosed with varying amounts of warfarin for one, two or three days previously. Table 3.2.1 gives the results of prothrombin determinations using the two assays. There is good correlation between the assays ($r = 0.991$) though the E. carinatus assay

Table 3.2.1 : Comparison of the prothrombin concentrations determined in plasma samples from warfarin-treated rats, using the Taipan one-stage and E. carinatus one-stage assays.

Group	Warfarin Concentration	No. of Doses	Percentage Prothrombin*	
			Taipan Assay	E. carinatus Assay
1	CONTROL	1	88.0 102.0	100.0 98.0
		2	115.0 96.0	108.0 105.0
		3	118.0 110.0	104.0 103.0
2	1 mg/kg BODY WEIGHT	1	17.0 20.0	23.0 25.5
		2	2.0 1.5	10.0 11.0
		3	1.5	11.5
3	2 mg/kg BODY WEIGHT	1	18.0 22.0	26.0 24.5
		2	1.8 2.1	10.5 11.5
		3	1.5	19.0
4	5 mg/kg BODY WEIGHT	1	21.0 15.0	24.5 21.0
		2	2.4 1.6	11.5 11.5
		3	1.5 1.5	13.0 13.0

*Prothrombin percentage calculated from respective calibration curves

generally gives higher estimates of prothrombin concentration than the Taipan assay. This is especially noticeable in anticoagulant-treated animals. Possible explanations are that "ecarin"-generated thrombin is more active towards fibrinogen or less affected by physiological inhibitors than Taipan-generated thrombin. It is more likely however that the E. carinatus assay is picking up small amounts of precursor prothrombin. Carlisle et al (1975) conducted a survey of abnormal prothrombins in the plasma of several species. Following barium sulphate adsorption, they found a very small amount of residual activity, assayable by a two-stage E. carinatus assay, in the plasma of anticoagulated rats. More recently, whilst investigating humoral influences on plasma prothrombin levels, Shah et al (1979) found inconsistency between the results of conventional two-stage assays and those of both the two-stage E. carinatus assay and an immunological assay. These observations again reflect the presence of small amounts of biologically inactive prothrombin-related material in the plasma of anticoagulated rats.

3.2.2 Assay of Liver Fractions

When the one-stage clotting assays were applied to liver fractions, major difficulties were encountered because the end-point was masked by the turbidity of the samples. Table 3.2.2 shows the results of one such experiment. Qualitatively, the E. carinatus assay reveals consistently higher levels of prothrombin-related material than the Taipan assay.

Neither assay gave particularly satisfactory end-points. The

samples could not be diluted, because the prothrombin concentration was already at the lower limit of detection. Therefore the nonionic detergent Triton X-100 was added in an effort to solubilise the samples and make it easier to observe clot formation. The effect on the E. carinatus assay was a dramatic shortening of clotting time. On the other hand, the response of the Taipan assay was variable, probably due to the disruption of phospholipid by the detergent. In Table 3.2.3 results of the E. carinatus assay of microsomal fractions in the presence and absence of Triton X-100, are compared. Prior to Triton X-100 treatment, the control samples give shorter clotting times than the samples from warfarin-treated rats. Following treatment, this situation is reversed. It seems likely that the detergent breaks down membranes making more prothrombin-related material available to the venom.

LIVER FRACTION	TAIPAN ASSAY		E. carinatus ASSAY	
	Clotting Time (sec)	Percentage Prothrombin	Clotting Time (sec)	Percentage Prothrombin
Homogenate	75.5	1.7	50.0	11.6
Supernatant-1	82.0	1.0	50.0	11.5
Microsomal Fraction	120.0	1.0	73.0	6.0
Supernatant-2	63.5	2.4	49.0	12.0

Table 3.2.2 : Comparison of the prothrombin concentrations determined in liver fractions using the Taipan one-stage and E.carinatus one-stage assays.

Group	Hours post dosing	% Prothrombin by E. carinatus Assay	
		no TX-100	plus TX-100
Control	24	7.0	15.6
	48	7.0	14.7
	72	6.5	14.8
Test	24	6.5	19.5
	48	6.0	19.5
	72	5.5	20.5

Table 3.2.3 : The effect of Triton X-100 on the E. carinatus assay of microsomal samples prepared from control and warfarin-treated rats (1 mg/kg body weight).

3.3 Application of the Chromogenic Assay to Plasma and Liver Fractions

Arising from studies on the interaction between thrombin and fibrinogen, synthetic substrates have been developed, which imitate the natural substrate. The present generation of synthetic substrates consist of tripeptides joined through an amide linkage to the chromogenic group, p-nitro-aniline. The p-nitroaniline released by enzymic hydrolysis is proportional to enzyme activity and is measured spectrophotometrically.

A prothrombin assay was developed (see Appendix 2) using the chromogenic reagent 'Chromozym TH' (see Figure 3.3.1) and *E. carinatus* venom. Having compared it with the one-stage assays of section 3.2, the chromogenic assay was used to investigate the distribution of prothrombin in plasma and liver fractions.

3.3.1 Comparison of the Chromogenic Assay with Clotting Assays

Plasma samples were collected from rats dosed with varying amounts of warfarin for 1, 2 or 3 days previously. Prothrombin determinations were carried out using the *E. carinatus* one-stage assay and the chromogenic assay (see Figure 3.3.2). There is good correlation between the two assays ($r = 0.987$).

In a second experiment, plasma samples taken from warfarin-dosed rats, were assayed using the Taipan one-stage assay and the chromogenic assay (see Table 3.3.1). Again there is good correlation between the assays ($r = 0.947$). As noted in section 3.2.2 *E. carinatus* activation leads to higher estimates of prothrombin concentration than activation by

Taipan venom in samples derived from warfarinised animals.

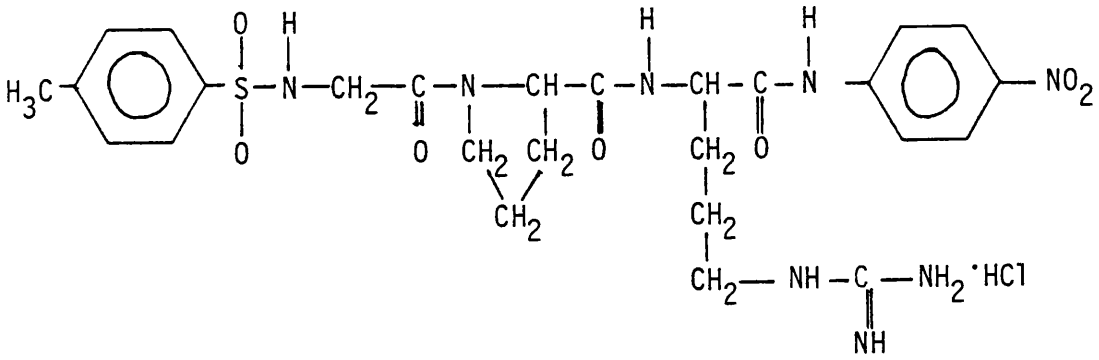


Figure 3.3.1 : The structure of 'Chromozym TH' (Tos-Gly-Pro-Arg.pNA)

3.3.2 Application of the Chromogenic Assay to Liver Fractions

When Triton X-100 was added to liver fractions, the prothrombin-related material measured by the E. carinatus clotting assay was greatly increased (section 3.2.2). With this in mind, the effect of the detergent on the chromogenic assay was investigated. Surprisingly, no increase in assayable activity was observed.

During pre-incubation of liver fractions with E. carinatus venom in the absence of detergent, a fall in optical density was regularly recorded (see Figure 3.3.3) and substantial clearing of turbidity occurred. Electron micrographs taken before and after snake venom treatment of microsomes revealed considerable breakdown of vesicles by the venom (see Plates 3.3.1 and 3.3.2). This action is attributed to a cholinesterase activity in E. carinatus venom (Bhattacharya and Gaitonde, 1979). The initial fall in optical density could be abolished by pre-treatment of the microsomes with Triton X-100.

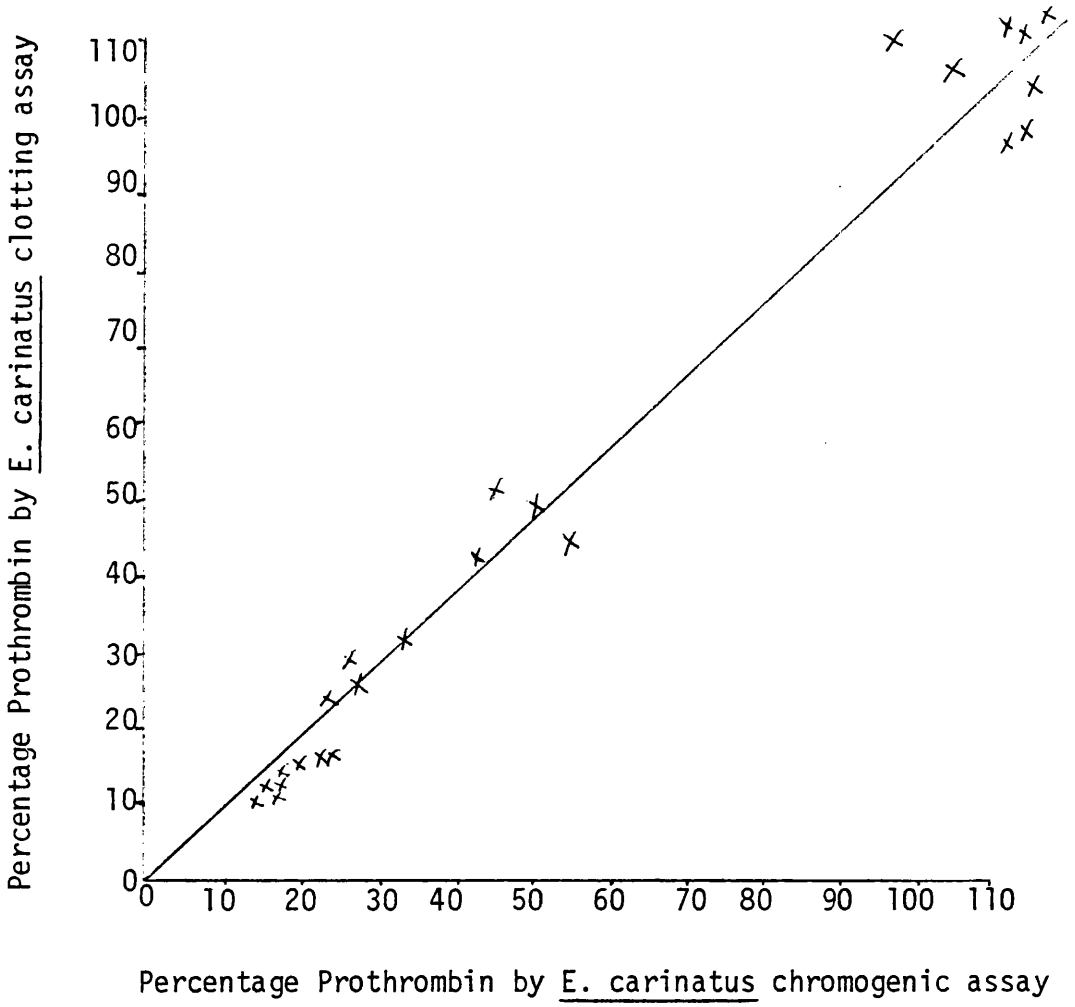


Figure 3.3.2 : Comparison of the E. carinatus clotting assay and chromogenic assay applied to plasma samples from control and warfarin-treated rats.

Group	Animal No.	Percentage Prothrombin	
		Taipan clotting assay	*E. carinatus* chromogenic assay
1 Control	1	76.0	97.6
	2	102.0	115.0
	3	100.0	116.0
	4	96.0	101.0
2 1 mg/kg	5	74.0	71.8
	6	76.0	64.5
	7	83.0	67.1
3 10 mg/kg	8	28.0	32.6
	9	20.0	28.5
	10	23.0	31.1
	11	12.0	25.0

Table 3.3.1 : Comparison of the Taipan clotting assay and the chromogenic assay applied to plasma samples from control and warfarin-treated rats.

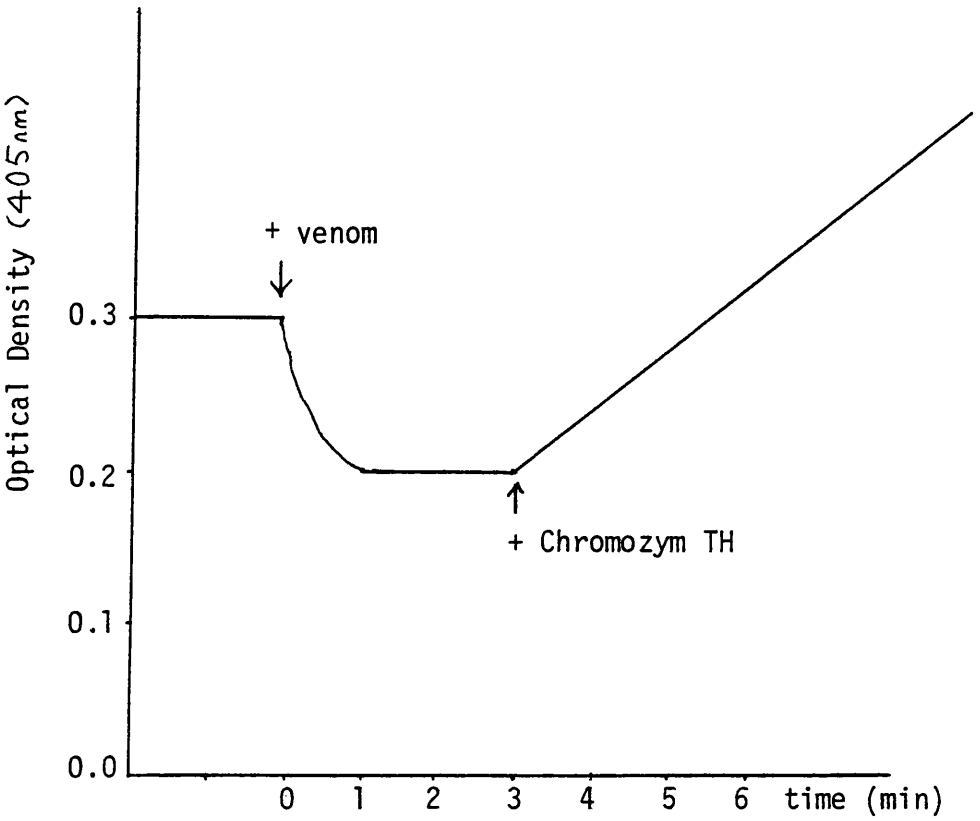


Figure 3.3.3 : Schematic representation of the change in optical density during chromogenic assay of microsomal fractions.

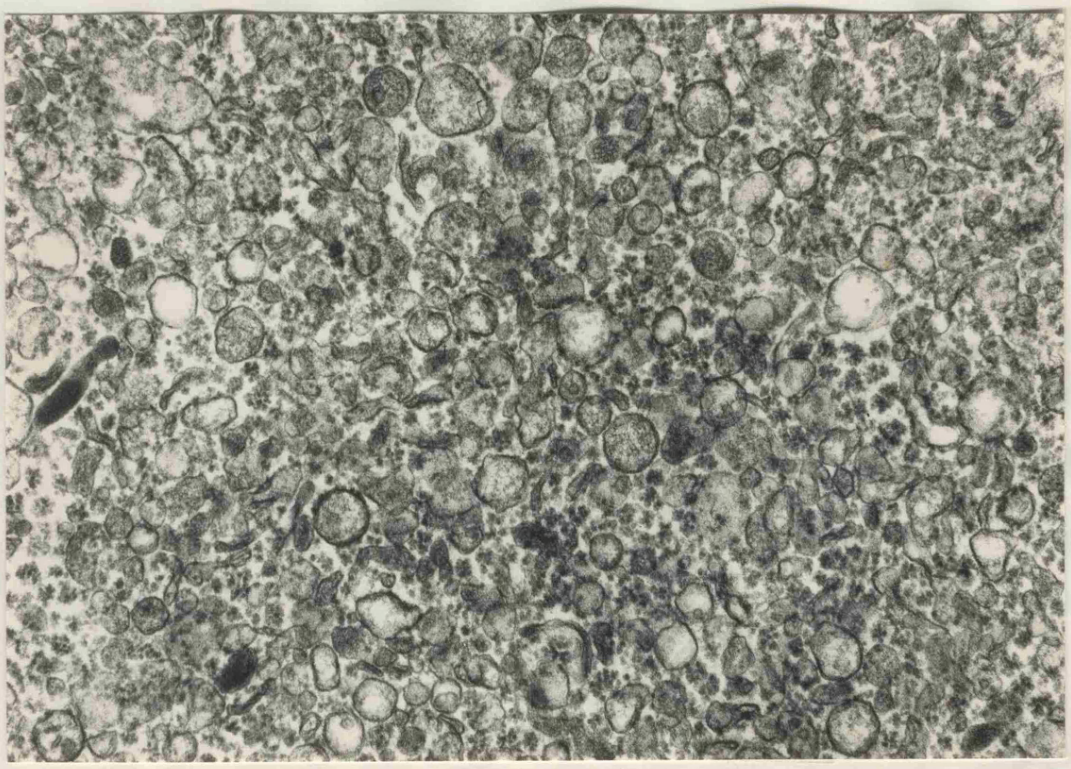


Plate 3.3.1 : Electron micrograph of control microsomes (60,000 X)

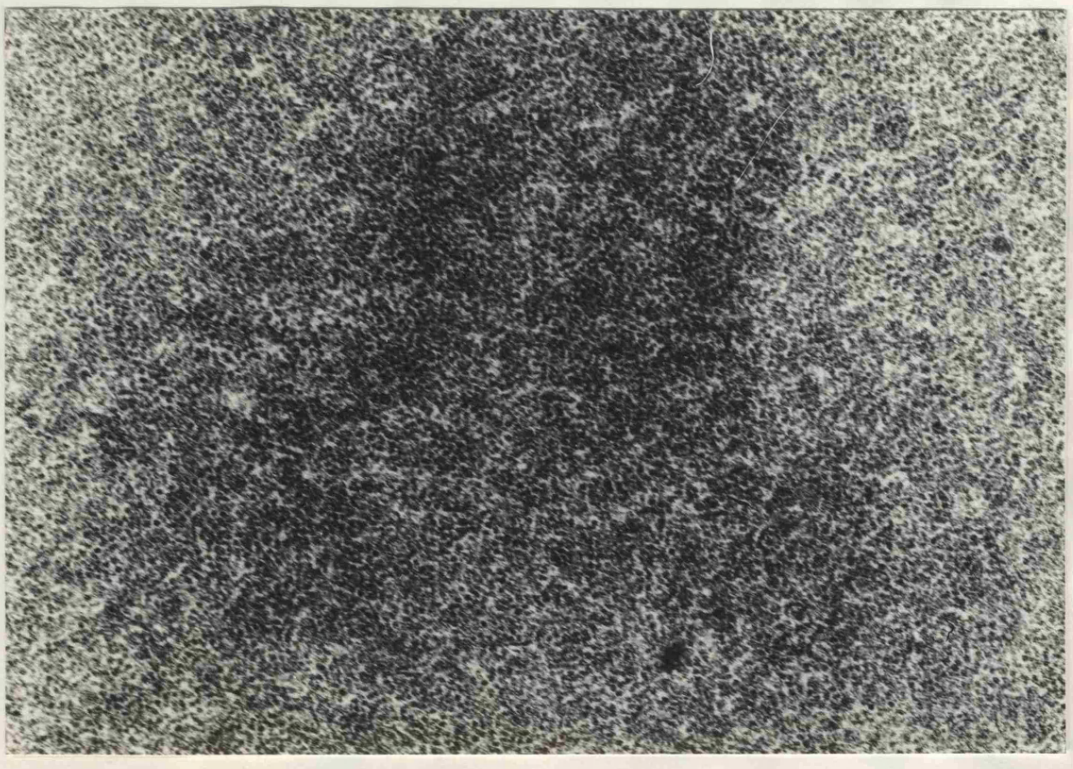


Plate 3.3.2 : Electron micrograph of microsomes treated with E. carinatus snake venom (60,000 x)

Table 3.3.2 shows the results of an experiment where liver fractions were prepared from warfarinised rats, and the prothrombin assayed by the E. carinatus clotting assay and the chromogenic assay. The amount of prothrombin-related material measured by chromogenic assay is far higher than that of the clotting assay. This indicates that not all of the prothrombin-related material activated by E. carinatus venom in the Triton X-100 solubilised liver fraction is available to act on fibrinogen. Chromozym TH, being a smaller more soluble substrate, is more readily hydrolysed.

An increase in prothrombin-related material in microsomal fractions prepared from warfarinised rats was noted in Section 3.2.2. This increase is far more apparent with the chromogenic assay. Furthermore a concommitent decrease in the prothrombin-related material of supernatant-2 is evident.

3.4 Investigation of Warfarin Dose-Dependent Changes in the Prothrombin-Related Material of Rat Liver Fractions

The relationship between warfarin dose and its effect on plasma and liver fractions was studied. (This work has been published. See Beecroft and Sanderson, 1979.)

Figures 3.4.1., 3.4.2., and 3.4.3 show the relationship between prothrombin-related material and warfarin dosage in plasma, microsomal fractions and supernatant-2 fractions from male and female

GROUP NO.	LIVER FRACTIONS	PERCENTAGE PROTHROMBIN	
		E. carinatus clotting assay	*E. carinatus* chromogenic assay
1	Homogenate	23.6	69.0
2		18.2	90.6
3		10.0	90.6
4		10.0	108.4
1	Supernatant-1	14.0	52.0
2		11.5	78.2
3		5.0	72.8
4		5.0	87.9
1	Microsomal Fraction	10.5	33.5
2		15.5	54.9
3		15.5	70.1
4		15.5	77.3
1	Supernatant-2	10.5	22.4
2		9.4	19.4
3		3.5	6.8
4		4.0	5.3

Table 3.3.2 : Comparison of the *E. carinatus* clotting and chromogenic assay applied to liver fractions from warfarin-treated rats (Group 1 - control; Group 2 - 0.1 mg/kg; Group 3 - 0.5 mg/kg; Group 4 - 1.0 mg/kg body weight)

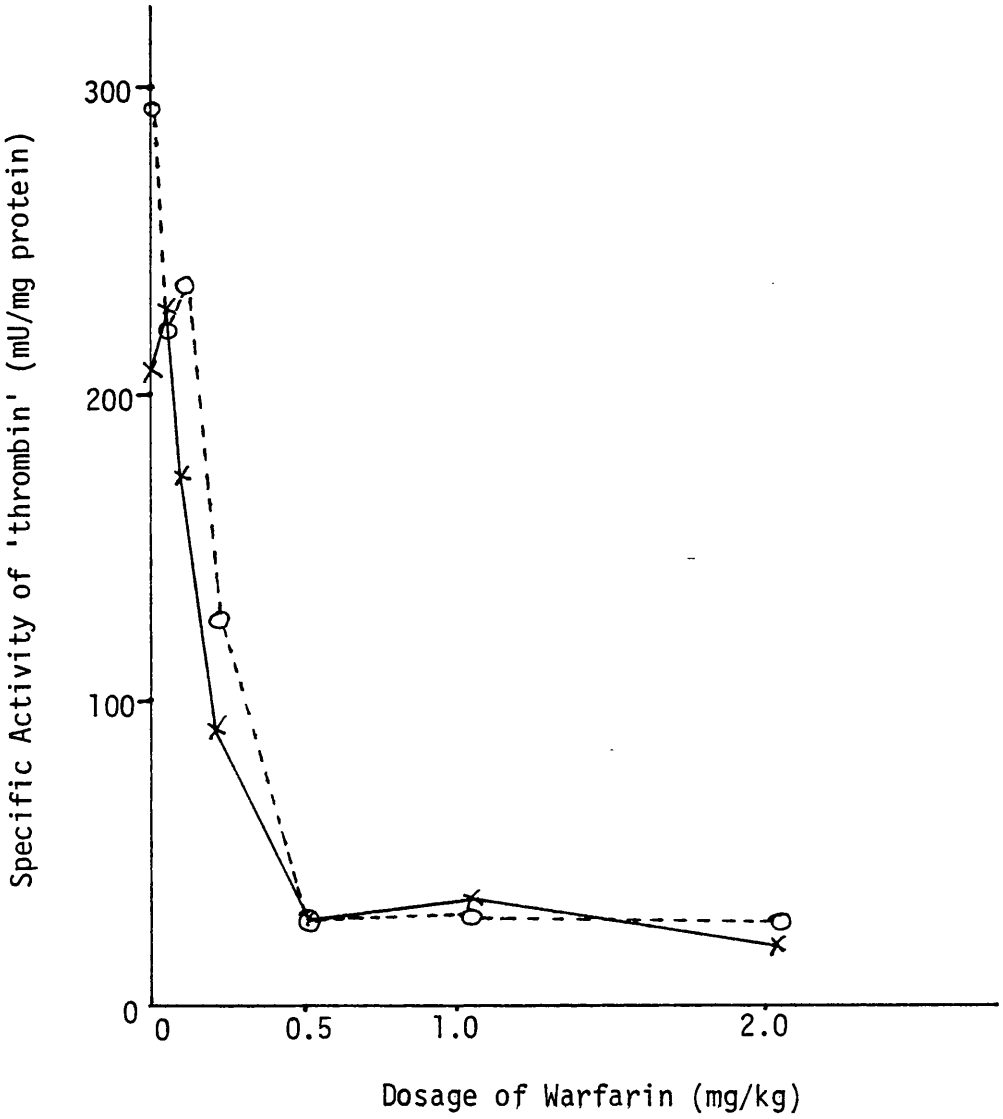


Figure 3.4.1 : Relationship between Prothrombin-related Material and warfarin dosage in Plasma samples (o--o, female rats, x—x, male rats)

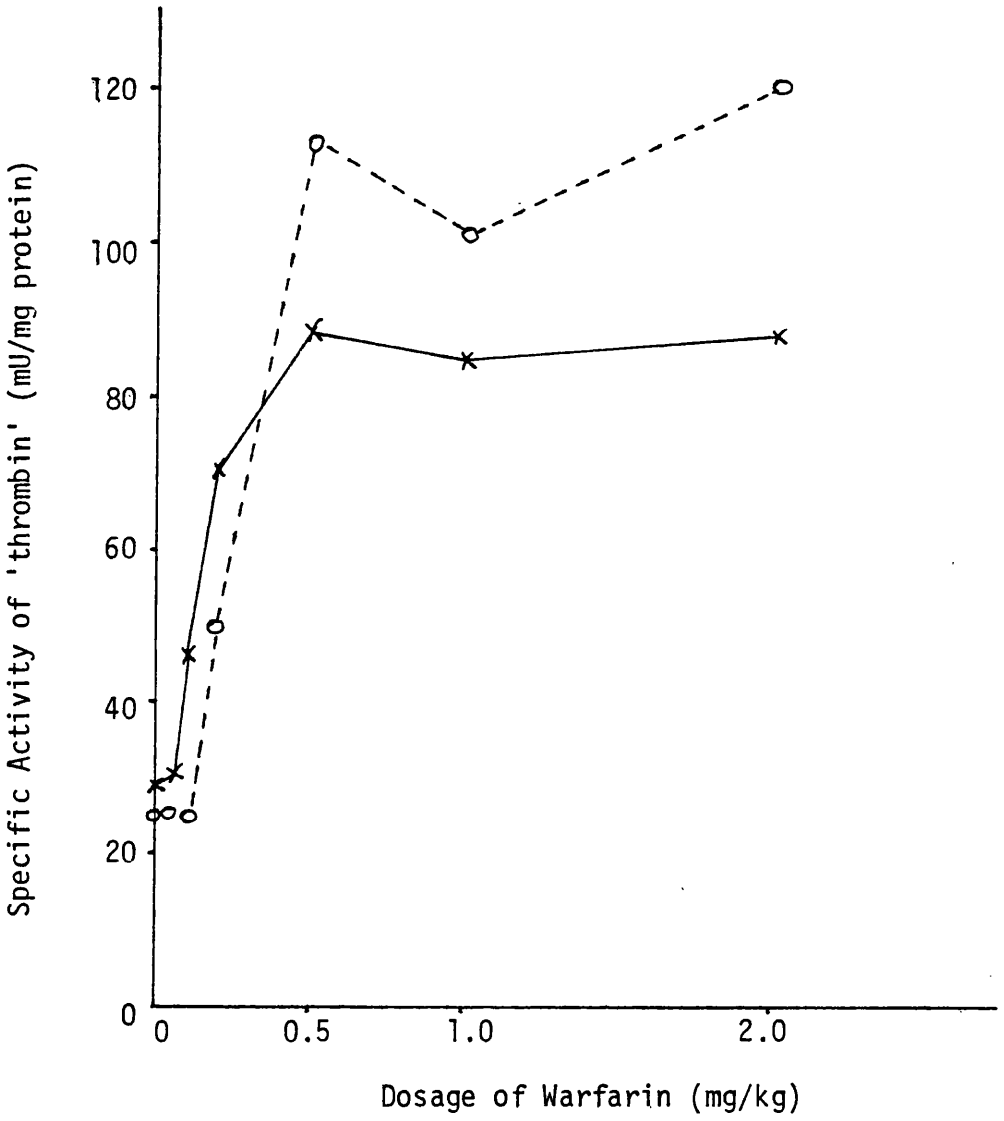


Figure 3.4.2 : Relationship between Prothrombin-related Material and warfarin dosage in Microsomal fractions (o---o, female rats, x——x, male rats)

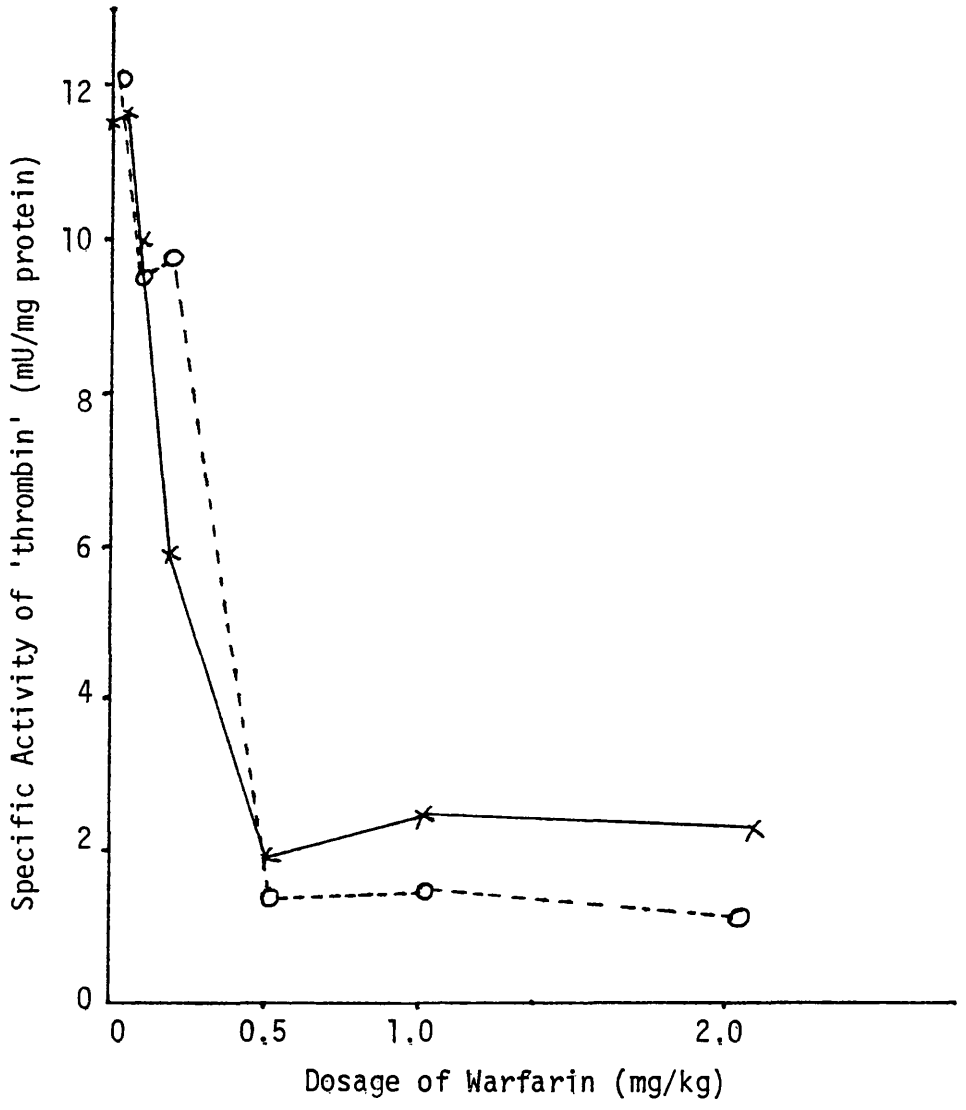


Figure 3.4.3 : Relationship between Prothrombin-related Material and warfarin dosage in Supernatant-2 samples (o---o, female rats, x——x, male rats)

rats. The results indicate a sigmoidal relationship between dose and response with little difference between sexes. At low levels of warfarin, no significant change in PRM is observed. With increasing dose levels, more and more PRM is found in the microsomal fraction and correspondingly less is found in supernatant-2. At the higher doses, the PRM of the microsomal fraction reaches a plateau (the level in females being slightly higher than that in males).

The dramatic shift in PRM from supernatant-2 (presumably soluble forms) to microsomal fractions (microsome-bound forms) caused by warfarin is attributed to the inhibition of vitamin K-dependent carboxylation. This leads to a build up of PIVKA protein in the rat liver. The dose-dependent changes in plasma and microsomal fraction levels of PRM determined using the chromogenic assay, confirm previous observations using clotting assays (Shah et al, 1973). Carlisle et al, (1975) reported no measurable activity in the postmicrosomal supernatants of control or warfarin-treated rats, indicating that the levels are below detection by conventional assays.

3.5 Application of Prothrombin Assays to Plasma Samples Taken From Warfarin-Treated Patients

Results from Section 3.4 suggest that the chromogenic assay measures not only prothrombin but also the related PIVKA protein. In the rat, PIVKA protein remains in the liver. In the bovine and human systems, little accumulation of liver precursors occurs. Instead, abnormal prothrombin is found in the plasma. Several factor II assays were carried out on plasma isolated from warfarin-treated humans to reveal whether or not the chromogenic assay picked up the abnormal

prothrombin. The human samples were kindly provided by Manchester Royal Infirmary.

The factor II assays used were the prothrombin time screening test, the one-stage Taipan assay, the one-stage E. carinatus assay and the chromogenic assay. Samples taken from six patients on anticoagulant therapy were tested by each method and the results are given in Table 3.5.1. Results of the Thrombotest (a whole blood screening test carried out by the MRI) are given for comparison.

The prothrombin time screening test correlates well with the Thrombotest ($r = 0.995$) and the one-stage Taipan assay ($r = 0.988$), providing that the results of patient 3 are discounted. Although the chromogenic assay correlates well with the one-stage E. carinatus assay ($r = 0.985$), both give significantly higher estimations of factor II activity than the other clotting assays. Correlation between the Taipan assay and the chromogenic assay, for example, is poor ($r = 0.444$). It is evident that the E. carinatus assays measure not only prothrombin but also the abnormal PIVKA protein. The anomolous results of sample 3 require some comment. When the clinical details of the patients were studied (Table 3.5.2) it was noted that on the day prior to collection of the plasma, patient 3 received 6.0 mg of warfarin. This would cause the excessive increase in PIVKA protein indicated by the E. carinatus assays. The low level of prothrombin estimated by the Taipan assay might be due to the inhibitory effect of large amounts of PIVKA on this test.

Table 3.5.1 : Comparison of percentage factor II concentrations of plasma samples from six anticoagulant-treated patients determined by several different factor II assays

Sample No.	Thrombotest (Reported by MRI)	Prothrombin Time	One-stage Taipan Assay	One-stage E. carinatus Assay	Chromogenic Assay
1	23	44.8	49.2	64.0	61.2
2	19	35.0	41.9	55.8	52.4
3	19	44.8	28.5	75.7	81.0
4	11	24.2	28.5	45.6	46.4
5	9	19.0	18.6	40.8	38.8
6	7	15.8	18.3	39.4	38.8

Table 3.5.2 : Details of patients' case histories

Patient No.	Sex	Clinical Details	Warfarin Dosage
1	♀	Long term patient from Huddersfield	2.5 mg Mon, Thur, 2.0 mg all other days
2	♀	Long term patient from Barrow	3.0 mg Sun, Wed 4.0 mg all other days
3	♀	Patient at Burnley since February 1978	For 7 days prior to sample: 3.0,3.0,6.0,3.0,4.0,4.0,6.0mg
4	♂	Patient at Crumpsall since November 1977	4.0 mg Mon, Thur, 3.0 mg all other days
5	♀	Long term patient since January 1977	2.0 mg Mon, Fri, 1.0 mg all other days
6	♀	New patient, 30th September 1978	3.0 mg daily

3.6 Conclusion

Clotting assays utilising Taipan and E. carinatus venoms can be used in parallel to determine the prothrombin and PRM, respectively of plasma samples. Application of these assays to liver fractions is less satisfactory because the end point is difficult to see. Furthermore not all the prothrombin appears to be available to act on fibrinogen, even in the presence of detergent.

The chromogenic assay can easily be applied to plasma and liver fractions. The addition of detergent is not necessary because of the cholinesterase activity endogenous to the E. carinatus venom. Also, the molecular size of the chromogenic reagent makes the activated prothrombin more accessible. The assumption that E. carinatus venom activates both abnormal PIVKA and prothrombin is supported by the observations on samples from humans treated with warfarin. The level of PRM measured using E. carinatus venom as activator is greater than that determined by conventional assays.

Using the chromogenic assay, clear warfarin dose-dependent changes in the PRM of plasma and liver fractions are observed. In particular, a change in the partition of PRM between supernatant-2 and the microsomal fraction is evident.

CHAPTER 4

PROTHROMBIN PURIFICATION AND THE KINETIC PROPERTIES OF THROMBIN

4.1 Introduction

Chapter 3 described how the chromogenic assay was used to determine prothrombin-related material in *ex vivo* samples. The assay is equally applicable to non-biological samples and was used to assess the degree of purity of prothrombin isolated from rat plasma. It was further used to investigate the kinetic properties of *E. carinatus*-generated thrombin.

4.2 Assessment of Prothrombin Purity :

4.2.1 By Chromogenic Assay

At each stage in the prothrombin purification procedure (see Section 2.10) a sample was retained for chromogenic assay and protein estimation in order to monitor the specific activity. The results of a typical analysis are given in Table 4.2.1. The most significant increase in purity is achieved with barium citrate adsorption. This step utilises the ability of vitamin K-dependent proteins to bind to divalent cations. Separation of prothrombin from the other vitamin K-dependent proteins is then achieved by differential salting out and ion exchange chromatography.

At several stages in the procedure, serine protease inhibitors were added to prevent prothrombin from being inadvertently activated (either by factor Xa or by thrombin). Since prothrombin determinations in the presence of these inhibitors were readily carried out, it seems probable that ecarin-

Purification Step	$\Delta A/min$	Dilution factor	Thrombin Activity mU/ml	Protein Concentration µg protein/ml	Specific Activity units/mg protein
1) Original Plasma	0.1263	50	14,875	86,000	0.17
2) Pooled plasma	0.1278	50	15,000	76,000	0.20
3) Ba citrate adsorption	0.4353	100	102,500	3,000	34.2
4) 1st NH_4SO_4 fractionation	0.4353	50	51,300	1,700	30.16
5) 2nd NH_4SO_4 fractionation	0.4503	200	212,000	6,500	32.6
6) Dialysis	0.3003	200	141,500	6,100	23.6
7) QAE-Sephadex column	0.1008	200	47,500	700	67.75

Table 4.2.1 : Purification regime for prothrombin

generated thrombin is unaffected by heparin, benzamidine HCl and soyabean trypsin inhibitor.

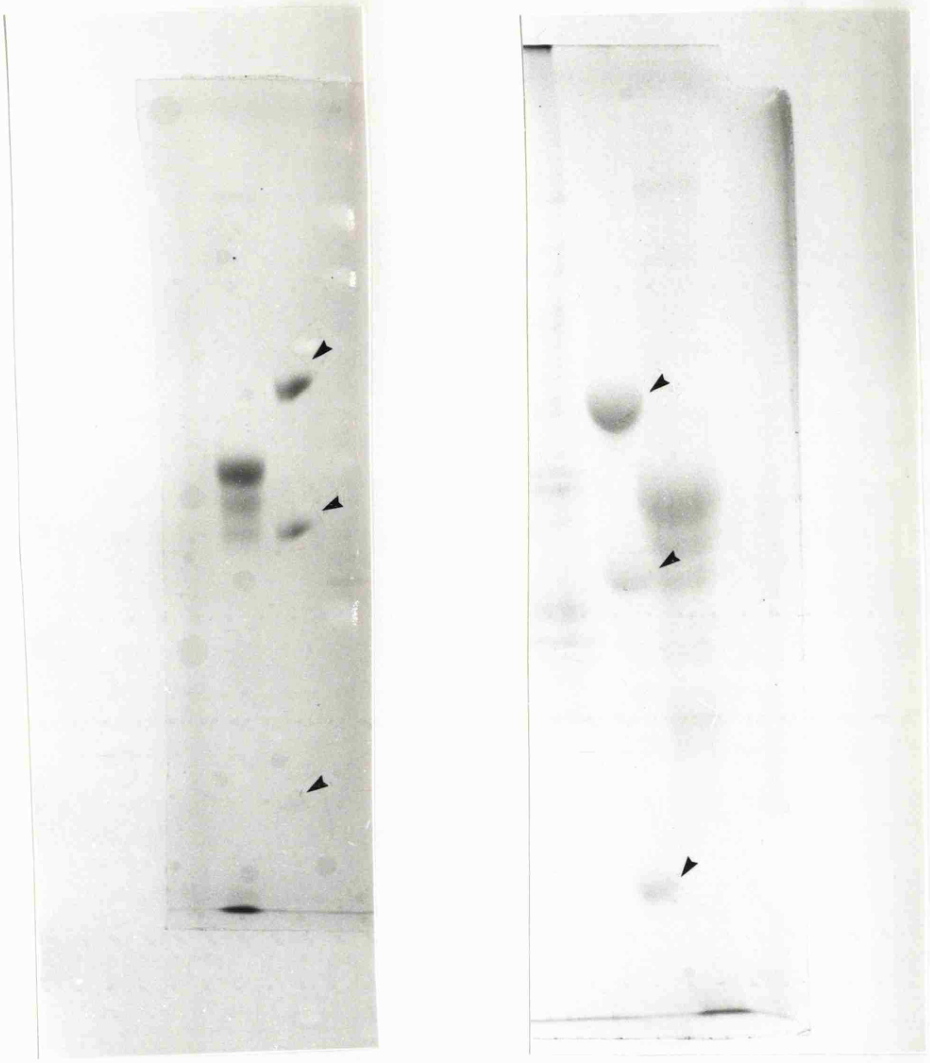
4.2.2 By SDS-PAGE (See Section 2.16)

Before the prothrombin concentrate could be run on a gel, it had to be extensively dialysed against 0.03 M Tris, pH 7.4, to remove the ammonium chloride ions. When the dialysate was run on the SDS gel, one major protein band was observed with a molecular weight of 75 - 80,000 (average of several determinations). This is in close agreement with Grant and Suttie (1976a).

On storage of the prothrombin concentrate at -20°C, minor proteins of approximately 58,000 and 28,000 molecular weight appeared indicating that the preparation was unstable. (See Plate 4.2.1a) Grant and Suttie (1976a) attribute the instability of some prothrombin preparations to the presence of trace amounts of thrombin. Prothrombin, when subjected to auto-catalysis by thrombin, yields prethrombin-1 and fragment-1 breakdown products of 58,000 and 25,000 molecular weight, respectively. (See Figure 4.2.2.)

To determine whether the 58,000 and 75,000 molecular weight species were related, partial peptide digestion (see Section 2.17) was undertaken, using V8, papain and chymotrypsin. Plate 4.2.2 clearly shows that the two proteins have common peptides.

To further characterise the prothrombin concentrate a second stain, specific for carbohydrate-containing proteins (Glossman and Neville, 1971) was applied to an SDS gel. The gel was



(a) (b)

Plate 4.2.1 : SDS polyacrylamide gel electrophoresis of prothrombin concentrate. Arrows indicate prothrombin, prethrombin-1 and fragment-1 respectively (from top to bottom).
(a) Visualised with protein stain.
(b) Visualised with carbohydrate stain.

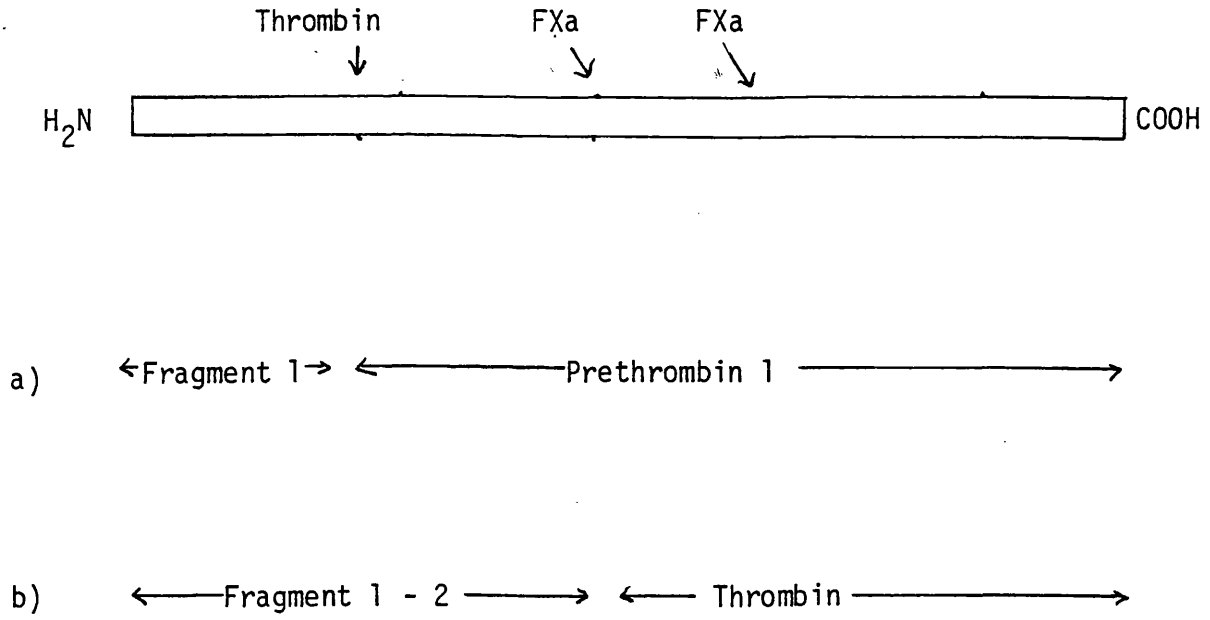


Figure 4.2.2 : Schematic representation of the breakdown products of prothrombin when it is cleaved a) by thrombin and b) by factor Xa

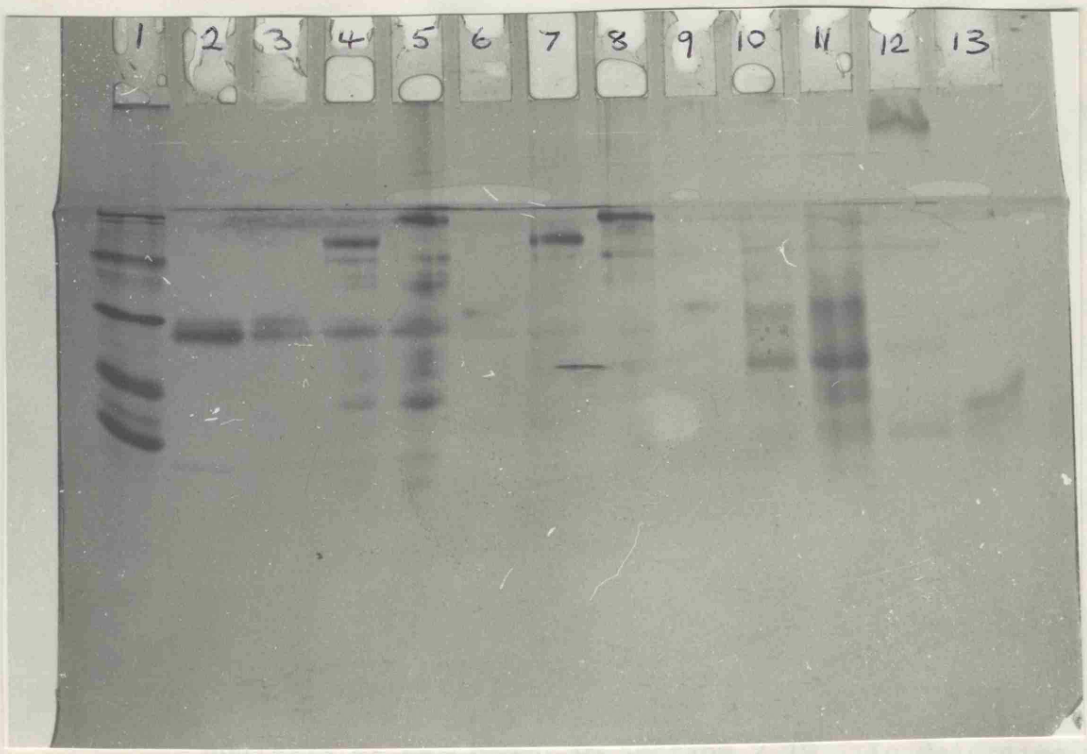


Plate 4.2.2 : Partial peptide digestion of prothrombin, prethrombin-1 and fragment-1 using chymotrypsin, V8 and papain.
Tracks loaded as follows:

1. Molecular weight markers
2. V8 protease
3. Fragment-1 treated with V8
4. Prethrombin-1 treated with V8
5. Prothrombin treated with V8
6. Fragment-1 treated with chymotrypsin
7. Prethrombin-1 treated with "
8. Prothrombin treated with "
9. Fragment-1 treated with papain
10. Prethrombin-1 treated with "
11. Prothrombin treated with "
12. Papain
13. Chymotrypsin

washed for 24 hr. in several changes of 40% methanol, 7% acetic acid, oxidised for 1 hr. at $4^{o}C$ in the dark with 1% periodic acid in 7% acetic acid, washed for a further 24 hr with 7% acetic acid and then incubated in Schiffs Reagent for 1 hr. at $4^{o}C$ in the dark. Finally, the gel was washed with 0.05 M sodium metabisulphate in 0.1 M HCl, and the pink-stained glycoprotein bands appeared. Using this stain the three bands of 75,000, 58,000 and 28,000 molecular weight were again observed. (See Plate 4.2.1b.)

4.2.3 By Amino Acid Analysis

2 mg of the purified prothrombin were subjected to amino acid analysis as described in Section 2.19. To determine the empirical formula of the protein, the normalised data were divided by the lowest common denominator (i.e. n moles of methionine). Having treated the analyses reported by Grant and Suttie (1976a) and Li and Olson (1967) in a similar manner, the three were compared in Table 4.2.2. Although tryptophan was not quantitated in the present study, 1 mg prothrombin was hydrolysed in 1 ml 3 M para-toluene sulphonic acid containing 0.2% 3-(2-aminoethyl)-indole. Following amino acid analysis, the relative proportions of methionine and tryptophan were found to agree, qualitatively with the reported analyses.

The empirical formula determined in the present study agrees more closely with that of Li and Olson (1967) than that of Grant and Suttie (1976a). However, it must be noted that Li and Olson found prothrombin to have a molecular weight of 86,000.

Residue	Empirical formula of protein		
	a	b	c
Asp	10.96	7.50	6.53
Thr	6.83	5.25	3.82
Ser	8.50	4.30	3.51
Glu	13.30	7.80	5.95
Pro	6.90	3.30	ND
Gly	10.70	4.60	5.58
Ala	5.30	2.80	3.30
Val	5.70	3.90	3.10
Met	1.00	1.00	1.00
Ile	4.09	2.25	1.83
Leu	8.07	4.40	4.35
Tyr	2.76	1.90	2.36
Phe	3.93	3.20	2.64
His	2.48	1.50	1.55
Lys	5.46	3.50	3.05
Trp	2.85	1.50	ND
Arg	6.65	2.90	3.00

Table 4.2.2 : Empirical Formula of Rat Prothrombin
a) from Grant and Suttie (1976a)
b) from Li and Olson (1967)
c) average of two analyses, this study.

4.3 Kinetic Analysis of Purified Prothrombin

4.3.1

In order to investigate the kinetics of the chromogenic assay, the Chromozym TH concentration was varied for a fixed prothrombin concentration and the initial reaction rates were measured (see Table 4.3.1). The curve obtained by plotting initial reaction rate (v_0) against initial substrate concentration (S_0) (see Figure 4.3.1) was converted to linearity using the Lineweaver-Burk transformation where the reciprocal of the initial reaction rate is plotted against the reciprocal of the initial substrate concentration. (See Figure 4.3.2.) From this graph, the Michaelis constant (Km) was calculated to be 6.8×10^{-6} M and the Vmax to be 4.1 nmol/ml/min. The Km value agrees closely with that of Jackson's group from Washington University (personal communication). The kinetic analysis was carried out in response to a questionnaire distributed by the Subcommittee on Synthetic Substrates (for the International Committee on Thrombosis and Haemostasis).

4.3.2

Since conventional clotting assays are affected by the presence of physiological inhibitors such as heparin and antithrombin III, the effect of heparin on the kinetic parameters of the chromogenic assay was investigated. The Chromozym TH concentration was varied as before in the presence and absence of heparin and the results of the Lineweaver-Burk transformation are shown in Figure 4.3.2. Table 4.3.2 gives a comparison of the kinetic parameters derived from all the kinetic analyses. Although the Km did not change between

Substrate Concentration (mM) (S)	1/(S)	A/min	Reaction Velocity nmole/ml/min (V)	1/(V)
0.153	6.53	0.0438	4.1	2.439
0.122	8.17	0.0429	4.0	2.500
0.092	10.89	0.0417	3.9	2.578
0.061	16.33	0.0408	3.8	2.635
0.031	32.67	0.0315	3.3	3.063
0.015	65.33	0.0300	2.8	3.583
0.008	130.67	0.0237	2.2	4.536
0.005	196.00	0.0195	1.8	5.510

Table 4.3.1 : Kinetic Data for Chromogenic Assay (experiment 1)

Analysis	Heparin	Km (M)	Vmax (nmol/ml/min)
4.3.1	-	$6.8\ 10^{-6}$	4.1
4.3.2	-	$6.9\ 10^{-6}$	3.0
4.3.2	+	$8.8\ 10^{-6}$	2.7

Table 4.3.2 : Enzyme characteristics calculated from Lineweaver-Burk transformations of Figure 4.3

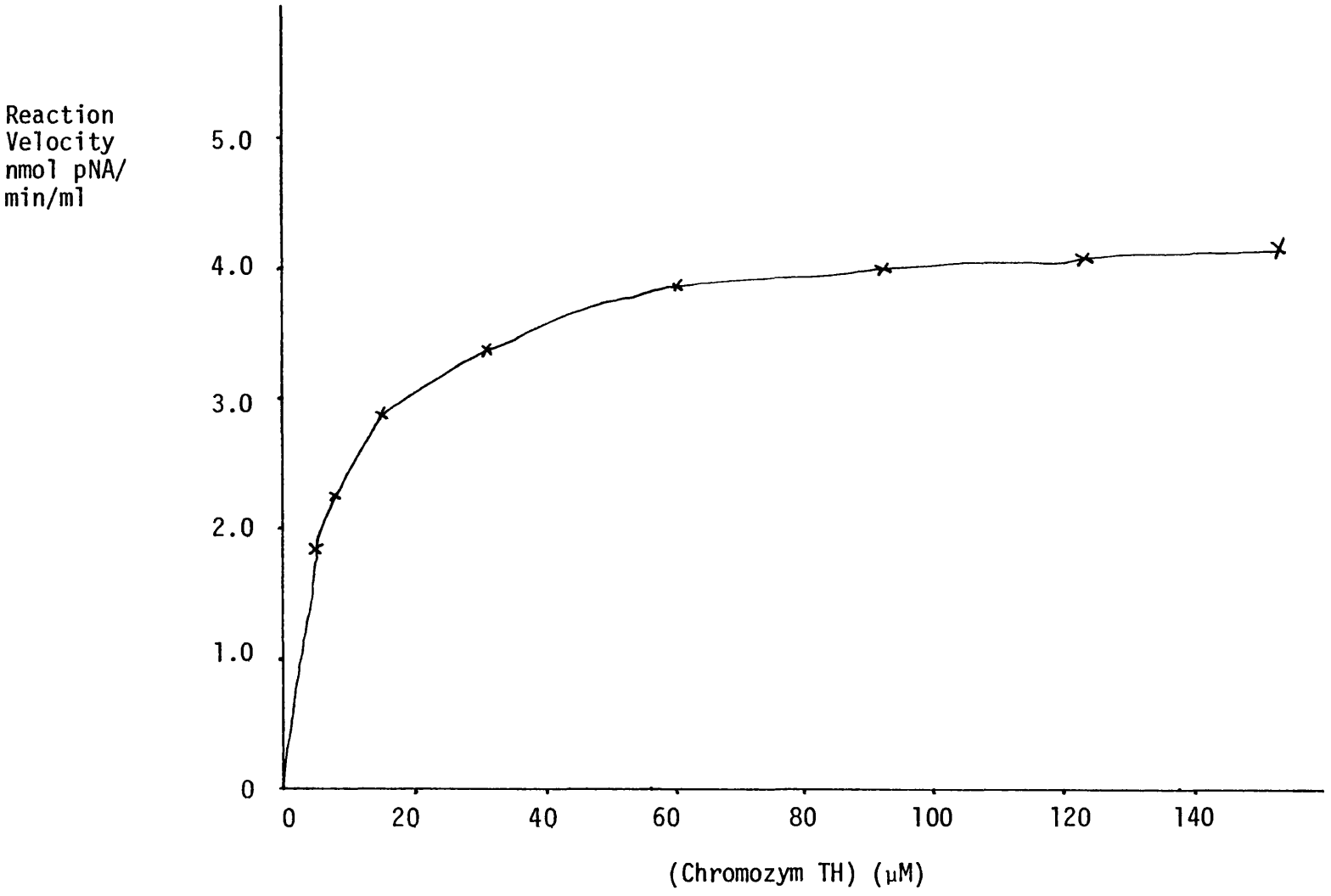


Figure 4.3.1 : Relationship between initial reaction rate and initial substrate concentration

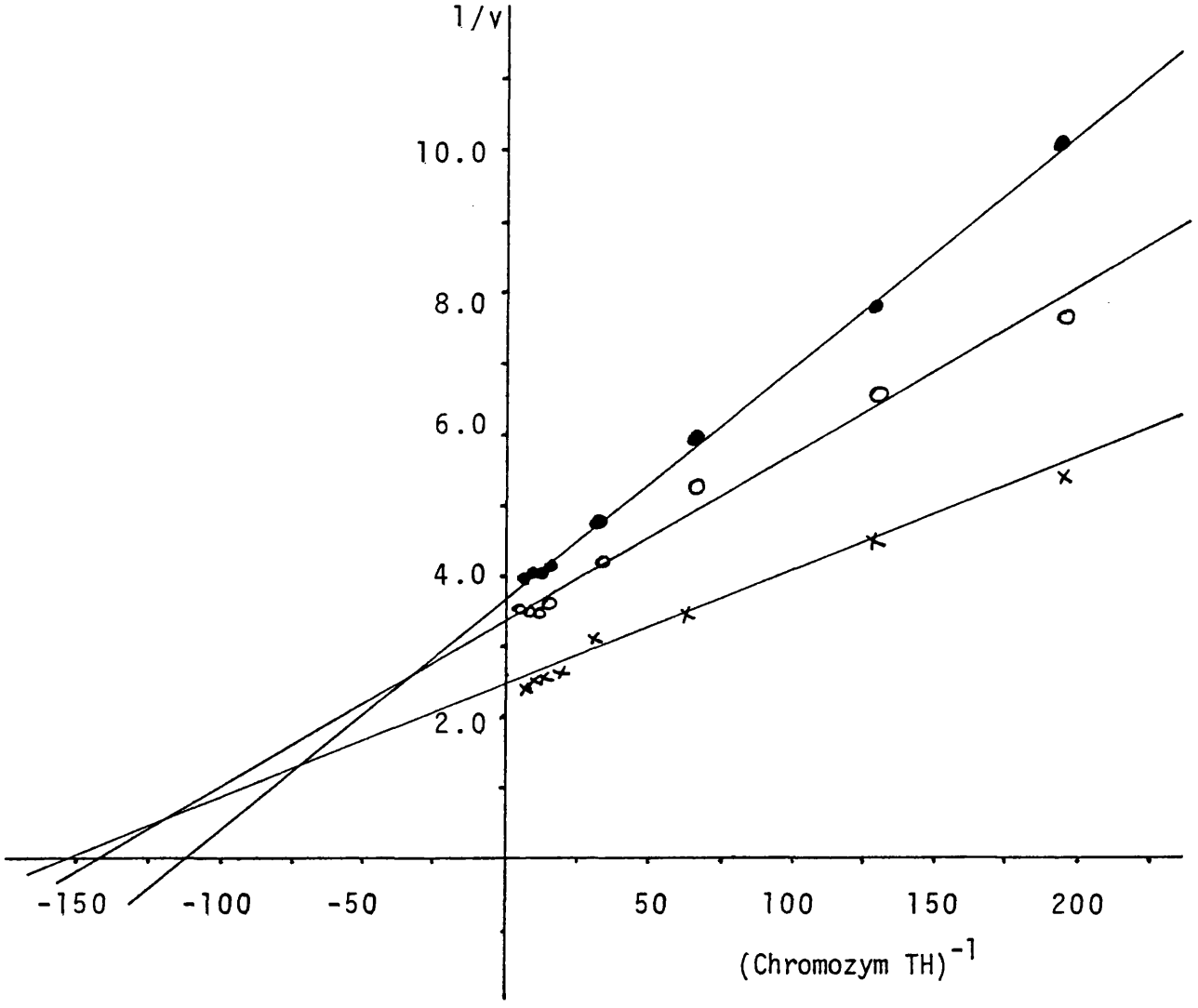


Figure 4.3.2 : Lineweaver-Burk transformation of kinetic analyses.
i) (x—x) freshly thawed prothrombin concentrate (see Section 4.3.1
ii) (o—o) prothrombin concentrate kept on ice for 2 hr.
iii) (•—•) as ii) but assayed in the presence of heparin ($5x10^{-5}$U)

experiment 4.3.1 and experiment 4.3.2, the Vmax fell significantly. This effect on an enzyme assay is indicative of noncompetitive inhibition. The same enzyme solution was used in both experiments, but it was kept on ice for about two hours before the second experiment was undertaken. It is assumed that the instability of the prothrombin solution led to the formation of breakdown products during this time, which inhibit the action of thrombin on Chromozym TH. The addition of heparin to the assay caused an increase in the Km. This effect is indicative of competitive inhibition. (The decrease in Vmax is probably due to further breakdown of prothrombin on standing.)

4.4 Effect of heparin on plasma samples

Roth and Haarsma (1977) found that mixing increasing amounts of heparin with plasma resulted in a progressive loss of thrombin activity as measured by chromogenic assay. In their system, human plasma was used and thrombin activity was generated by thromboplastin.

In an attempt to reproduce their results using rat plasma, varying amounts of heparin (from porcine gastric mucosa) were incorporated in the pre-incubation stage of the assay, so that thrombin was generated in the presence of heparin. Thrombin activity was measured at two Chromozym TH concentrations (at substrate saturation and at a concentration slightly below the Km) and the results are shown in Figure 4.4.1. Over a wide range of heparin concentrations and at both substrate concentrations, little inhibition could be demonstrated in this system. Nordenman and Bjork (1978) and Kowalski and Finlay

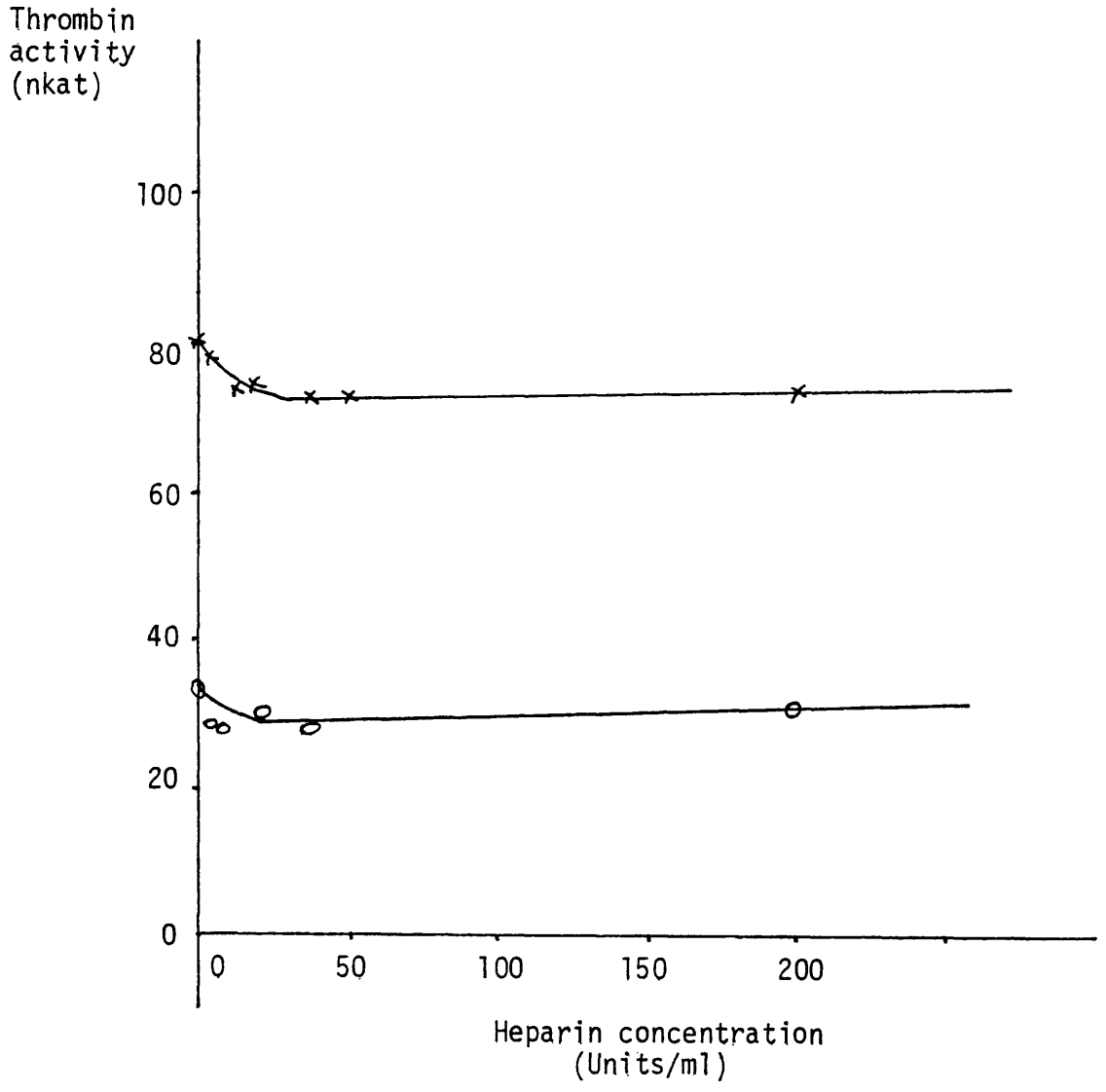


Figure 4.4.1 : The effect of heparin on thrombin amidase activity assayed at a substrate concentration of 15.3 μM (x—x), and 4.1 μM (o—o)

(1979) have both found that thrombin is largely unaffected by heparin alone. Antithrombin III must be present for heparins anticoagulant activity to be fully expressed.

Since heparin and antithrombin III might be expected to be endogenous to some biological samples, they could work in concert to inhibit thrombin as it was formed during the pre-incubation stage. Only when enough enzyme had been produced to combine with all the endogenous inhibitors would further enzyme molecules remain active. If this was the case, then the addition of a heparin inhibitor at the pre-incubation step would cause an increase in apparent thrombin activity. When the heparin inhibitor, polybrene, was incorporated into the assay no such stimulation was observed. This implies that i) there is too little endogenous heparin to be picked up by this procedure or ii) that ecarin-generated thrombin is not affected by the presence of heparin with antithrombin III.

4.5 Conclusion

The chromogenic assay was very useful in assessing the specific activity of the prothrombin preparation at each step in the purification procedure. The final prothrombin concentrate gave a single band on SDS gel electrophoresis with a molecular weight of 75 - 80,000. On storage of the concentrate at -20^{o}C, two breakdown products of molecular weight 58,000 and 28,000 were formed. All three protein bands could be visualised using a stain specific for carbohydrate-containing proteins. These observations agree well with Grant and Suttie (1976a). Li and Olson (1967) reported an 86,000 molecular weight species in addition to a 73,000 dalton form, and suggested that the smaller molecule is an autodigestion product of

the larger. No indication of an 86,000 dalton species was noted in the present study. It is interesting to note, however, that the amino acid analysis yielded an empirical formula more akin to that of Li and Olson (1967) than that of Grant and Suttie (1976a).

The enzyme characteristics of ecarin-generated thrombin were determined using the prothrombin concentrate. The enzyme has a high affinity for the chromogenic substrate ($Km = 6.8 \times 10^{-6}$). On storage of the concentrate at $4^{o}C$ noncompetitive inhibitors of thrombin were formed. This was probably due to the presence of trace amounts of thrombin in the concentrate. Addition of heparin to the assay caused a slight increase in the Km, indicating that it inhibits thrombin in a competitive manner. Longas et al (1980) found heparin to be a mixed-competitive inhibitor of human thrombin. This discrepancy could be due to the fact that the thrombin was generated by a different method.

The inhibition of thrombin by heparin, observed in this present study is probably not of physiological importance. When plasma samples were assayed in the presence of heparin, very little inhibition was demonstrated. This reflects the involvement of antithrombin III in heparin's anticoagulant activity (Kowalski and Finlay, 1979). Since polybrene failed to increase the amount of thrombin assayable, the effect of endogenous antithrombin III together with heparin would appear to be negligible. Fulton et al (1979) have suggested that the presence of ecarin in the reaction vessel prevents the inactivation of ecarin-thrombin by antithrombin III. If this is the case, then the chromogenic assay utilising E. carinatus venom will give a true estimate of prothrombin concentration, unlike conventional assays

which are affected by endogenous inhibitors.

CHAPTER 5

ANTIBODY CHARACTERISATION AND APPLICATION TO MICROSOMAL FRACTIONS

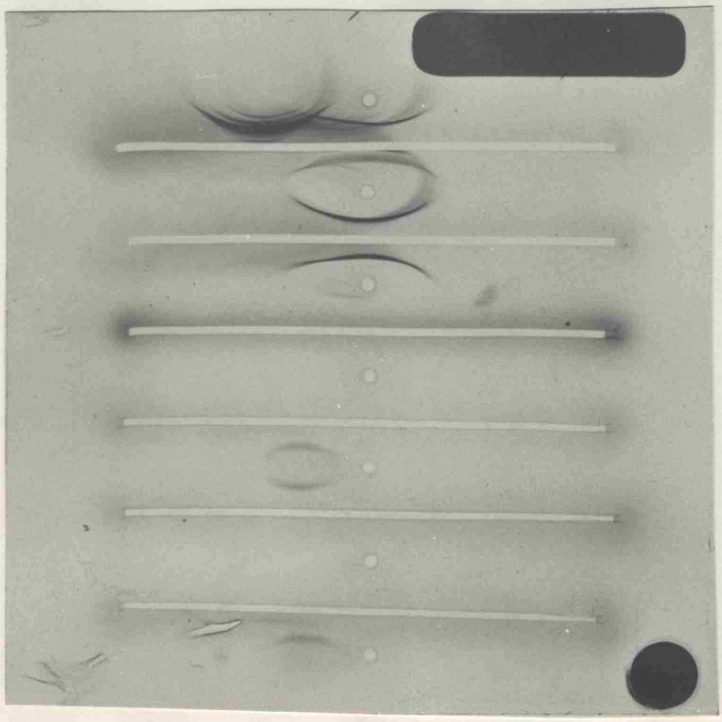
5.1 Introduction

The prothrombin concentrate, characterised in Chapter 4, was used to raise antibody against rat prothrombin in the rabbit. (See Section 2.11). The antibody was characterised using standard immunological techniques. It was further characterised functionally using the chromogenic assay. Finally the interaction between antibody and microsomal fractions isolated from rats treated with warfarin was investigated.

5.2 Characterisation of the Anti-prothrombin Antibody

5.2.1 By Immunoelectrophoresis (Section 2.12.1)

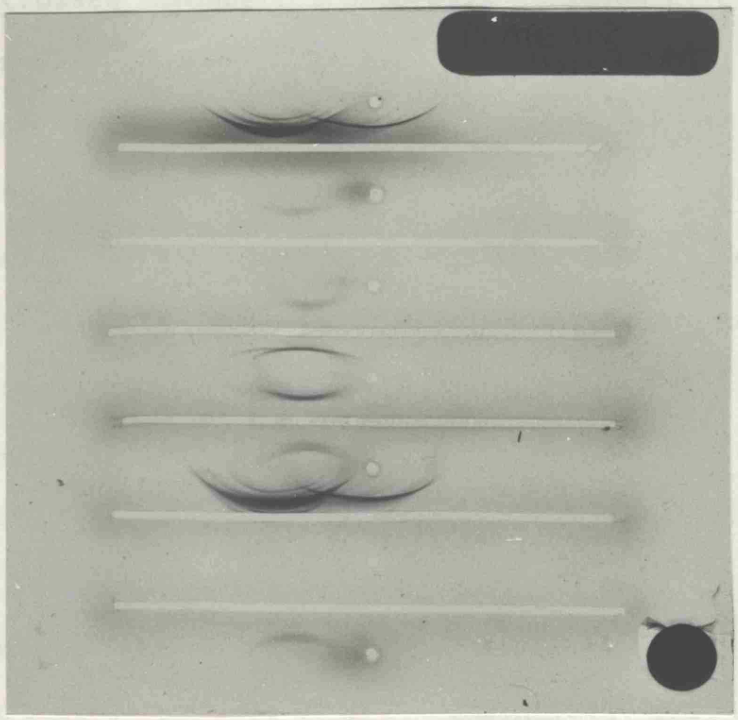
The anti-prothrombin antisera collected from the two rabbits, "Woodstock" and "Peanuts", were diffused against normal rat plasma, rat IgG and prothrombin concentrate as antigen sources. The resulting precipitin lines were compared with appropriate controls using anti-rat serum antibody, anti-rat IgG antibody and anti-IgM antibody. As a further control, anti-human prothrombin antibody was run against human plasma. The results are shown in Plates 5.2.1 and 5.2.2. Only one precipitin band was observed when Woodstock or Peanuts antisera were diffused against normal rat plasma, but anti-rat serum antibody gave multiple precipitin lines against this antigen source. The position of the precipitin band was closer to the anode than that of the IgG and IgM control bands, but in the same region as the human prothrombin control band.



+ -

o NRP	@ Rat serum
o Rat IgG	@ Rat IgG
o NRP	@ Rat IgM
o Rat IgG	@ Prothrombin (Woodstock)
o NRP	@ Prothrombin (Peanuts)
o Rat IgG	@ Prothrombin (Woodstock)
o NRP	

Plate 5.2.1 : Characterisation of the anti-prothrombin antibody by immunoelectrophoresis against normal rat plasma (NRP) and Rat IgG



+		-
o	NRP	@ Rat serum
o	Human plasma	@ Human prothrombin
o	NRP	@ Prothrombin (Woodstock)
o	PT concentrate	@ Prothrombin (Peanuts)
o	NRP	@ Rat serum
o	PT concentrate	@ Human prothrombin
o	Human plasma	

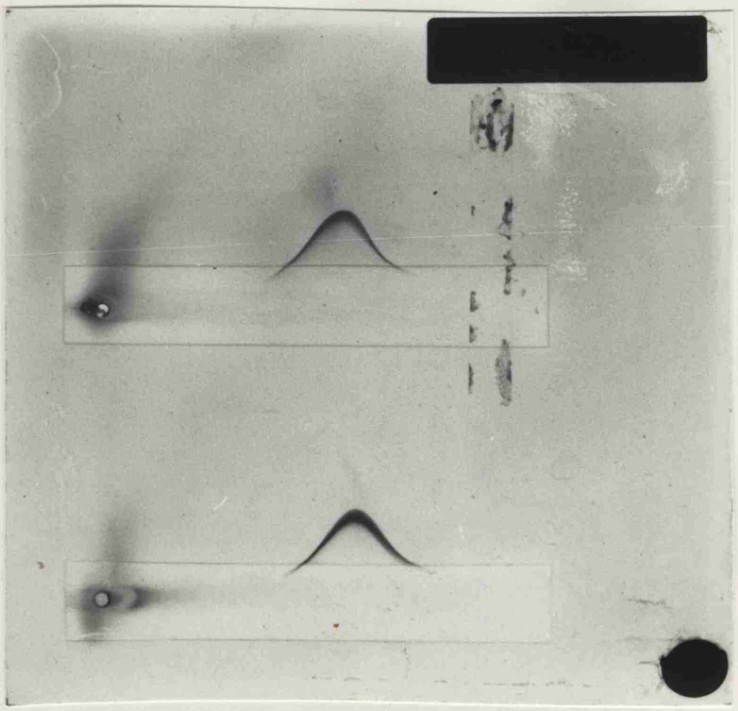
Plate 5.2.2 : Characterisation of the anti-prothrombin antibodies by immunoelectrophoresis against normal rat plasma (NRP) and prothrombin (PT) concentrate.

5.2.2 By Crossed Immunoelectrophoresis (Section 2.12.2)

Normal rat plasma, EDTA plasma (from blood collected into 1 mg/ml EDTA) and prothrombin concentrate were the antigen sources subjected to electrophoretic separation in the first dimension. In the second dimension, electrophoresis in a gel containing anti-prothrombin antibody was conducted. The results are shown in Plates 5.2.3 and 5.2.4. In all cases, clean precipitin arcs were seen. Both EDTA plasma and citrated plasma produced a precipitin arc in the same region of the gel as the major rocket formed by the prothrombin concentrate. The shoulder arc may have been produced by a degradation product of prothrombin (e.g. prethrombin-1). To produce rockets of a reasonable size, the antisera had to be greatly diluted indicating that they are of high titre. Minor contaminating antibodies which formed faint rockets at higher antiserum concentration were diluted out by this procedure.

5.2.3 By Chromogenic Assay

Following the formation of immune complexes between plasma and anti-prothrombin and their immunoprecipitation using protein A-Sephadex, the supernatant was assayed chromogenically as described in Section 2.12.4. As a positive control, anti-prothrombin antibody was replaced by triethanolamine buffer. As a negative control, to check for non-specific antibody binding, anti-prothrombin antiserum was replaced by anti-rat IgG antiserum. The controls were processed in an analogous manner to the test samples. The results are given in Table 5.2.1. Antiserum from both Woodstock and Peanuts was found



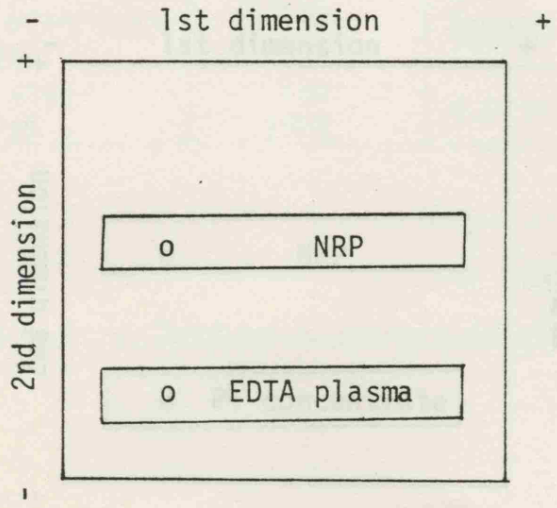
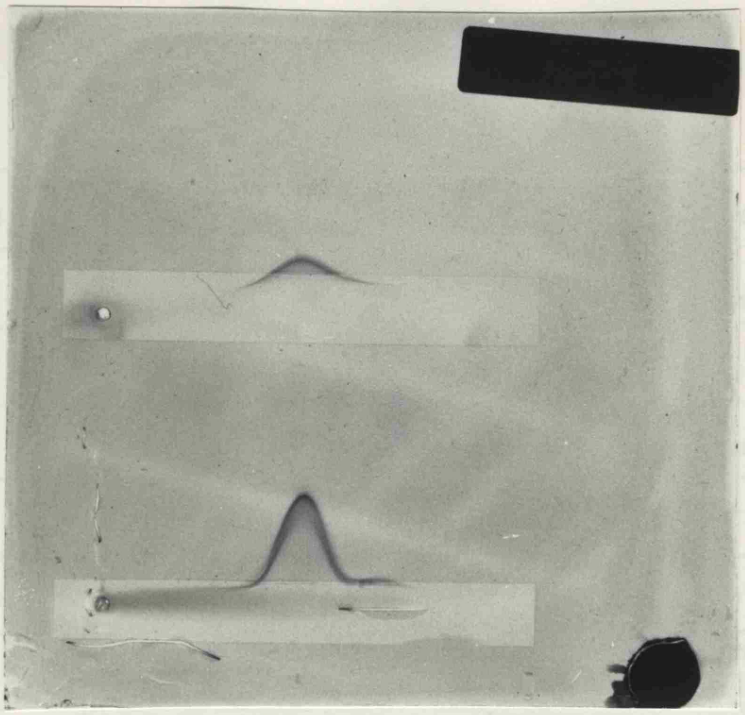
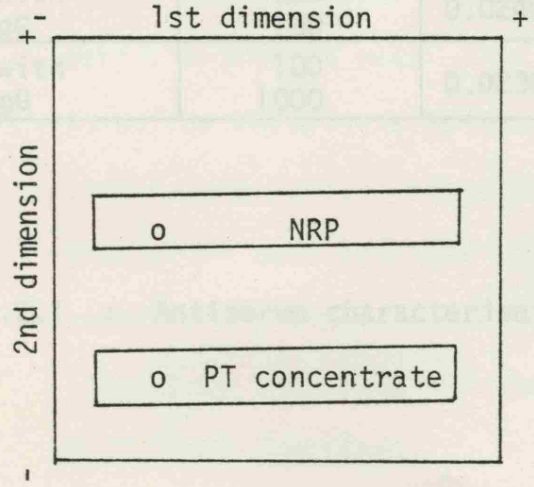


Plate 5.2.3 : Characterisation of the anti-prothrombin antibody by crossed immunoelectrophoresis against normal rat plasma (NRP) and EDTA plasma.





agarose containing 5 µl/ml
Peanuts anti-prothrombin antiserum

Plate 5.2.4 : Characterisation of the anti-prothrombin antibody by crossed immunoelectrophoresis against normal rat plasma (NRP) and prothrombin (PT) concentrate.

INCUBATION	DILUTION	Δ A/MIN	COMMENTS
Plasma alone	100	0.0230	Positive control (100%)
Woodstock antiserum	100	0.0003	Woodstock control - no endogenous prothrombin
Plasma with Woodstock @	100 100	0.0005	100% loss of activity in supernatant
Plasma with Woodstock @	100 1000	0.0205	10% loss of activity in supernatant
Peanuts antiserum	100	0.0062	Peanuts control - prothrombin present
Plasma with Peanuts @	100 100	0.0053	100% loss of rat prothrombin activity in supernatant
@ rat IgG	100	0.0005	Negative control
Plasma with @ rat IgG	100 100	0.0240	100% activity in supernatant
Plasma with @ rat IgG	100 1000	0.0235	100% activity in supernatant

Table 5.2.1 : Antiserum characterisation using the chromogenic assay

to remove all prothrombin activity from the supernatant in this system, whilst anti-rat IgG had no effect.

It was found that prothrombin endogenous to the rabbit antiserum was activated by E. carinatus venom and picked up by the chromogenic assay. Suitable control assays on the antisera were therefore carried out. Following this discovery, subsequent batches of antiserum were routinely treated with barium citrate to remove endogenous prothrombin.

5.3 Immunological Investigation of Microsomal Fractions

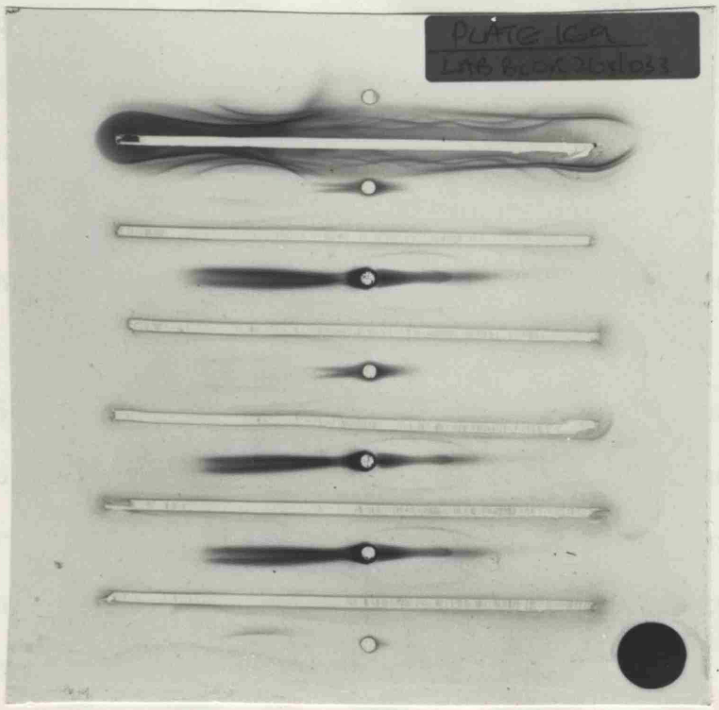
5.3.1 By Immunoelectrophoresis

The anti-prothrombin antisera were diffused against normal rat plasma and microsomal fractions prepared from control and warfarin-treated rats. The microsomal fractions were solubilised in buffer containing 1% Nonidet P40 before they were loaded into sample wells. Using the Biorad agarose system, the precipitin band obtained when the antiserum was run against normal rat plasma was further from the origin (on the anodal side) than that obtained when the antiserum was run against microsomal fractions.

When the experiment was repeated using the sodium barbitone buffer-agar system, a precipitin band on the cathodal side of the origin was observed when the antiserum was run against control and test microsomes. (See Plate 5.3.1.) The precipitin line against normal rat plasma remained on the anodal side.

5.3.2 By Crossed Immunoelectrophoresis

Normal rat plasma and microsomal fractions prepared from control



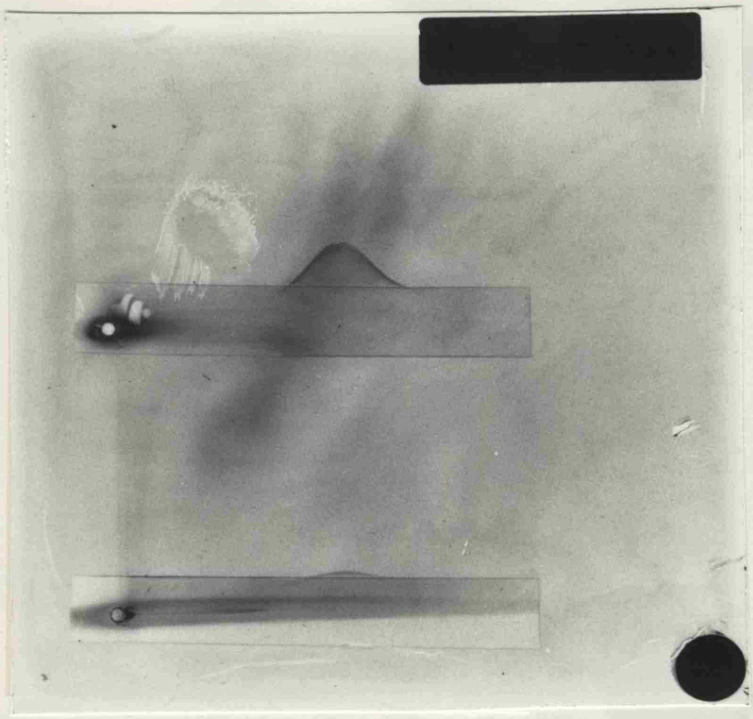
+	o	NRP	-
	o	NRP	@ Rat serum
	o	Control microsomes	@ Prothrombin (Woodstock)
	o	NRP	@ Prothrombin (")
	o	Test microsomes	@ Prothrombin (")
	o	Control microsomes	@ Prothrombin (")
	o	NRP	@ Prothrombin (")

Plate 5.3.1 : Immunological investigation of microsomal fractions using immunoelectrophoresis.
(Test microsomes were prepared from rats dosed with 10 mg warfarin/kg body weight.)

and warfarin-treated rats (and concentrated 5x) were the antigen sources subjected to electrophoretic separation in the first dimension. On electrophoresis into the anti-prothrombin antibody-containing gel, no rockets were seen against the microsomal antigen sources at the higher antibody concentrations. When the antibody concentration was decreased to 5 µl per ml, a small rocket was detected with the control microsomal fraction. (See plate 5.3.2.) However, even at this low antibody concentration, no rocket was observed for the test microsomal fraction. This indicates that no antigen was recognised in the sample derived from warfarin-treated rats under these conditions.

5.3.3 By Single Radial Immunodiffusion

Serial dilutions of normal rat plasma and control and test microsomes were loaded in the origin wells of the antiserum-containing gel (see Section 2.12.3). The results of a typical gel are shown in Plate 5.3.3. The concentration of PRM in the microsomal fractions was determined from the standard curve. No precipitin rings were observed round the wells containing control microsomes. It is possible that the excessive amount of contaminating protein in the samples masked or interfered with precipitin line formation. In microsomes prepared from warfarin-treated rats, precipitin lines were clearly discernable. The average concentration of PRM in the test microsomes was estimated to be between 75 and 80%. This estimate is in close agreement with determinations of PRM made using the chromogenic assay and demonstrates that the antibody is capable of recognising incomplete precursor forms of prothrombin.



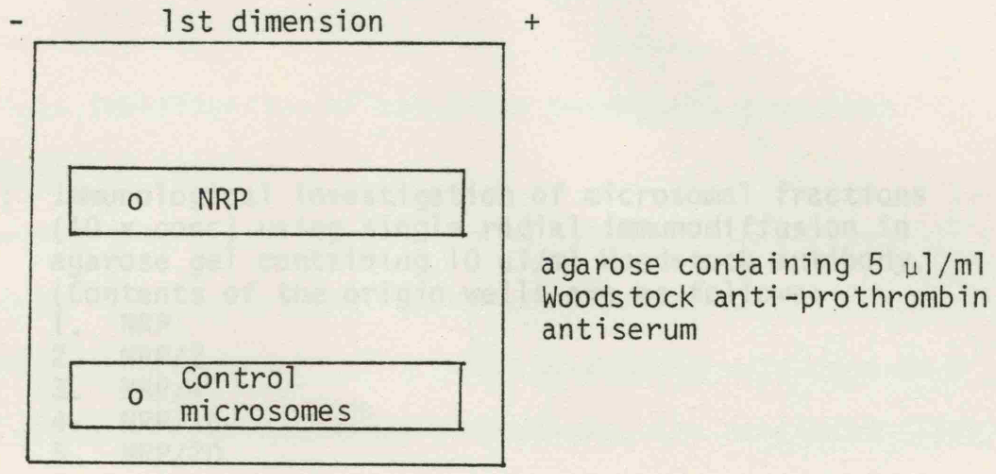


Plate 5.3.2 : Immunological investigation of microsomal fractions using crossed immunoelectrophoresis

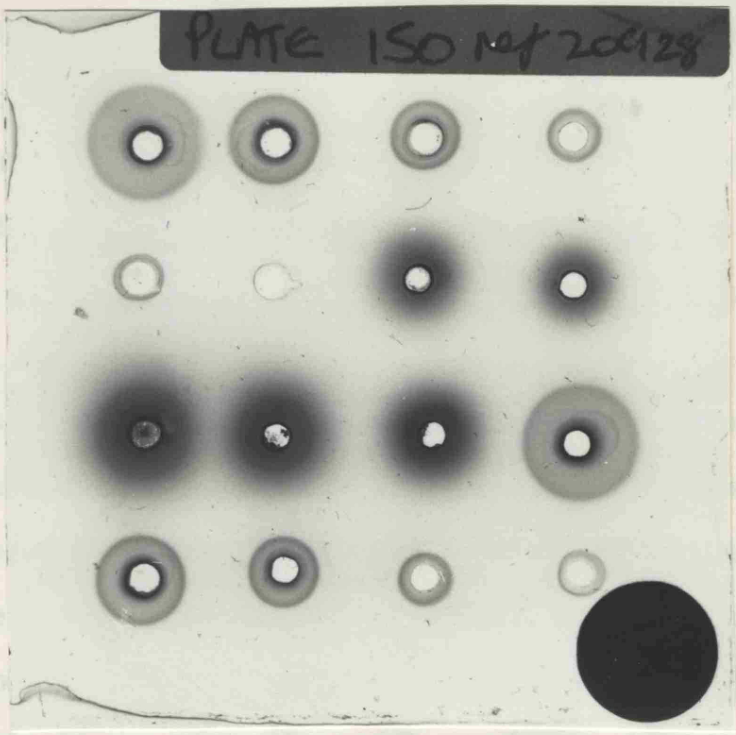


Plate 5.3.3 : Immunological investigation of microsomal fractions (10 x conc) using single radial immunodiffusion in agarose gel containing 10 µl/ml Woodstock antibody. (Contents of the origin wells are as follows:

1. NRP
2. NRP/2
3. NRP/4
4. NRP/10
5. NRP/20
6. -
7. Control microsomes (5 x conc)
8. Control microsomes (2.5 x conc)
9. Test microsomes (10 x conc)
10. Test microsomes (5 x conc)
11. Test microsomes (2.5 x conc)
12. NRP
13. NRP/2
14. NRP/4
15. NRP/10
16. NRP/20.)

5.4 Discussion

The antiserum, raised against prothrombin concentrate, gave a single precipitin line on immunoelectrophoresis against rat plasma or prothrombin concentrate. The position of the precipitin line, on the anodal side of the origin, was analogous to that of the human control using anti-human prothrombin and human plasma.

On crossed immunoelectrophoresis, the rocket formed by rat plasma had the same mobility as the major peak formed by prothrombin concentrate though an additional shoulder peak was evident in the latter case. When the immune complexes formed between rat plasma and the antiserum were removed by immunoprecipitation, no prothrombin activity was detectable in the supernatant. These observations led to the conclusion that the antiserum had antiprothrombin specificity.

Immunological investigation of rat liver microsomal fractions yielded anomalous results.
On immunoelectrophoresis, using a sodium barbitone buffer-agar system, cross-reacting material (CRM) was observed on the cathodal side of the origin when control or test microsomes were used as a source of antigen. Against normal rat plasma, the precipitin line was found on the anodal side of the origin.

On crossed immunoelectrophoresis, a small amount of CRM was detected in the control microsomal fraction which had the same mobility as the rocket formed against rat plasma. No antigen was recognised in the sample derived from warfarin-treated rats under these conditions.

Carlisle and Suttie (1980) recently published their immunological characterisation of rat liver prothrombin precursors. In control microsomes they identified a second cross-reacting form, in addition to the form identified here, with a slightly reduced mobility. They suggested that this might represent an intermediate in prothrombin biosynthesis. They also identified two forms of CRM in microsomes prepared from warfarin-treated rats, which had even more reduced mobilities.

The precursors detected by Carlisle and Suttie (1980) were isolated by a more extreme procedure than that used in the present study. Firstly, the microsomes were solubilised in 2.5% Triton X-100. Then the supernatant was passed through a heparin-agarose column and concentrated prior to immunological characterisation. It seems likely that the precursors were neither solubilised nor concentrated enough to be detected by crossed immunoelectrophoresis in the present study.

Using single radial diffusion, CRM was detected in test microsomes but not in control microsomes. The level of prothrombin-related material was estimated to be between 75 and 80%, in close agreement with chromogenic determinations. This demonstrated that the antiserum is capable of recognising incomplete precursor forms of prothrombin under certain conditions.

CHAPTER 6

VITAMIN K-DEPENDENT CARBOXYLATION

6.1 Introduction

To investigate the post-translational modification of prothrombin, an *in vitro* vitamin K-dependent carboxylation assay was set up using both non-solubilised and solubilised microsomes. (See Section 2.9 and Appendix 3.) The anticoagulant activities of benzyl-3,5-dichloro-2, 6-difluoro-4-pyridyl ether (PP888) and the related compound 2,6-difluoro-3,5-dichloro-pyridinol (PP493) on the *in vitro* system were compared with that of warfarin. Because of the sex effect noted with PP888, its action on microsomes prepared from both male and female rats was observed. Finally, the incorporation of radioactivity into immunoprecipitable protein was investigated using anti-prothrombin antiserum.

6.2 Strain difference in response to the carboxylation assay

Microsomes prepared from Sprague Dawley and Wistar-derived rats were included in the non-solubilised vitamin K-dependent carboxylation assay. Table 6.2.1 shows the counts incorporated into acid-precipitable protein in the presence and absence of vitamin K. Each assay was carried out in duplicate. Microsomes prepared from Wistar rats showed twice the activity of those prepared from Sprague Dawley rats. There are several possible explanations for this. If the Sprague Dawley rats were less susceptible to the warfarin (10 mg/kg body weight) administered 18 hours prior to microsome preparation, then there could

STRAIN	DISINTEGRATIONS/MIN/ML MICROSOMES	
	NO VITAMIN K	+ VITAMIN K
Wistar	300	2,250
Sprague Dawley	250	950

Table 6.2.1 : Incorporation of radioactivity into acid-precipitable protein in the presence and absence of vitamin K using microsomes prepared from two strains of rats.

ASSAY No.	INCUBATION DETAILS	DPM/ML MICROSOMES		COMMENTS
		♂	♀	
1	No vitamin K	120	370	-ve control
2	vitamin K 100 µg	230	670	+ve control
3	" 10 µg	180	420	
4	" 1 µg	170	420	
5	warfarin 10.0 mg/ml	100	437	inhibition
6	" 1.0 mg/ml	250	790	slight stimulation
7	" 0.1 mg/ml	390	725	
8	PP888 10.0 mg/ml	280	655	no effect
9	" 1.0 mg/ml	275	690	"
10	" 0.1 mg/ml	275	600	"
11	PP493 10.0 mg/ml	65	40	inhibition
12	" 1.0 mg/ml	145	375	"
13	" 0.1 mg/ml	115	335	"

Table 6.3.1 : Incorporation of radioactivity into acid-precipitable protein in the presence of various concentrations of vitamin K and anticoagulants. (100 µg of vitamin K was present in all assays containing anticoagulants)

have been a smaller build up of precursors in the liver. This is unlikely since chromogenic assay of the two microsomal preparations indicated similar levels of PRM in each sample.

Alternatively, the carboxylase system may not have been induced to the same extent in the Sprague Dawley rats. Shah and Suttie (1978) showed that the protein carboxylase activity reflects the level of precursor substrates in the liver.

Wistar-derived rats were used for all assays involving non-solubilised microsomes.

6.3 Effect of anticoagulants on the non-solubilised carboxylation assay

Microsomes were prepared from male and female rats, previously dosed with 10 mg warfarin per kg body weight. The microsomes were washed to remove endogenous warfarin and soluble PRM. Vitamin K-dependent carboxylation was then carried out in the presence of various concentrations of vitamin K, warfarin, PP888 and PP493. The results are given in Table 6.3.1 and several comments can be made. Radioactive incorporation was higher in microsomes derived from female rats than from male rats. Siegfried et al (1979) have shown that the levels of precursor prothrombin are higher in female warfarin treated rats than in male warfarin-treated rats. (See also Chapter 3, Section 3.4.) Warfarin inhibited carboxylation at the highest concentration but at lower concentrations, the inhibition was overcome by the presence of a relatively high level of vitamin K. This confirms the observations of Sadowski et al (1976). PP888 had no inhibitory effect

on the carboxylation assay whilst PP493 inhibited at all concentrations tested. This implies that it is a metabolite of PP888 and not the compound itself which is the active anticoagulant. It is possible that the sex effect noted with PP888 is due to variation in the degree or mechanism of metabolism between male and female rats.

6.4 Effect of oral administration of PP888 on microsomal precursor levels

Because of the lack of anticoagulant activity of PP888 in the *in vitro* system, its action *in vivo* was investigated. The effect of a single dose of PP888 (100 mg/kg body weight) was compared with that of a single dose of warfarin (5 mg/kg body weight). PP888 was administered in corn oil because it was found to be insoluble in aqueous buffer or the carrier, "Lissatan". Appropriate controls dosed with corn oil or "Lissatan" alone were included in the experiment. Table 6.4.1 indicated that a single dose of PP888 was not sufficient to cause an increase in the level of PRM in the microsomal fraction.

6.5 Effect of PP493 on the solubilised carboxylation assay

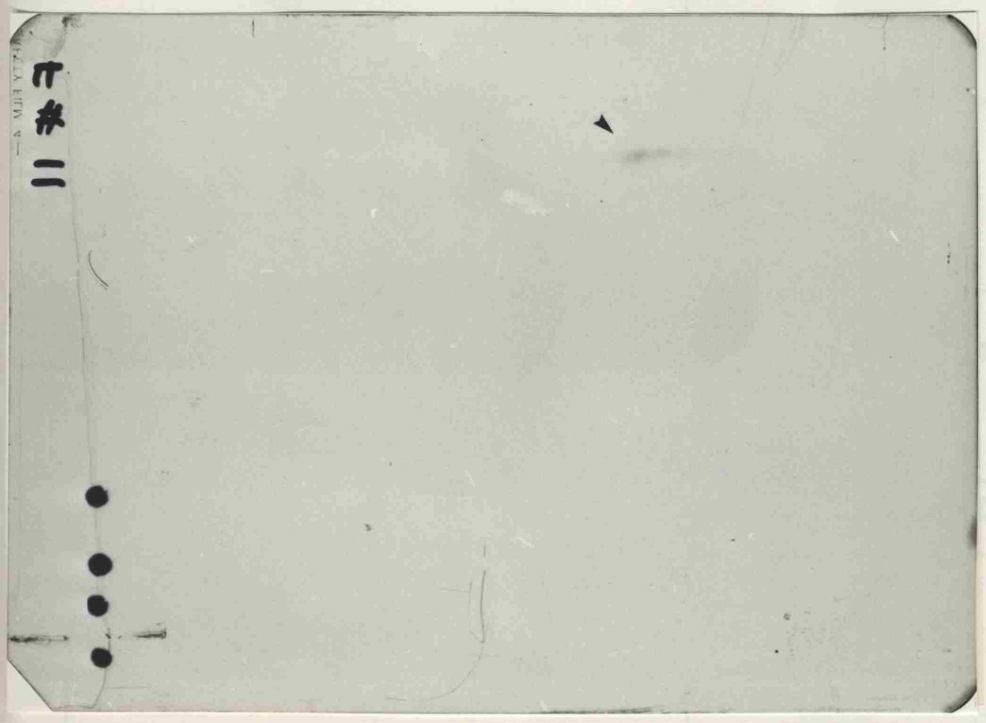
Esmon and Suttie (1976) showed that warfarin is unable to inhibit the solubilised carboxylase system. Friedman and Griep (1980) found that tetrachloropyridinol - structurally related to PP493 - is a potent inhibitor of the solubilised carboxylase, therefore the effect of PP493 on this system was investigated. Table 6.5.1 shows that PP493 is a potent inhibitor of the solubilised vitamin K-dependent carboxylation assay.

6.6 Effect of Preabsorption of the Microsomes with Anti-prothrombin antibody on the Solubilised Carboxylation Assay

Microsomes were solubilised in the presence of anti-prothrombin antibody (30 µl of antiserum per ml microsomes) and SAC cells (20 µl per ml microsomes) at $4^{o}C$ for 30 min. Centrifugation at 105,000 g for 1 hr removed not only insoluble microsomal components, but also the immune complexes bound to SAC cells. The preadsorbed microsomal fraction was incorporated into the vitamin K-dependent carboxylation assay. Table 6.6.1 compares the amount of radioactivity incorporated into TCA-precipitable protein by treated and untreated microsomes in the presence and absence of vitamin K. The results indicate that pre-treatment of the microsomes with antiprothrombin antibody reduces the background incorporation of $H^{14}CO_3^-$ into microsomal protein by about 25%. Stimulation of radioactive incorporation on the addition of vitamin K is not affected by pretreatment of the microsomes with this level of antibody.

6.7 Post-absorption of the Carboxylation Assay with Anti-prothrombin Antibody

Following vitamin K-dependent incorporation of $H^{14}CO_3^-$ into microsomal protein (using the solubilised system) the incubation mix was subjected to immunoprecipitation. 0.2 ml of anti-prothrombin antiserum was added and the mixture allowed to stand overnight at $4^{o}C$. After the addition of 100 µl of SAC cells, the immune complexes were collected as described in Section 2.15. Instead of resuspending the pellet in Staph. A gel buffer it was resuspended in 2 x 20 µl of lysis buffer. The sample was then analysed by two-dimensional electrophoresis as described in Section 2.18. The result is shown in Plate 6.7.1. A



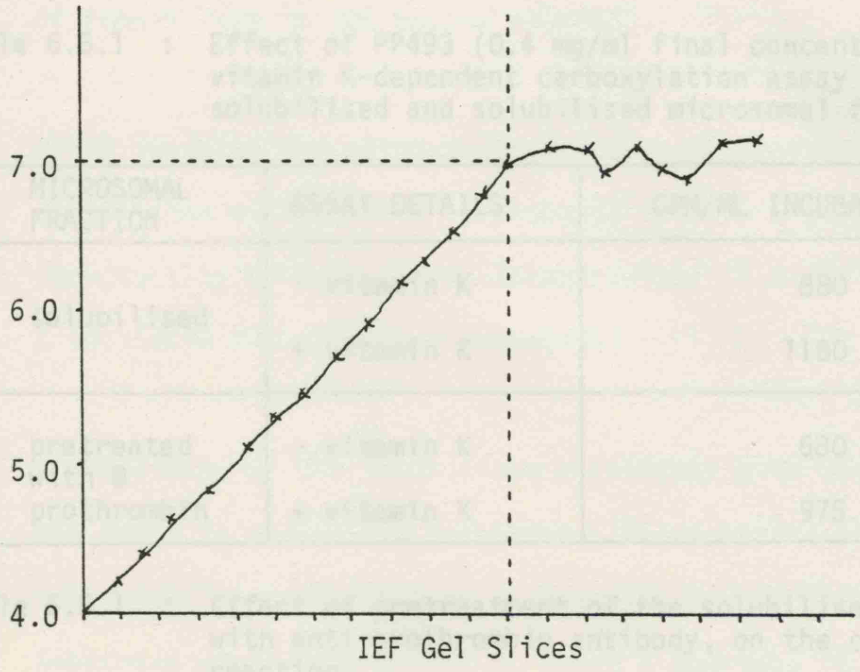


Plate 6.7.1 : Two-dimensional electrophoresis of the immunoprecipitate obtained after vitamin K-dependent carboxylation.

GROUP	DOSE REGIME	PERCENTAGE PRM BY CHROMOGENIC ASSAY
1	Corn oil (5 ml/kg)	30
2	Corn oil with PP888 (100 mg/kg)	30
1	Lissatan (5 ml/kg)	28
2	Lissatan with warfarin (5 mg/kg)	90

Table 6.4.1 : Effect of single doses of PP888 and warfarin on the PRM of microsomal fractions.

MICROSOMAL FRACTION	ASSAY DETAILS	CPM/ML INCUBATION MIX
non-solubilised	+ vitamin K	2550
	- vitamin K	470
	+ PP493 + vitamin K	400
solubilised	+ vitamin K	3600
	- vitamin K	1900
	+PP493 + vitamin K	2000

Table 6.5.1 : Effect of PP493 (0.4 mg/ml final concentration) on the vitamin K-dependent carboxylation assay using non-solubilised and solubilised microsomal fractions.

MICROSOMAL FRACTION	ASSAY DETAILS	CPM/ML INCUBATION MIX
solubilised	- vitamin K	880
	+ vitamin K	1180
pretreated with @ prothrombin	- vitamin K	680
	+ vitamin K	975

Table 6.6.1 : Effect of pretreatment of the solubilised microsomes with anti-prothrombin antibody, on the carboxylation reaction.

single radioactively labelled product was demonstrated having a molecular weight of between 70,000 and 80,000, and an isoelectric point (pI) of 7.1 ± 0.2.

6.8 Conclusion

PP888 was found not to be a useful tool for the investigation of vitamin K-dependent carboxylation. It had far less anticoagulant activity than warfarin in *in vivo* studies, and showed no inhibitory activity in the *in vitro* system. It was concluded that a metabolite of PP888 was the active anticoagulant. The difference in response of male and female rats to PP888 might have been due to hormonal effects on its metabolic pathway. Jolly *et al* (1977) have shown that oestrogen facilitates the intestinal absorption of vitamin K. This might reduce the sensitivity of female rats to the action of PP888.

PP493 inhibits the carboxylation reaction in both solubilised and non-solubilised microsomes. It probably acts in a similar way to the structurally related tetrachloropyridinol (i.e. by inhibiting vitamin K epoxidase and vitamin K reductase).

Pretreatment of the solubilised microsomes with anti-prothrombin antibody was found to reduce the background incorporation of radioactivity. This was rather surprising since a reduction in the level of endogenous substrate would be expected to cause a decrease in the vitamin K-dependent carboxylation reaction. It is probable that the amounts of antibody and SAC added were insufficient to remove all the PRM from

the microsomes. Suttie et al (1980) found that specific removal of precursor prothrombin with antiprothrombin-Sepharose reduced vitamin K-dependent carboxylation activity by 20 - 25%. They failed to mention whether or not background incorporation was monitored. The carboxylation which occurs after removal of PRM is due to the presence of the other vitamin K-dependent clotting factor precursors. In addition, Wallin and Suttie (1980) have reported that a pyridine nucleotide-dependent carboxylation reaction occurs in the microsomal system. This may account for some of the background incorporation of radioactivity.

The existence of several different isoelectric forms of precursor prothrombin has been recognised for several years. Esmon et al (1975_a) purified a precursor of pI 5.8 and Grant and Suttie (1976) purified a second, more basic form (pI 7.2). More recently, using immunoadsorption techniques, Graves et al (1979) identified five different isoelectric forms of the precursor having pI values of 7.2, 6.7, 6.2, 5.8 and 5.5 respectively. Willingham et al (1979) suggested that the 6.7 and 7.2 precursor species serve as the substrate and product, respectively for the vitamin K-dependent carboxylase reaction. Two-dimensional electrophoresis of the immunoprecipitated product of vitamin K-dependent carboxylation, in the present study appears to confirm this suggestion. A single product of isoelectric point 7.1 was identified. Had the microsomal fraction been capable of further modification of the carboxylated protein, a range of isoelectric forms of precursor would have been expected on two-dimensional analysis.

Recently, Carlisle and Suttie (1980) have presented evidence that the 7.2 precursor is non-carboxylated and that on vitamin K-dependent carboxylation it is converted to a more acidic form. Graves et al (1980) have suggested that both the 7.2 and 6.7 precursor forms might be capable of participation in the carboxylation reaction.

The development of soluble peptide substrates for the vitamin K-dependent carboxylase assay (Suttie et al (1976) has greatly aided the investigation of the nature of the carboxylated products. Finnan and Suttie (1979) have found that the pentapeptide undergoes an unidentified postcarboxylational modification in the assay. This additional modification event may account for the discrepancy between the results of various workers over the precursor forms involved in carboxylation.

CHAPTER 7

CHARACTERISATION OF THE PRIMARY PROTHROMBIN-RELATED GENE PRODUCT

7.1 Introduction

Because of the complexity of the processing events associated with the production of prothrombin from its precursors *in vivo*, the primary unmodified translation product was isolated and attempts were made to modify it *in vitro*. (See Figure 7.1.1.) mRNA was isolated from rat liver (see Appendix 4 and Section 2.13) and translated in a heterologous *in vitro* translation system (see Appendix 5 and Section 2.14). The prothrombin-related translation product was isolated by immunoprecipitation using anti-prothrombin antibody and was then characterised. Finally, its modification in the presence of microsomes was investigated.

7.2 Characterisation of the Translation Product

7.2.1 By SDS PAGE

The immune complexes formed when anti-prothrombin antibody was added to the translation mix were adsorbed onto SAC cells. (See Section 2.15.2.) On recovery of the immune complexes in Staph A gel buffer, the prothrombin-related translation product was analysed by SDS polyacrylamide gel electrophoresis to determine its molecular weight. To take account of non-specifically adsorbed translation products, a second translation mix was treated with non-immune rabbit serum and processed in parallel. Two translation products were specifically precipitated with anti-prothrombin antibody.

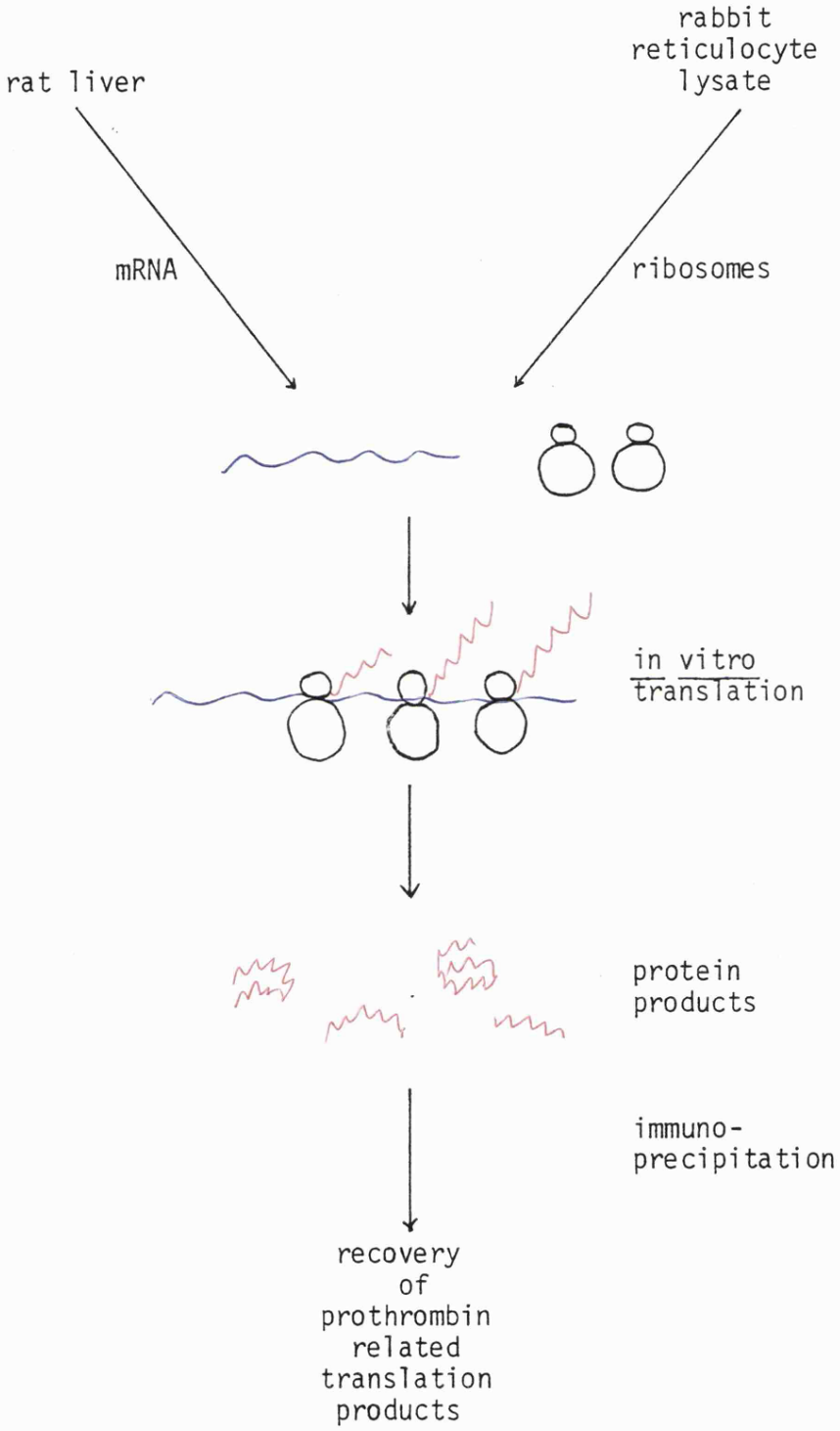


Figure 7.1.1 : Protocol for the production and isolation of prothrombin-related translation products.

These were found by SDS gel analysis to have molecular weights of 70 - 75,000 and 47 - 52,000 respectively. (See Plate 7.2.1.)

7.2.2 By Competitive Immunoprecipitation

The prothrombin-related translation products were immunoprecipitated with various concentrations of anti-prothrombin antibody in the presence and absence of prothrombin concentrate. At the lowest antibody concentration used, the presence of prothrombin concentrate prevented the immunoprecipitation of the translation products. (See Plate 7.2.2.)

7.2.3 By Partial Peptide Digestion

To determine whether the smaller molecular weight translation product was related to the larger, partial peptide digestion of the two products was carried out using V8 and papain proteases. Peptides common to both proteins were identified by autoradiography. (See Plate 7.2.3.)

Prothrombin, purified from rat plasma was labelled *in vitro* using iodoacetamide (see Section 2.22). The partial peptide map generated by digestion of prothrombin was compared with that of the translation products. No common peptides were observed. (See Plate 7.2.4.) This is probably because prothrombin is a glycoprotein (containing 15% by weight of carbohydrate (Grant and Suttie, 1976a)) but the translation products are not glycosylated.

7.2.4 By Tryptic Digest

In an effort to identify peptides common to both prothrombin

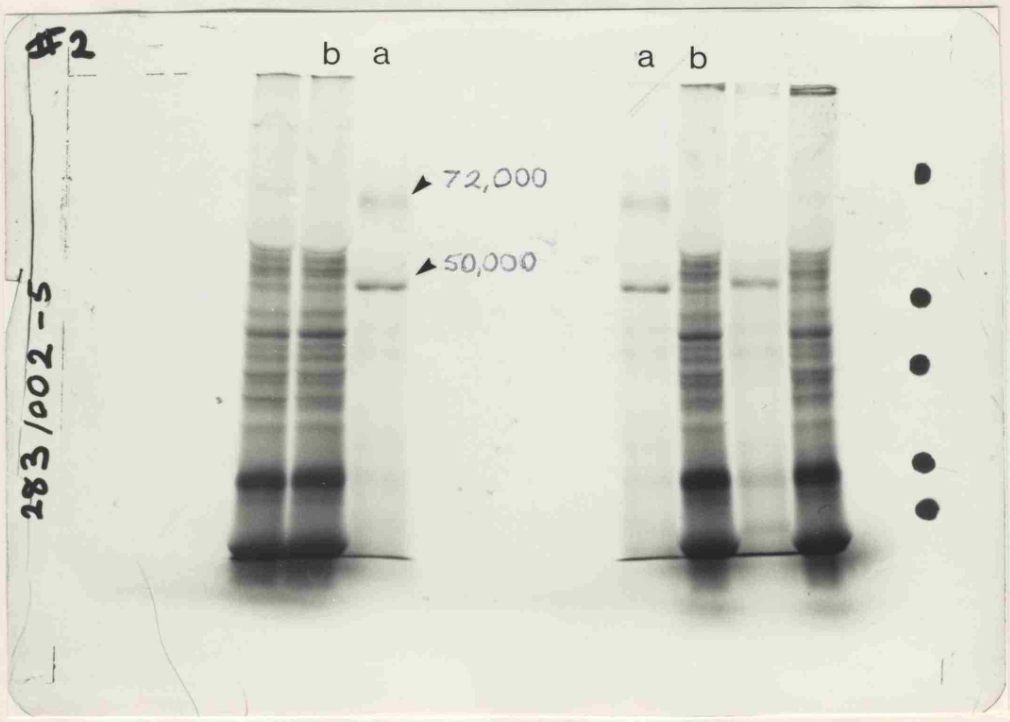


Plate 7.2.1 : SDS polyacrylamide gel electrophoresis of the prothrombin-related translation products (track (a)) obtained by immunoprecipitation, and the remaining supernatant (track (b)).

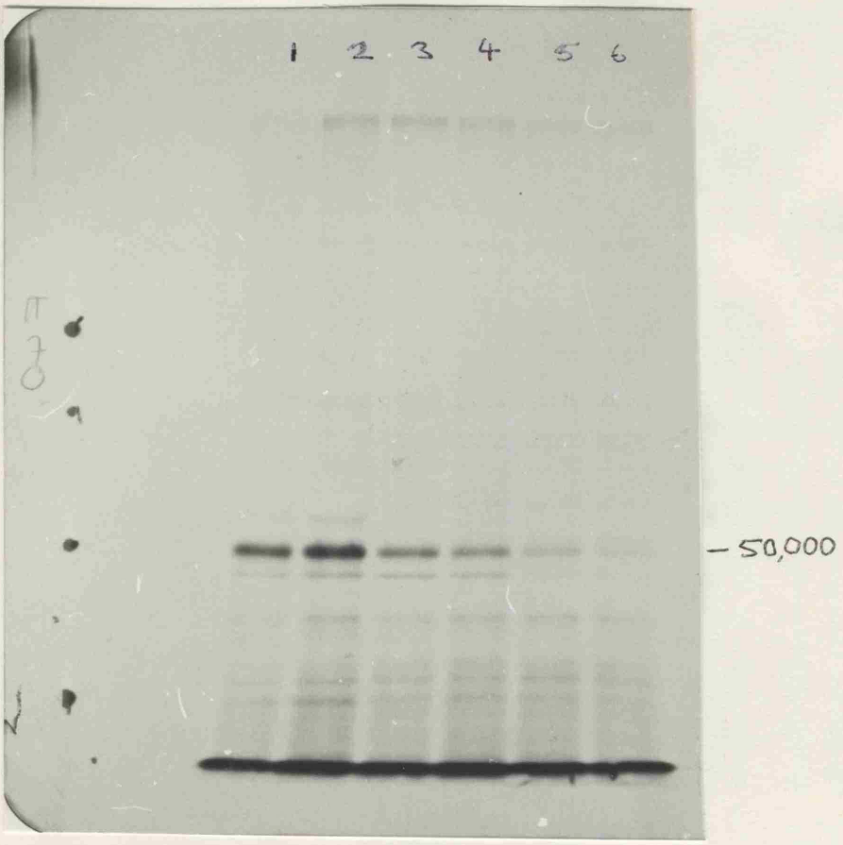
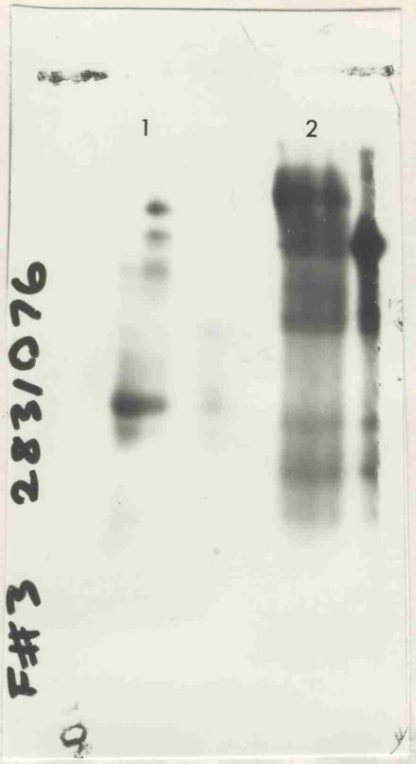


Plate 7.2.2 : Competitive immunoprecipitation of the translation products in the presence and absence of prothrombin concentrate, at various anti-prothrombin antibody concentrations. (Immunoprecipitates were generated as follows:
1. with undiluted antibody
2. with undiluted antibody in the presence of prothrombin
3. with antibody diluted 1 in 20
4. with antibody (1 in 20) in the presence of prothrombin
5. with antibody diluted 1 in 100
6. with antibody (1 in 100) in the presence of prothrombin.)

Plate 7.2.3 : Partial peptide digestion of the 72,000 and 50,000 molecular weight translation products using papain and V8 proteases.
(Tracks are as follows:
1. 72,000 translation product after papain digestion
2. 50,000 translation product after papain digestion
3. 72,000 translation product after V8 digestion
4. 50,000 translation product after V8 digestion.)



2.4 : Partial peptide digestion of prothrombin (1) and the 72,000 molecular weight translation product (2) using papain.

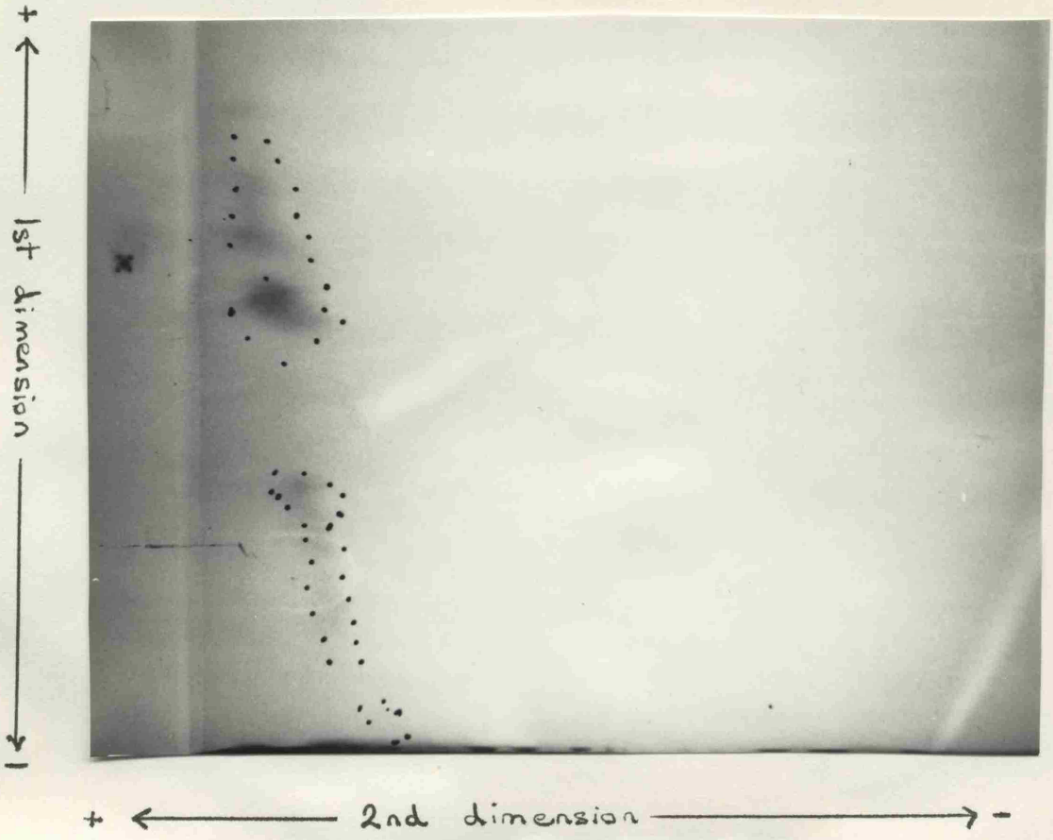


Plate 7.2.4.1 : Autoradiograph of tryptic digest of translation product in presence of prothrombin. 'x' marks the origin. Dots indicate boundaries of fluorescent stain on chromatogram.

and the translation products, a total tryptic digest was undertaken (see Section 2.20). The peptides derived from both prothrombin and the translation products were separated using high voltage paper electrophoresis. (See Section 2.21.) Radioactively-labelled peptides were detected by autoradiography of the unstained chromatogram. Peptides were then detected by fluorescamine staining, the spots being visualised with ultra-violet light. Peptides detected by autoradiography closely matched those detected by protein stain. (See Plate 7.2.4.1)

7.2.5 By Two-Dimensional Gel Electrophoresis

Immune complexes formed when anti-prothrombin antibody was added to the translation mix were adsorbed onto SAC cells and recovered using lysis buffer. 2-D analysis of the translation products precipitated by anti-prothrombin antibody revealed more than fifty spots on the fluorograph (see Plate 7.2.5). It was suggested that the greater denaturing capacity of lysis buffer compared with Staph. A gel buffer caused the release of tightly bound non-specifically absorbed, radioactively-labelled proteins. To assess the non-specific binding to SAC cells, the experiment was repeated using non-immune serum. The resulting fluorograph is shown in Plate 7.2.6. Comparison of Plates 7.2.5 and 7.2.6 reveal one protein of about 50,000 molecular weight which is specifically precipitated with anti-prothrombin antibody. This protein, which is assumed to be the smaller translation product referred to in Section 7.2.1, has a pI of 5.6 $\pm$ 0.2.

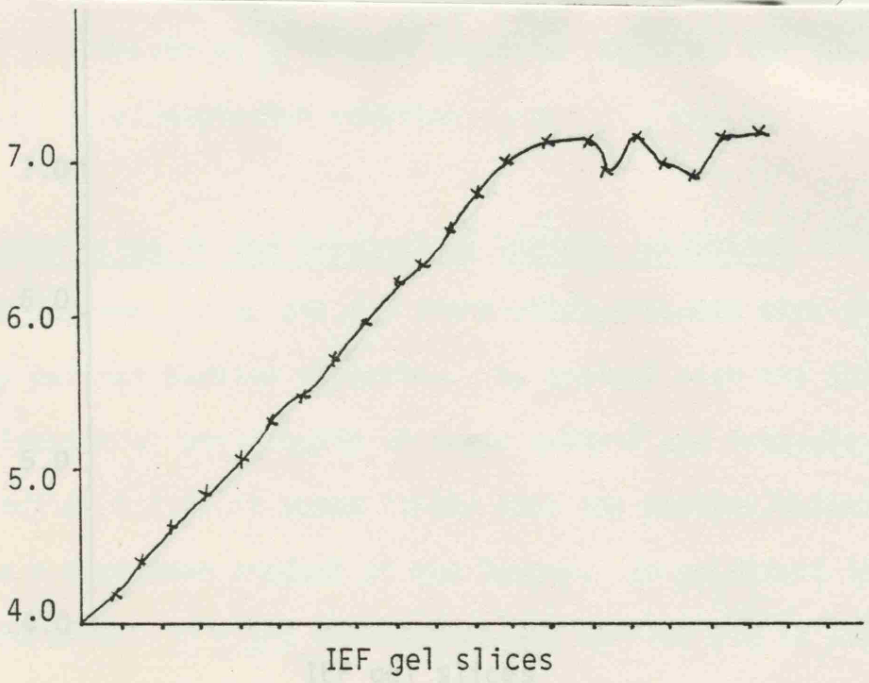


Plate 7.2.5 : Two-dimensional electrophoresis of the immunoprecipitate obtained after <u>in vitro</u> translation of rat liver mRNA.
Compare region marked (▲) with Plate 7.2.6

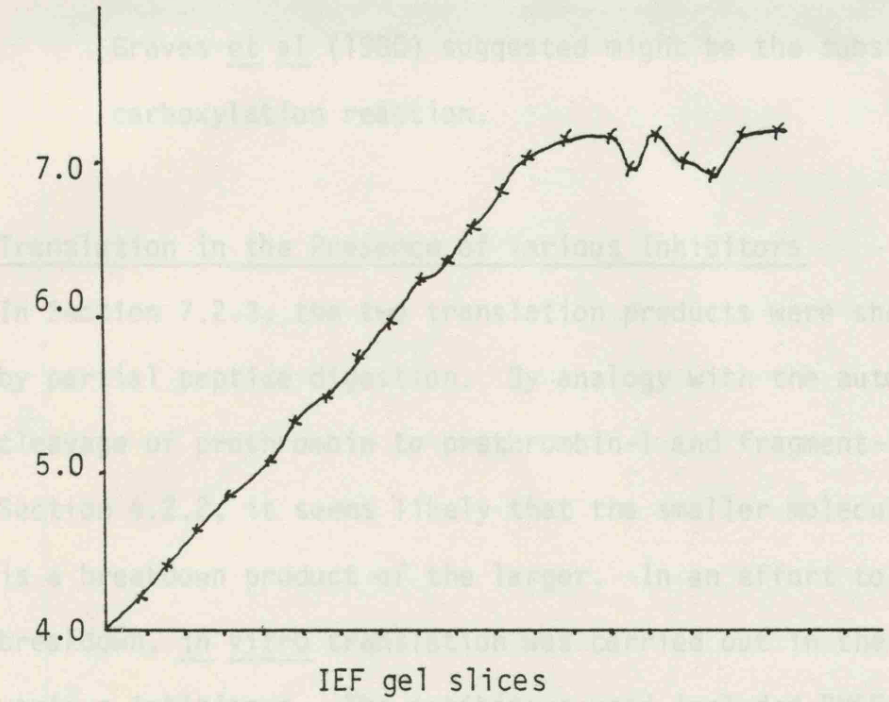
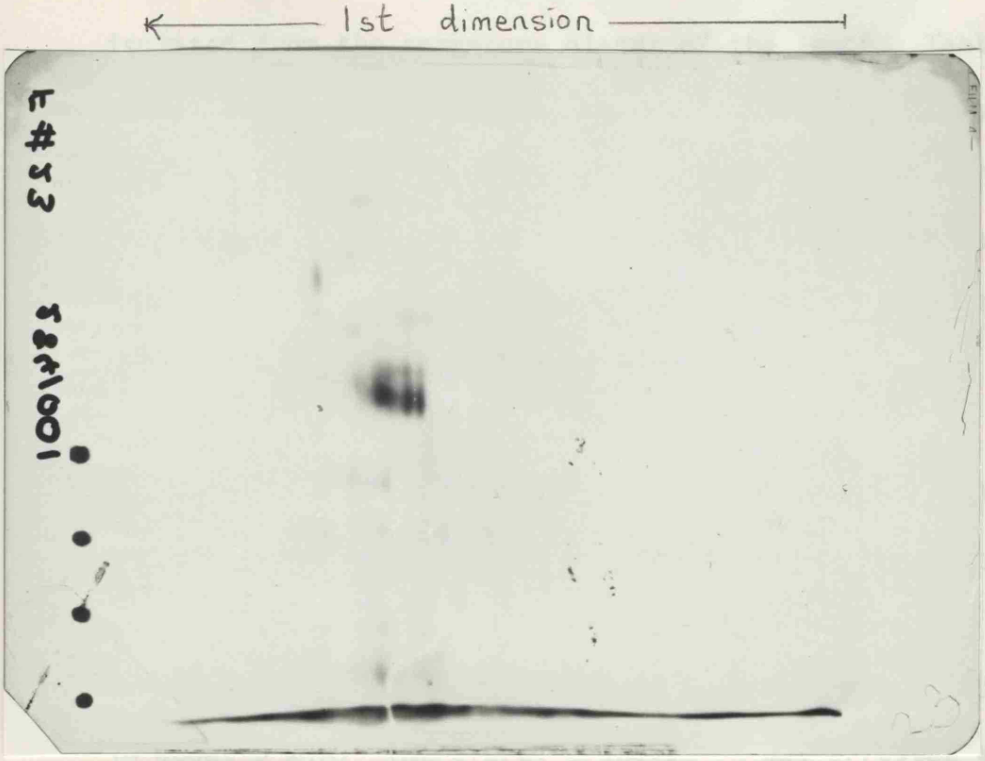


Plate 7.2.6 : Two-dimensional electrophoresis of the non-immune precipitate obtained after <u>in vitro</u> translation of rat liver mRNA.

Because of the large number of spots with molecular weights in the 70 - 80,000 range, it was impossible to identify the larger molecular weight translation product of Section 7.2.1. In an effort to reduce the number of spots due to non-specific binding, the translation mix was pre-treated with non-immune serum and SAC cells, for 15 min at $4^{o}C$. Following removal of the SAC cells by centrifugation, the translation mix was incubated overnight with anti-prothrombin antibody as previously described. On two-dimensional analysis, a much clearer fluorograph was obtained (see Plate 7.2.7), but it was still impossible to identify the larger molecular weight translation product. No spot was observed with a pI of 6.7 which is the isoelectric form of precursor prothrombin which Graves et al (1980) suggested might be the substrate for the carboxylation reaction.

7.3 Translation in the Presence of Various Inhibitors

In Section 7.2.3, the two translation products were shown to be related by partial peptide digestion. By analogy with the autocatalytic cleavage of prothrombin to prethrombin-1 and fragment-1, observed in Section 4.2.2, it seems likely that the smaller molecular weight form is a breakdown product of the larger. In an effort to prevent this breakdown, in vitro translation was carried out in the presence of various inhibitors. The inhibitors used included PMSF, benzamidine hydrochloride, soya bean trypsin inhibitor, heparin and hirudin. PMSF and soya bean trypsin inhibitor are general serine protease inhibitors. Benzamidine hydrochloride is a specific factor Xa



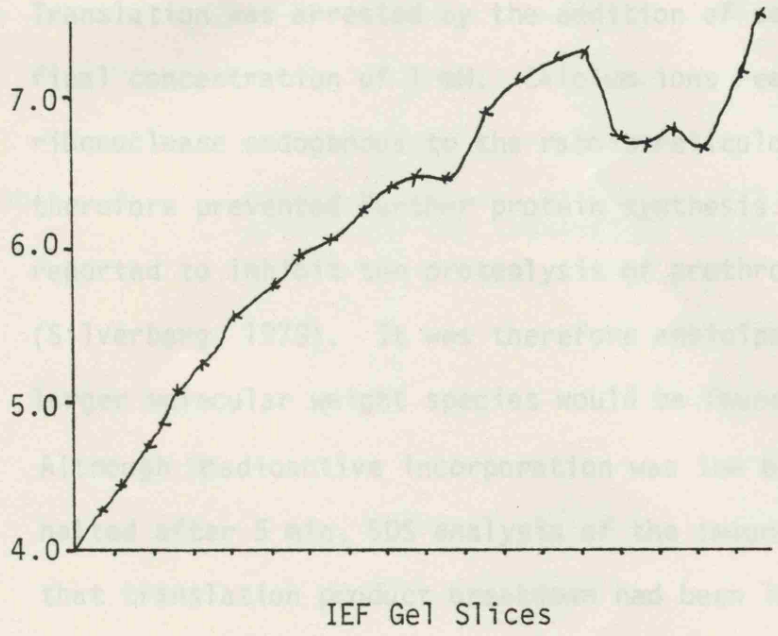


Plate 7.2.7 : Two-dimensional electrophoresis of the immunoprecipitate obtained after pre-absorption of the in vitro translation mix with non-immune serum.

inhibitor. Heparin is an anti-thrombin agent which acts in conjunction with antithrombin III, whilst hirudin is a specific thrombin inhibitor isolated from the secretory glands of the leech. Table 7.3.1 shows the effect of each inhibitor on translation efficiency and on the appearance of both the 72,000 and the 50,000 molecular weight species. None was found to appreciably reduce the amount of the smaller product formed in the translation assay. Most inhibitors drastically reduced the efficiency of the translation reaction.

If the smaller translation product arises by autocatalysis, then it is likely that breakdown would only commence once complete prothrombin precursors had been produced in the translation mix. It was reasoned that a molecule of about 72,000 molecular weight would be synthesised in about 5 min. Therefore, translation was arrested after 5 min and samples were removed for immunoprecipitation over the next 90 min. Translation was arrested by the addition of calcium chloride to a final concentration of 1 mM. Calcium ions reactivated the ribonuclease endogenous to the rabbit reticulocyte lysate and therefore prevented further protein synthesis. Calcium has also been reported to inhibit the proteolysis of prothrombin by thrombin (Silverberg, 1979). It was therefore anticipated that only the larger molecular weight species would be found on immunoprecipitation. Although radioactive incorporation was low because translation was halted after 5 min, SDS analysis of the immunoprecipitate revealed that translation product breakdown had been largely prevented (see Plate 7.3.1). Only trace amounts of the smaller molecular weight species ($\simeq$ 45,000) were observed. On the other hand, a doublet appeared in the region of the larger molecular weight form, the

INHIBITOR	CONCENTRATION	PERCENTAGE INHIBITION OF TRANSLATION	INHIBITION OF TRANSLATION PRODUCT BREAKDOWN
PMSF	1 μm	25	-
Soya bean trypsin inhibitor	20 μg/ml	30	-
Benzamidine HCl	1 mM	33	-
Heparin (in HCl)	0.17 unit 1.70 unit	75 100	- -
Hirudin (in NaCl)	0.1 unit 1.0 unit	0 100	- -

Table 7.3.1 : Effect of several protease inhibitors on translation efficiency and translation product breakdown.

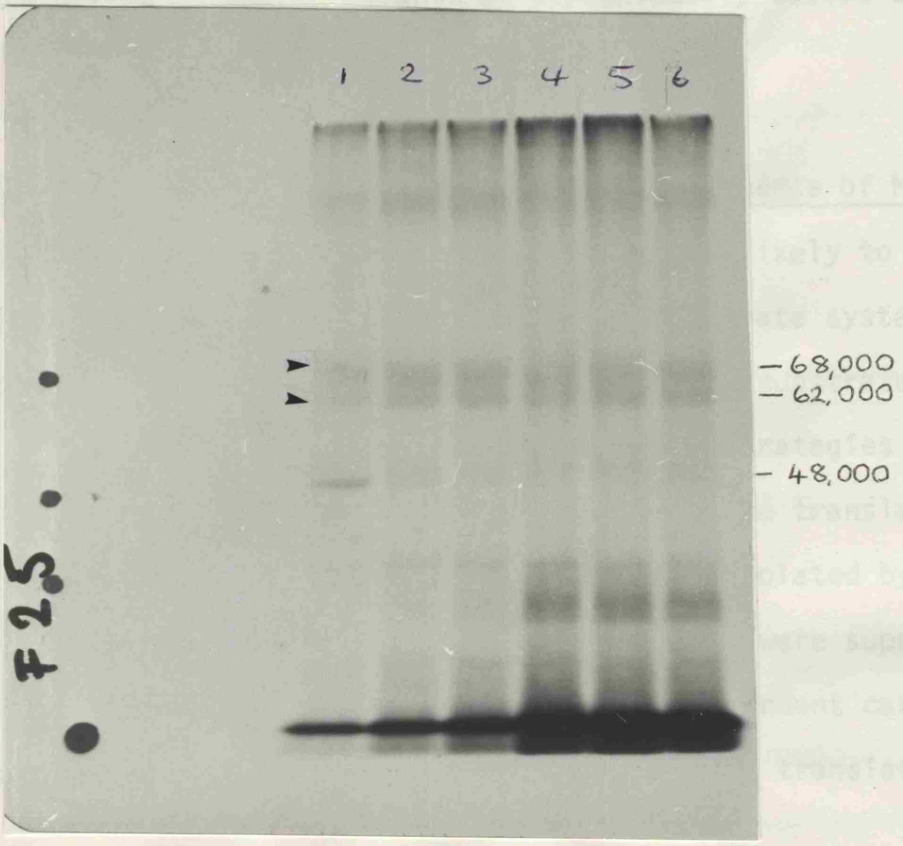


Plate 7.3.1 : Immunoprecipitates collected at various times after the addition of calcium ions to the translation mix. (t = 5) The doublet at about 70,000 is indicated with arrows. (Tracks are as follows:
1. t = 5
2. t = 10
3. t = 15
4. t = 30
5. t = 60
6. t = 90.)

components having molecular weights of approximately 62,000 and 68,000.

7.4 Modification of the Translation Products in the Presence of Microsomes

The prothrombin-related translation product is not likely to be modified in the heterologous rabbit reticulocyte lysate system. It should therefore be recognised by microsomes as the substrate for vitamin K-dependent carboxylation. Two different strategies were devised in an attempt to achieve carboxylation of the translation product. In the first, the translation products, isolated by immunoprecipitation, were added to microsomes which were supplemented with all the components necessary for vitamin K-dependent carboxylation. In the second, microsomes were added to the *in vitro* translation system.

7.4.1 Addition of Translation Products to Microsomes

The immune complexes formed when anti-prothrombin antibody was added to the translation mix were adsorbed onto SAC cells. (See Section 2.15.2.) The immune complexes were released by the addition of 1 M potassium chloride. (Analysis of the radioactivity released by high salt treatment indicated that this is a satisfactory method for the recovery of translation products.) The translation products were added in a small volume (10 µl) of 1 M KCl to the solubilised vitamin K-dependent carboxylation assay mixture. (See Section 2.9.2.) Carboxylation was carried out using cold HCO_3^{2-} instead of $H^{14}CO_3^{2-}$. When carboxylation was over, the incubation mix was immunoprecipitated using anti-prothrombin antibody and analysed using two-dimensional electrophoresis. Even after

six weeks of exposure, no spots were observed on the resulting fluorographs. It was decided that endogenous PRM had diluted out the small amount of translation product added to the microsomes. Therefore, the experiment was repeated using microsomes pretreated with anti-prothrombin antibody (see Section 6.6) and adding ten times more translation product (i.e. 0.1 ml). Again, nothing was visible on the resulting fluorographs.

7.4.2 Addition of Microsomes to the Translation System

Das et al (1980) described a system capable of in vitro protein synthesis together with glycosylation. They found that partial disruption of the microsomal membranes with non-ionic detergent was essential for co-translational modification of the newly synthesised protein. Their system employed microsomes derived from hen oviduct and solubilised with NP-40. In the hope that the vitamin K-dependent carboxylation enzymes would be viable in a system capable of glycosylation, microsomes derived from rat liver, and solubilised with Triton X-100 were added to the translation incubation mix. Concentrations of microsomes and detergent closely approximating the optimum conditions determined by Das et al (1980) were chosen.

Unlike the system described by Das et al (1980), the source of microsomes in this experiment is the same as that of the mRNA. In an effort to ensure that the mRNA endogenous to the microsomes was not translated in the in vitro translation

system, the microsomes were preincubated with 1 mM ATP and 0.1 mM GTP so that all endogenous protein synthesis was completed. At the same time, endogenous PRM was removed by adding anti-prothrombin antibody and SAC cells to the microsomes. Finally, before the microsomes were added to the translation mixture, the Mg^{2+} ion concentration was adjusted to 2 mM and 1 mM EDTA was added to prevent accidental activation of micrococcal nuclease.

Three translation incubation mixes were set up (see Table 7.4.1). Microsomes were added at a concentration of 0.5 A_{260} units of membrane per ml of total incubation mix. This gave a final Triton X-100 concentration of 0.083%. The addition of microsomes to the translation mix markedly reduced the counts incorporated in the presence of mRNA. (See Table 7.4.2.) Counts incorporated when microsomes were added in the absence of mRNA were only slightly less than this indicating that the microsomes might be stimulating radioactive incorporation.

The experiemnt was repeated using microsomes at a concentration of 0.2 A_{260} units of membrane per ml of total incubation mix, in an effort to increase translation efficiency. Analysis of the immunoprecipitates on SDS-PAGE indicated that only mRNA-containing incubation mixes produced prothrombin-related translation products. However, when the immunopreicpitates were analysed by two-dimensional electrophoresis, similar patterns were obtained for both the mRNA + microsomes sample,

COMPONENT	ASSAY NUMBER		
	1	2	3
Lysate	100	100	100
^{335}S-methionine	25	25	25
mRNA (1 g/ml)	12.5	12.5	-
Microsomes	-	12.5	12.5
Vitamin K (1 mg/ml)	1	1	1
NADH (50 mg/ml)	2	2	2
HCO_3 (0.034 M)	2	2	2
Buffer A	12.5	-	-
DEP-water	-	-	12.5

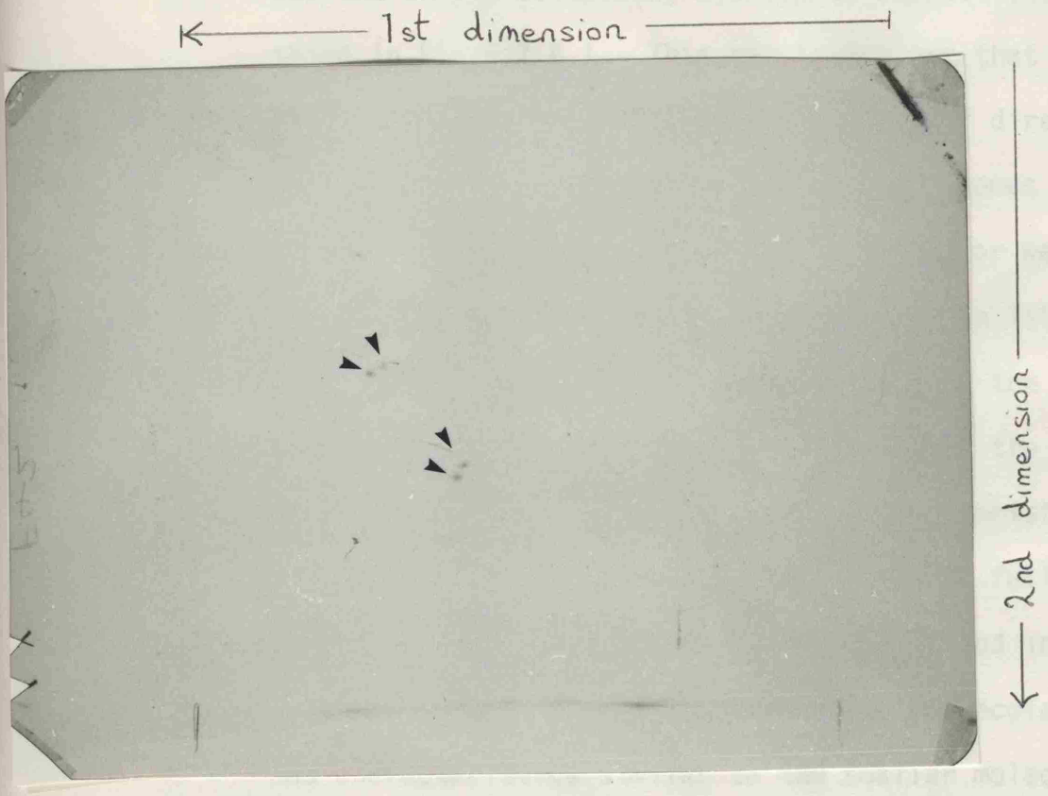
Table 7.4.1 : Translation assays set up to investigate the effect of microsomes on the translation products.

Assay Number	Counts incorporated (cpm/µl incubation)
1	18,000
2	6,500
3	5,500

Table 7.4.2 : Counts incorporated into TCA-precipitable protein in the assays described in Table 7.4.2.

Spot No.	RF value	molecular weight	pI
1	0.475	73,000	5.0
2	0.480	72,000	5.2
3	0.625	48,000	5.55
4	0.640	46,000	5.65

Table 7.4.3 : Characteristics of the spots revealed on two-dimensional electrophoresis of the immunoprecipitates of assays 2 and 3 from Table 7.4.1.



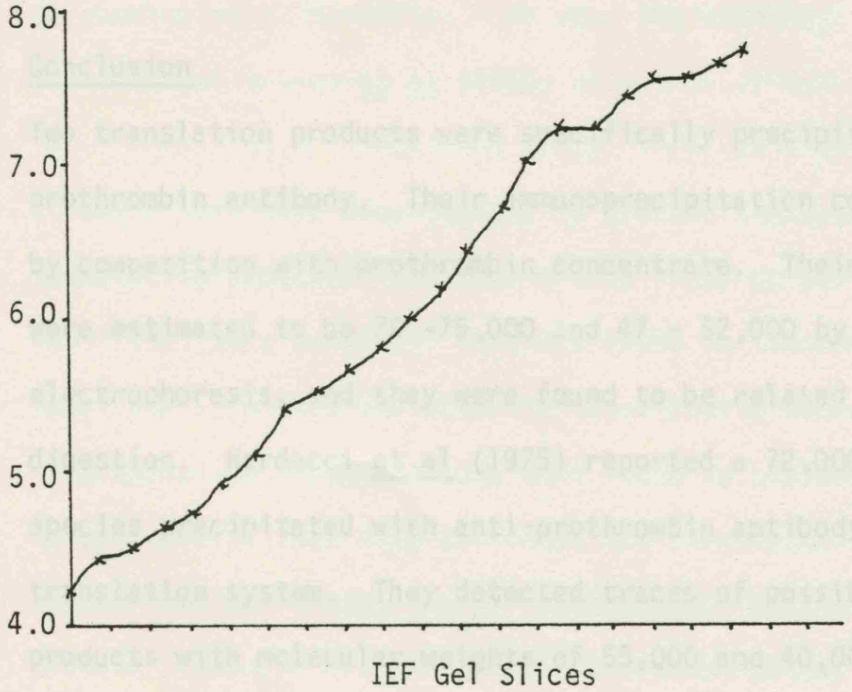


Plate 7.4.1 : Two-dimensional electrophoresis of the immunoprecipitate obtained following in vitro protein translation in the presence of rat liver microsomes and the absence of exogenous mRNA.

and the sample containing microsomes alone. The latter is shown in Plate 7.4.1. This result implies that mRNA endogenous to the microsomes was capable of directing protein synthesis whilst the presence of the microsomes inhibited translation of the exogenous mRNA. Molecular weights and isoelectric points of the spots are given in Table 7.4.3. The molecular weights indicate breakdown of the larger form to the smaller form. The isoelectric point of the 72,000 molecular weight form (pI = 5.0) is characteristic of prothrombin though it seems unlikely that a fully carboxylated and glycosylated protein would be synthesised in the in vitro system. On the other hand, the smaller molecular weight form has characteristics similar to the smaller molecular weight translation product of Section 7.2.5.

7.5 Conclusion

Two translation products were specifically precipitated with anti-prothrombin antibody. Their immunoprecipitation could be inhibited by competition with prothrombin concentrate. Their molecular weights were estimated to be 70 -75,000 and 47 - 52,000 by SDS gel electrophoresis, and they were found to be related by partial peptide digestion. Nardacci et al (1975) reported a 72,000 molecular weight species precipitated with anti-prothrombin antibody in their translation system. They detected traces of possible degradation products with molecular weights of 55,000 and 40,000.

The two translation products were not found to have common peptides with

prothrombin and prethrombin-1 on partial peptide digestion. This is probably because, unlike the serum-derived proteins, the translation products are neither glycosylated nor carboxylated. To overcome this problem, it would be necessary to decarboxylate prothrombin (e.g. by the method of Poser and Price, 1979) and also to deglycosylate it (e.g. enzymatically using endo N-acetyl glucosaminidases).

When complete tryptic digestion of prothrombin and the translation products was undertaken, the resulting peptide map closely matched the pattern of spots on the autoradiograph. This indicates that the translation products are related to prothrombin.

On two-dimensional analysis of the translation products, the smaller one was found to have a pI of 5.6 $\pm$ 0.2. The larger spot could not be conclusively identified. No spot representing the carboxylase substrate of Graves et al (1980) with a pI of 6.7 was observed.

It would have been advantageous to prevent the breakdown of the larger translation product. Although a wide range of serine protease inhibitors were screened, none was found to inhibit the breakdown specifically whilst the majority dramatically decreased the efficiency of translation. Calcium ions were found to prevent degradation, but unfortunately, they also activated the micrococcal nuclease and caused complete inhibition of translation. Not only did calcium prevent breakdown of the larger translation product, but SDS gel analysis of the immunoprecipitate revealed two bands differing in molecular weight by approximately 5,000 daltons. Blobel and

Dobberstein (1975) predicted that secretory proteins contain an NH_2-terminal extension or signal sequence which is cleaved as the protein is fed through the rough endoplasmic reticulum. The larger protein of the doublet could have its signal sequence intact.

Two different strategies for translation product modification were attempted. In the first, the translation products were added to microsomes, capable of vitamin K-dependent carboxylation. Unfortunately, carboxylation of the translation products was not achieved by this method. This may have been due to the fact that the translation products were diluted out by, or unable to compete with, endogenous substrate for the carboxylation reaction. It is also possible that the carboxylase enzymes were unable to recognise the fully formed translation product with its tertiary structure intact. In vivo the nascent protein may be co-translationally modified. With this in mind, microsomes, together with the components of the carboxylase assay, were added to the in vitro translation system. It was hoped that the presence of the microsomes might prevent breakdown of the larger translation product to the smaller, and that the solubilised carboxylase enzymes would be capable of recognising and modifying the nascent prothrombin-related translation product.

Unfortunately, the addition of microsomes to the translation system inhibited the stimulation of protein synthesis by exogenous mRNA. mRNA "endogenous" to the microsomes did direct protein synthesis, however, and a protein with properties characteristic of the smaller

prothrombin-related translation product was revealed on two-dimensional electrophoresis of the immunoprecipitate. Additionally a protein of 72,000 was observed having a pI of 5.0.

CHAPTER 8

DISCUSSION

8.1 Prothrombin Determinations

The original objective of the study was to investigate the effect of anti-coagulants on prothrombin production in the liver and therefore assays which could be applied to turbid solutions had to be devised. Ideally, assays for the determination of prothrombin alone and total prothrombin-related material (PRM) were needed so that prothrombin and its precursors could be quantitatively distinguished.

To begin with, conventional clotting assays, using Taipan venom (to activate prothrombin only) and E. carinatus venom (to activate both prothrombin and its precursors) were assessed. One-stage assays were used because they are technically simpler and less time-consuming than two-stage assays. When applied to plasma samples derived from control and warfarin-treated rats, the assays yielded comparable results as expected. This was not the case when the assays were applied to liver fractions, but problems were encountered because the end-points were not easily discernable. When Triton X-100 was added to the samples in an effort to solubilise the microsomes and make more prothrombin-related material available, this had an adverse effect on the Taipan assay.

Because of the difficulties experienced with the clotting assays, I turned to biochemical assays utilising the chromogenic reagent, Chromozym TH. A number of prothrombin activators were surveyed in the

hope that prothrombin and total PRM could be determined separately. The Tiger snake assay was the only one which showed promise as a means of assaying biologically complete prothrombin. However, the work of Esnouf and Prowse (1977) suggested that Tiger snake venom might be capable of activating partially carboxylated prothrombin molecules (those with more than 7 γ-carboxyglutamic acid residues). This cast doubt on the validity of the assay. The E. carinatus chromogenic assay showed good correlation with conventional clotting assays when plasma samples from control and warfarin-treated rats were tested. The "ecarin" activity was not purified prior to use (as has been done by other workers such as Latallo and Teisseyre, 1977) because the cholinesterase activity, endogenous to the venom, had a beneficial clearing action on the turbid liver fractions.

When the E. carinatus chromogenic assay was applied to turbid liver fractions, high levels of activity were recorded in the microsomal fractions of warfarin-treated rats, reflecting a build-up of precursors. A clear relationship between warfarin dosage and the increase in PRM in the microsomes was demonstrated. Also, a decrease in the level of PRM in supernatant-2 fractions with increasing warfarin dose was revealed.

To further demonstrate that the E. carinatus venom activates both prothrombin and its precursors, the response of the assay to plasma samples taken from patients on warfarin therapy was compared with that of conventional clotting assays. Higher levels of PRM were revealed by the E. carinatus activator.

The chromogenic assay was used in the kinetic analysis of ecarin-generated thrombin which was found to have a Km of 6.8×10^{-6}. Inhibitor studies indicated that heparin had very little effect on the assay. It seemed to act as a competitive inhibitor though the effect was probably not physiologically significant. Fulton et al (1979) found that ecarin in situ prevented inhibition of thrombin by antithrombin III and heparin. If this is the case, then the E. carinatus assay has a real advantage over conventional assays since it would be insensitive to the variable levels of inhibitors endogenous to the plasma samples under test and would give a true estimate of PRM.

The chromogenic assay was found to be very versatile. In addition to kinetic studies, it was used in the assessment of prothrombin purity at each stage in the purification procedure. This was achieved by monitoring the specific activity. It was also useful as a means of testing the anti-prothrombin specificity of antibody raised against the prothrombin concentrate.

The use of chromogenic reagents for factor assays is already a reality in some hospitals. The chromogenic assays are readily automated, releasing technicians for more important tasks. Since E. carinatus venom activates precursor prothrombin in addition to prothrombin, this assay would not be useful for monitoring the prothrombin status of patients on anticoagulant therapy. However, it would be very useful if used in conjunction with an assay for the determination of prothrombin alone.

Future work on the chromogenic assay would include a more extensive study on the effect of potential physiological inhibitors, and a continuation of the search for a prothrombin activator. It would be instructive to look at the action of Tiger snake venom and E. carinatus venom on isolated prothrombin precursors.

8.2 Prothrombin Characterisation

Prothrombin, purified from rat plasma was found to have a molecular weight of 75 - 80,000 in agreement with Grant and Suttie (1976a). No indication of the 86,000 dalton species reported by Li and Olson (1967) was noted in the present study. On storage of the prothrombin concentrate, degradation products of molecular weights 58,000 and 28,000 were observed. The instability of the preparation was attributed to the presence of trace amounts of thrombin. Thrombin autocatalytically cleaves prothrombin to yield prethrombin-1 and fragment-1. Partial peptide digestion confirmed that the 75 - 80,000 and 58,000 molecular weight species are related.

Grant and Suttie (1976a) reported that rat prothrombin is 15% carbohydrate by weight. Using a stain specific for glycoprotein, all three molecular weight species were found to contain carbohydrate. The presence of carbohydrate in fragment-1 of rat prothrombin is analogous to the bovine system where fragment-1 is 25% carbohydrate by weight (Pletcher et al, 1980).

8.3 Antibody Characterisation

The antibody raised against prothrombin concentrate was characterised using standard immunoelectrophoretic techniques. Several lines of

evidence indicated that the antibody possessed anti-prothrombin specificity. On immunoelectrophoresis, the precipitin line formed when the antibody was diffused against rat plasma or prothrombin concentrate was found at a position on the gel analogous to that of the human control. Crossed immunoelectrophoresis of the prothrombin concentrate against the antibody produced a rocket with the same mobility as the peak formed when rat plasma was cross-reacted with the antibody.

The third line of evidence was the functional test of antibody specificity using the chromogenic assay. Removal of the immune complexes formed between the antibody and rat plasma simultaneously caused the total loss of assayable prothrombin activity in the remaining supernatant.

8.4 Immunological Characterisation of Microsomal PRM

Immunoelectrophoresis of test and control microsomes against the anti-prothrombin antibody revealed cross-reacting material which moved cathodically from the origin when a sodium barbitone buffer-agar system was used. Normal rat plasma produced a precipitin line on the anodal side of the origin. Since biologically complete prothrombin carries more negative charges than its non-carboxylated precursors, it would be expected to run closer to the anode. Although the difference in mobilities between prothrombin and its precursors was surprisingly large, this may have been a function of the buffer-agar system used.

On crossed immunoelectrophoresis, only the small amount of prothrombin in control microsomal fractions was detected. No precursor forms with

altered mobility were recognised by the antibody. This is probably because the microsomal fractions were neither sufficiently solubilised nor concentrated enough. Janson and Helgeland (1975) used microsomes concentrated 45 times for their immunological studies, whilst Carlisle and Suttie (1980) purified the precursors before characterising them immunologically.

Single radial diffusion revealed that test microsomes contained cross-reacting material. Since the quantity of PRM detected by this method paralleled that of the chromogenic determination, it indicated that the antibody recognises precursor forms of prothrombin. Although a reaction was expected with control microsomes, no precipitin ring was observed. The large amount of contaminating protein in the microsomal fractions may have masked or interfered with the reaction.

8.5 Vitamin K-dependent Carboxylation and Anticoagulants

The anticoagulant action of PP888 was investigated both *in vivo* and *in vitro*. On oral administration of PP888 to rats, it was found to be far less active than warfarin. When *in vitro* studies revealed that PP888 had no inhibitory effect on vitamin K-dependent carboxylation, it was concluded that a metabolite of PP888 was probably the active anticoagulant. PP493, structurally related to PP888, inhibited *in vitro* vitamin K-dependent carboxylation in both solubilised and non-solubilised microsomes. Because of the structural similarity between PP493 and tetrachloropyridinol, it is likely that they share a common mechanism of action, attributable to inhibition of the carboxylase itself (Friedman and Griep, 1980).

8.6 Prothrombin Precursors

The primary prothrombin-related translation product was obtained by translating rat liver mRNA in an *in vitro* cell-free system. The mRNA was added to a heterologous rabbit reticulocyte lysate system so that the translation product would not be modified in any way. The prothrombin-related material was recovered by immunoprecipitation. On SDS gel electrophoresis of the immunoprecipitate, only two proteins were observed. This indicates that the antibody is relatively specific.

The two translation products had molecular weights of 72,000 and 50,000 respectively, and were shown to be related by partial peptide digestion. Two lines of evidence indicated that they were also related to prothrombin. Firstly, immunoprecipitation of the translation products could be competitively inhibited by the addition of prothrombin concentrate to the reaction vessel. Secondly, on total peptide digestion of prothrombin together with the translation products, closely matching maps of labelled and non-labelled peptides were obtained.

Prothrombin is a glycoprotein containing 15% carbohydrate by weight. The non-glycosylated precursor might be expected to have a molecular weight in the region of 68,000 daltons. Taking into account the presence of a signal sequence, the larger translation product (of molecular weight 72,000) is therefore the correct size for a putative prothrombin-related translation product. This implies that the 50,000 molecular weight translation product is a breakdown product of the larger form, or alternatively, that it is the primary

translation product of another vitamin K-dependent clotting factor. The latter possibility arises because the prothrombin concentrate against which antibody was raised, was likely to be contaminated with other vitamin K-dependent proteins.

Attempts to prevent the "breakdown" of the larger translation product by the addition of protease inhibitors to the translation system were largely unsuccessful. On the addition of calcium ions to the system, translation ceased and the appearance of the smaller molecular weight form was markedly reduced. Under these conditions, a doublet appeared in the region of the larger molecular weight translation product. It is possible that the high level of calcium stimulated proteolytic activity in the translation system.

On two-dimensional gel electrophoresis of the prothrombin-related translation products, the smaller molecular weight form could be identified both by its molecular weight and its specific immunoprecipitation. It exhibited a pI of 5.6 $\pm$ 0.2. The larger form was not identifiable because of the large number of spots in the region of 72,000 daltons.

Having characterised the primary translation products as far as possible, the product of vitamin K-dependent carboxylation was investigated.

Two-dimensional gel electrophoresis of the immunoprecipitate, obtained following vitamin K-dependent carboxylation, revealed a protein of pI 7.1 ± 0.2. This agrees with the work of Graves et al (1979) and Willingham et al (1979). Since only one form of radioactively labelled protein was observed, it appears that the conditions of the carboxylation assay did not allow further modification of the precursor.

Carlisle and Suttie (1980) suggested that a 7.2 form of precursor prothrombin might be the substrate for vitamin K-dependent carboxylation rather than the product of it. Graves et al (1980) have suggested that more than one precursor may be a substrate for vitamin K-dependent carboxylation. In the livers of rats treated with warfarin, at least five different isoelectric forms of precursor prothrombin have been identified (Graves et al 1979). These forms could arise by incomplete vitamin K-dependent carboxylation or by post-translational, vitamin K-independent modifications. (The unidentified post-carboxylational modification of pentapeptides observed by Finnan and Suttie (1979) may be an example of the latter). When vitamin K is administered to warfarin-treated rats, precursor levels fall and prothrombin is exported into the plasma (Suttie, 1980). It is possible that in vivo all precursors can be substrates for the carboxylation reaction.

In the studies of Graves et al (1979) and Carlisle and Suttie (1980) all prothrombin precursors were found to have a molecular weight identical to that of prothrombin itself. As discussed earlier, the

primary translation product would be expected to have a molecular weight of about 68,000 (excluding signal sequence) - significantly below that of prothrombin. This indicates a basic anomaly in the current understanding of precursor processing.

Isolation of the primary translation products provided an excellent opportunity to investigate the post-translational processing of precursors from a different angle - one, perhaps, more closely related to the *in vivo* situation. To begin with, the prothrombin-related translation products were added to microsomes capable of vitamin K-dependent carboxylation. No modification of the translation products was detected under these conditions and a number of possible explanations were considered. The endogenous non-labelled prothrombin precursors probably diluted the translation products and competed for sites on the carboxylase enzyme. If the translation product was broken down before it could be modified, or was lacking some co-translational modification, then the carboxylase system might not have recognised it.

With all these points in mind, the strategy was reversed. Solubilised rat liver microsomes and components of the vitamin K-dependent carboxylation assay were added to the *in vitro* translation system in an effort to glycosylate and carboxylate the translation products. Unfortunately, these additions greatly decreased translation efficiency and exogenous mRNA was not translated. However, in the presence of microsomes alone, prothrombin-related translation products were detected. These had similar molecular weights to the translation

products discussed earlier but they occurred as doublets. This may indicate a co-translational modification of the translation products. The smaller pair of proteins had isoelectric points in the region of 5.6 - again similar to the smaller translation product described earlier.

It is tempting to speculate that one of the larger pair of proteins synthesised in this system may be the primary translation product. Partial sequence analysis might show whether or not this is the case. Provided that conditions favourable for glycosylation and carboxylation could be achieved, it might be possible, using this system, to modify the primary translation product and mimic the *in vivo* processing pathway *in vitro*.

Now that rat liver mRNA and anti-prothrombin antibody have been isolated, it would not be unreasonable to consider the cloning of DNA coding for vitamin K-dependent proteins. This would potentially lead to the production of large amounts of the primary translation products and would compliment studies on precursor processing.

APPENDIX 1

DEVELOPMENT OF A ONE-STAGE CLOTTING ASSAY USING E. CARINATUS VENOM

The conventional one-stage clotting assay for prothrombin utilises Taipan or Oxyuranus scutellatus snake venom. Since this venom does not activate PIVKA, a simple one-stage assay using the venom of E. carinatus, the carpet viper, was developed. (E. carinatus venom activates both PIVKA and prothrombin.)

Once developed, the two assays could be used to investigate the relative proportions of PIVKA and prothrombin in biological samples.

A1.1 Determination of reaction conditions for the E. carinatus one-stage assay

A1.1.1 Order of addition of reactants

The components of the assay are a suitably diluted biological sample, a source of fibrinogen and the E. carinatus snake venom. Two possible assay procedures were available:

i) preincubation of the plasma with venom, followed by addition of the substrate

ii) pre-equilibration of the plasma with the substrate, followed by addition of the venom

Preincubation of plasma with the venom led to almost immediate clot formation on addition of absorbed bovine plasma (as source of fibrinogen) and was not therefore practical.

Pre-equilibration of plasma with substrate prior to triggering of the reaction by snake venom (a procedure which parallels the order of addition of reactants in the Taipan assay) was found to yield measurable clotting times. The concentration of the snake venom was varied until a 100% plasma sample (by convention, a 1 in 10 dilution) gave a clotting time of between 10 and 15 seconds.

The final procedure was as follows:

0.1 ml plasma dilution

0.1 ml fibrinogen source*

Equilibrated

at 37°C

+ 0.1 ml E. carinatus venom (1 mg/ml)

*Absorbed bovine plasma was later replaced by 1% clottable fibrinogen because the purity of the former was questionable.

A1.1.2 Modification of one-stage assay for application to human samples

When the assay procedure described in Section A1.1.1 was applied to control human plasma, the 100% sample gave a prolonged clotting time of about 30 sec. The relative proportion of plasma to the other components of the assay was therefore increased. The final procedure was as follows:

0.25 ml plasma dilution

0.05 ml fibrinogen source

Equilibrated

at 37°C

+ 0.05 ml *E. carinatus* venom (1 mg/ml)

A1.1.3 Modification of assay for application to liver fractions

All liver fractions were diluted in Buffer A (see Section 2.8) rather than imidazole buffer if dilution was required.

A1.2 Presentation of results

The Taipan and *E. carinatus* one-stage assays were carried out on a wide range of dilutions of control rat plasma (5 - 100%). Plasma dilution curves were generated by plotting plasma concentration versus clotting time (see Figure A1.2 and Table A1.2.1). Plasma dilution and reciprocal plasma concentration were plotted against clotting time on double log paper and linear graph paper respectively, to determine which treatment of the results yielded a straight line relationship. (See Figures A1.2.2 and A1.2.3.) The reciprocal concentration curve for *E. carinatus* assay was found to be steeper than that for the Taipan assay. This indicates that the *E. carinatus* assay is more sensitive because there is a greater change in clotting time for a given change in prothrombin concentration. In addition to this, the y-intercept for the *E. carinatus* assay was smaller, implying that less time is taken by *E. carinatus* venom to activate sufficient thrombin to form a fibrin clot. By convention the results of the Taipan assay

Prothrombin %	Clotting Time (s)	
	Taipan Venom	E. carinatus Venom
100	17.9	12.5
80	18.5	14.3
60	19.8	16.0
40	22.2	19.1
20	27.5	27.5
10	38.2	42.0
5	57.4	64.5

Table A1.2.1 : Comparison of clotting times for range of dilutions of female rat plasma, using the Taipan venom and E. carinatus one-stage assays

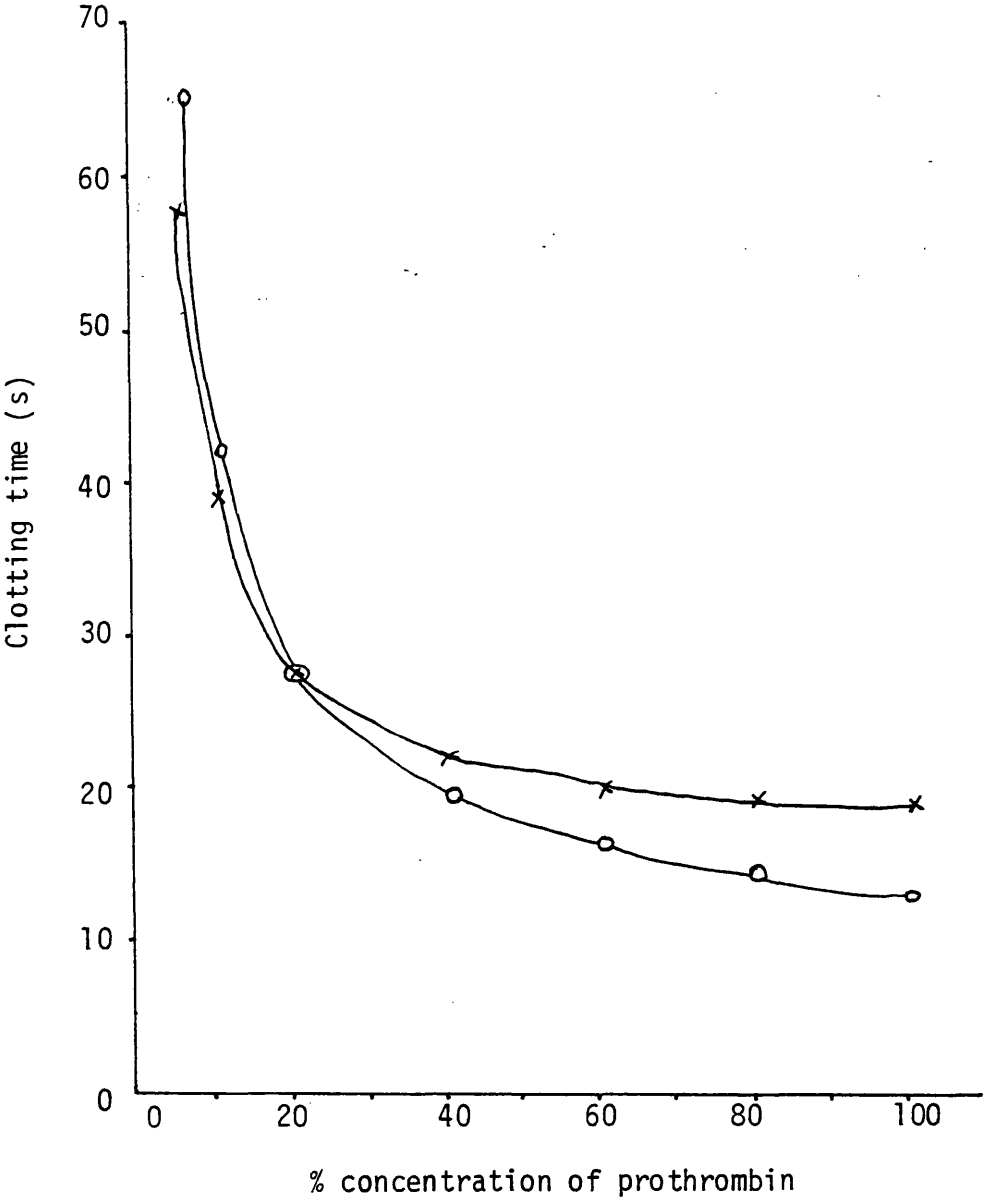


Figure A1.2.1 : Plasma dilution curve for the Taipan and E. carinatus one-stage assays. (x—x, Taipan assay, o—o, E. carinatus assay).

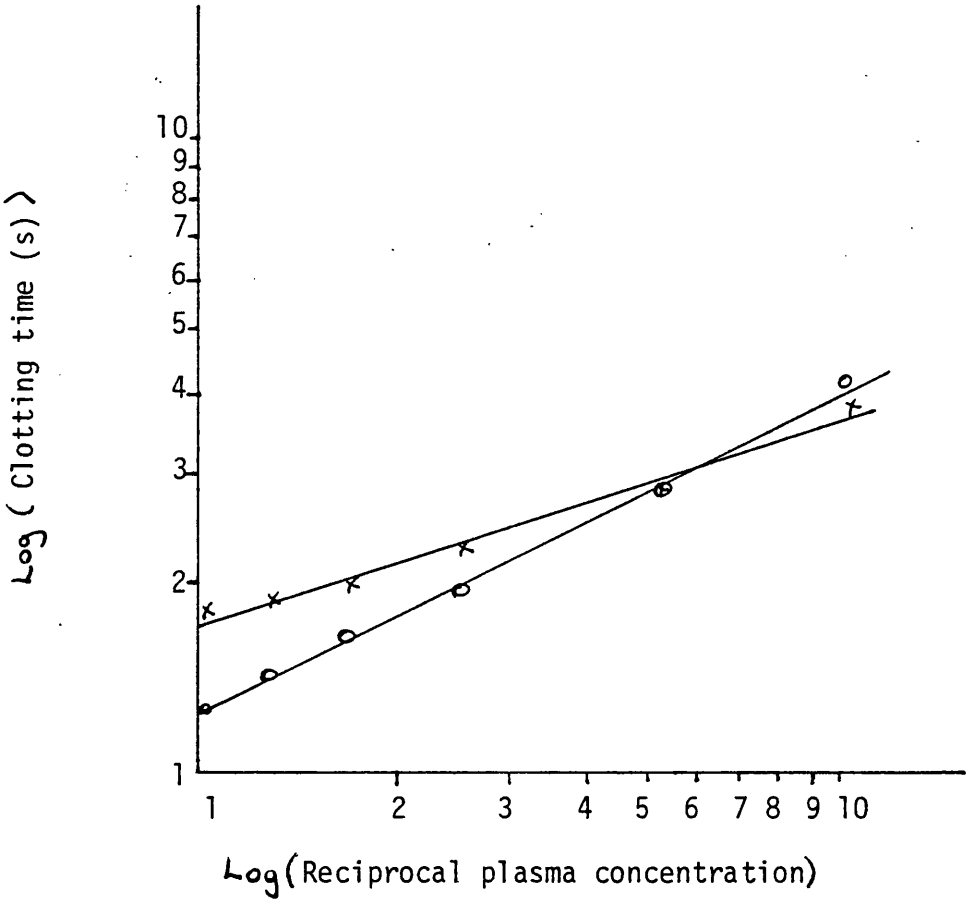


Figure A1.2.2 : Double log plot of the reciprocal concentration curves for the Taipan and E. carinatus assays.
(x—x, Taipan assay; o—o, E. carinatus assay)

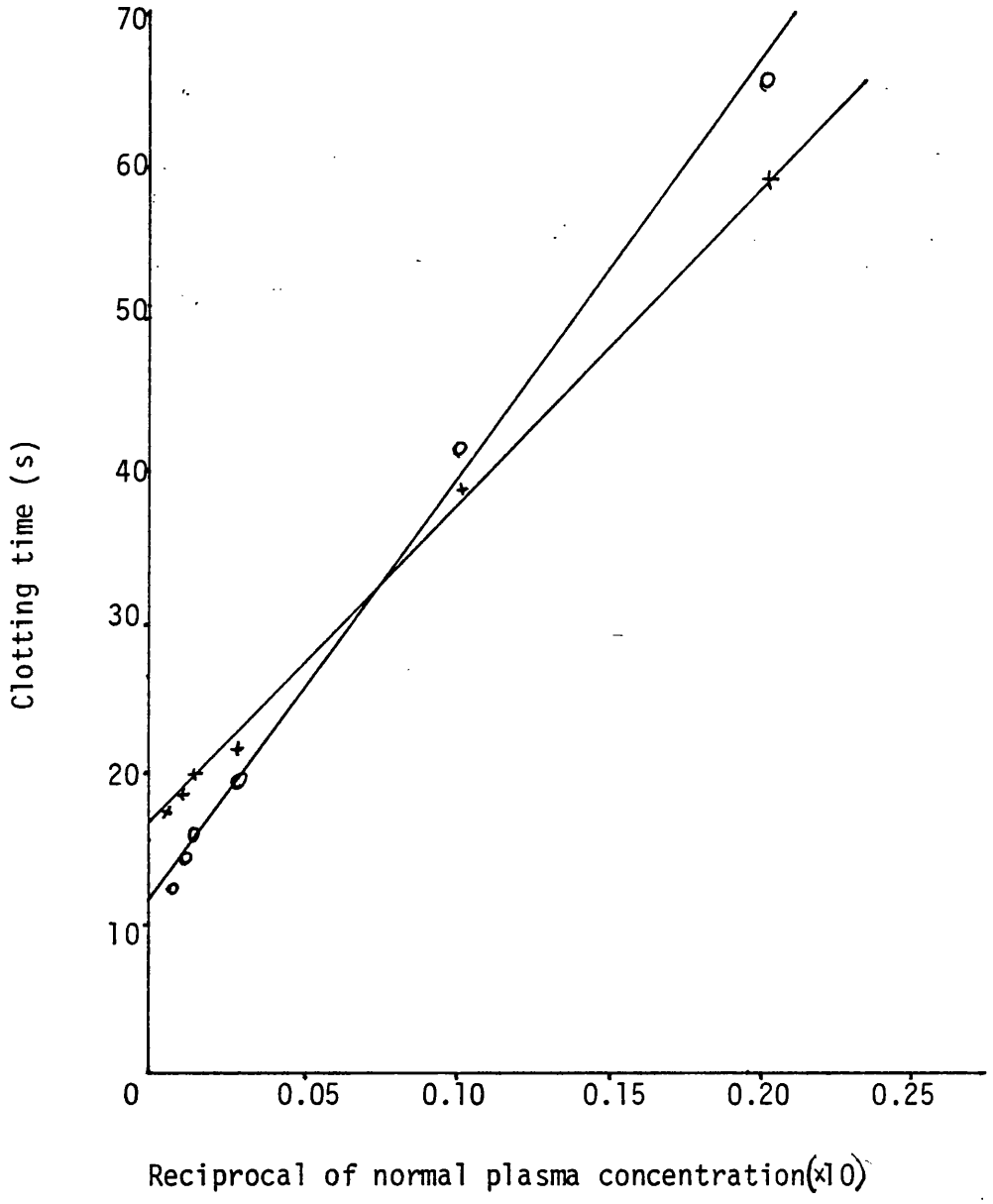


Figure A1.2.3 : Reciprocal concentration curves for the Taipan and E. carinatus one-stage assays.
(x—x, Taipan assay; o—o, E. carinatus assay)

are converted to linearity by plotting plasma dilution against clotting time on double log paper. The E. carinatus assay results were converted to linearity by plotting reciprocal plasma concentration against clotting time. For both assays, the level of prothrombin in test samples was expressed as a percentage of the normal plasma level by interpolation from the respective straight line graphs.

APPENDIX 2

DEVELOPMENT OF A CHROMOGENIC ASSAY FOR THE DETERMINATION OF PROTHROMBIN-RELATED MATERIAL

The clot formed at the end of conventional clotting assays is not easily seen if the sample under test is turbid. In addition, the very nature of the assay makes it subjective. Therefore, a biochemical assay was developed using the chromogenic reagent, Chromozym TH.

A wide range of activators of prothrombin was investigated but E. carinatus venom was found to be most versatile.

A2.1 Determination of Reaction Conditions for the E. carinatus Chromogenic Assay

Principle: The assay was carried out in two stages, on the lines of the assay described by Latallo and Teisseyre (1977). In the first stage, the proenzyme is converted into an active enzyme. The chromogenic substrate is then added and the reaction rate measured spectrophotometrically.

A2.1.1 Assay Procedure

After some initial experiments the following procedure was chosen:

0.1 ml plasma dilution

2.0 ml buffer

0.1 ml snake venom (1 mg/ml)

Incubated for set
period of time at 37°C

+ 0.25 ml Chromozym TH (1.5 mM)

ΔA/min followed at
405nm

Several experiments were carried out to optimise the conditions of the assay.

A2.1.2 Determination of Optimum Preincubation Time

The preincubation time was varied between 0 and 5 minutes and the initial reaction rates were compared. Incubation times of less than 3 minutes yielded curvilinear chart recordings (see Figure A2.1.1). This indicates that the incubation time was insufficient for complete activation of prothrombin. No increase in initial reaction rate was obtained if the preincubation time was extended beyond 3 minutes. Therefore, a 3 min incubation period was chosen as the optimum incubation time.

A2.1.3 Comparison of Alternative Buffer Systems

Latallo and Teisseyre (1977) used 0.15 M Tris buffer, pH 7.9 in their assay. The Boehringer Test Handbook, however, recommended the use of triethanolamine (TRA) buffer (0.1 M TRA, 0.2 M NaCl, pH 8.4) for the assay of thrombin. The two

buffer systems were therefore compared. TRA buffer was found to be a far better buffer. This probably reflects the pH dependence of the thrombin-Chromozym TH reaction. (See the Test Handbook.)

A2.1.4 Background Hydrolysis of Chromozym TH

When the assay was set up with the plasma replaced by buffer, a small residual amount of substrate hydrolysis was observed. This indicated that E. carinatus snake venom acts upon Chromozym TH. All determinations of absorbance change were therefore corrected for background hydrolysis.

A2.1.5 Investigation of the Effect of Aprotinin

The Boehringer test handbook recommends the addition of the protease inhibitor, aprotinin, to the assay buffer. This inhibits other arginine-specific proteases (e.g. trypsin, plasmin and kallikrein) which may be present in the system. No decrease in activity was found when rat plasma was assayed in the presence of aprotinin, but it was routinely included as a precaution. (Bang and Mattler, (1977) showed that Chromozym TH is hydrolysed by both plasmin and trypsin.)

A2.1.6 Investigation of the Effect of Acacia

It was suggested at the London Subcommittee meeting on Synthetic Substrates and Inhibitors of Coagulation Factors (1979), that acacia prevents thrombin from sticking to the sides of the reaction cuvette. (The amount of thrombin available for assay might otherwise be adversely affected.) To check whether or not this applied to the E. carinatus assay, a 3% solution of acacia in TRA buffer was prepared and the assay repeated. No enhancement of activity was noted and it was concluded that the use of plastic cuvettes prevented the adsorption of thrombin to the sides. Acacia was not therefore incorporated into the assay.

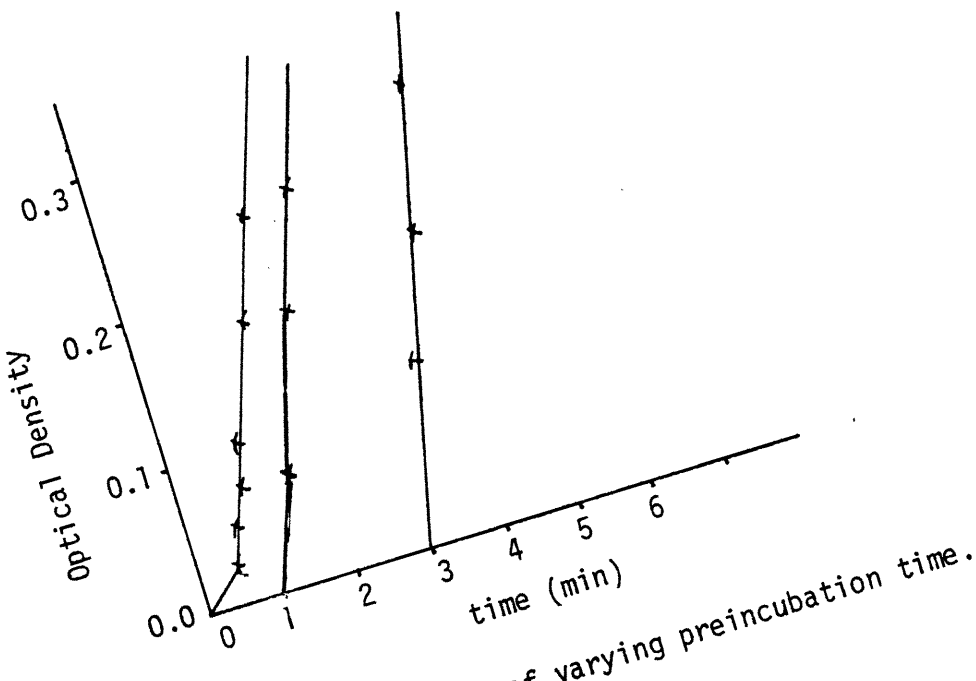


Figure A2.1.1 : The effect of varying preincubation time.

A2.1.7 Inhibition by Dithiothreitol

It was noted that dithiothreitol (DTT) had an inhibitory effect on the chromogenic assay. This could have been due to an effect on either thrombin or the snake venom, but was not investigated further.

A2.1.8 Construction of the calibration curve

The relationship between reaction rate and prothrombin percentage remained linear up to concentrations of 100%, but the $\Delta A/min$ at the higher concentrations was excessively rapid. Therefore, the calibration curve was constructed using a range of dilutions of control plasma from 5 to 50%. Test samples were suitably diluted so that their reaction rate fell within the range of the calibration curve.

A2.2 Investigation of Alternative Activators for the Chromogenic Assay

The E. carinatus assay measures both prothrombin and PIVKA. Several alternative activators (both physiological and non-physiological) were investigated in an effort to find one which would activate prothrombin only, yet would be applicable to both plasma and liver fractions. The two assays could then be used to determine the relative proportions of prothrombin and PIVKA in samples from warfarinised rats.

A2.2.1 Taipan Venom

The components of the Taipan chromogenic assay are a suitably diluted sample, platelet substitute, a Taipan-calcium chloride mixture and the substrate. Assay conditions as similar as possible to those of the E. carinatus assay were chosen so that results would be comparable.

After some initial experiments the following procedure was selected:

1.5 ml buffer

0.2 ml plasma dilution

0.2 ml 2 platelet substitute

0.2 ml Taipan-calcium chloride

Incubated for set period
at $37^{o}C$

+ 0.25 ml Chromozym TH (1.5 mM)

ΔA/min followed at 405 nm

Several observations were made on the assay. 1) In the absence of platelet substitute or in the presence of the detergent, Triton X-100, negligible activity was detectable. This indicates that the integrity of the phospholipid is crucial. 2) Activity was increased when buffer A, pH 7.5, was used instead of the Tris or TRA buffers. This probably reflects the pH dependence of the Taipan-prothrombin reaction. 3) A straight line relationship was obtained between absorbance change and time after a 3 minute preincubation. 4) All determinations had to be corrected for background hydrolysis. 5) The relationship between reaction rate and percentage prothrombin remained linear up to a concentration of about 70%.

GROUP	SAMPLE	PERCENTAGE PROTHROMBIN	
		E. CARINATUS ASSAY	TAIPAN ASSAY
Control 1	Homogenate	118.1	38.0
	Supernatant-1	70.9	50.9
	Microsomes	42.7	31.3
	Supernatant-2	20.5	5.9
Control 2	Homogenate	103.0	27.5
	Supernatant-1	59.3	22.3
	Microsomes	33.8	4.6
	Supernatant-2	29.9	7.6
Test 5 mg/kg	Homogenate	149.3	49.6
	Supernatant-1	65.5	11.9
	Microsomes	67.3	8.3
	Supernatant-2	1.0	1.0

Table A2.2.1 : Comparison of the prothrombin concentrations of liver fractions determined by the Taipan and E. carinatus chromogenic assays.

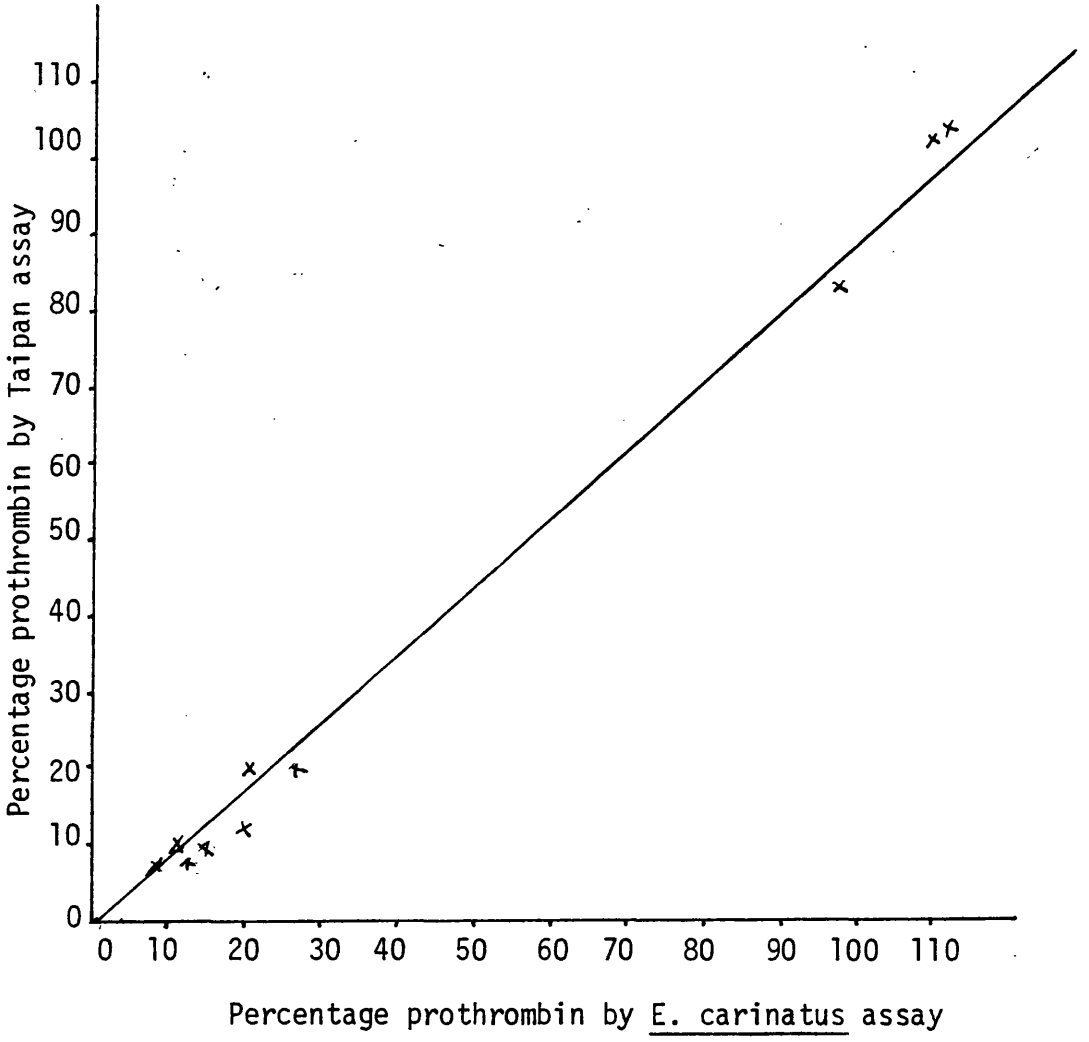


Figure A2.2.1 : Comparison of the prothrombin concentrations of plasma samples determined by the Taipan and E. carinatus assays

The most striking difference between the Taipan and E. carinatus chromogenic assays was that the reaction rates using the Taipan assay were about one third that of equivalent rates using E. carinatus venom. This implies that 'thrombin' generated by Taipan venom has less affinity for Chromozym TH than ecarin-generated 'thrombin'. The Taipan chromogenic assay was applied to plasma and liver samples isolated from warfarin-treated rats, and the results compared with those of the E. carinatus assay. (See Figure A2.2.1 and Table A2.2.1.) There was good correlation between the assays ($r = 0.99$) with respect to plasma samples. However the response of the Taipan assay to liver fractions was highly variable from one experiment to another, and showed little agreement with the E. carinatus assay.

A2.2.2 Activation by Factor Xa

Factor X, when activated by Russell's-viper venom (RVV), is able to activate prothrombin in the presence of Factor V, calcium and phospholipid. The simple addition of RVV as a trigger for the chromogenic assay of samples from anticoagulated rats is untenable, however, because they would contain insufficient endogenous Factor X. Therefore, an exogenous supply of Factor Xa is required. In the present study the Factor Xa, prepared by the method of Jesty and Esnouf (1973) from a human source, was a gift from Dr P Walton.

The following assay procedure was used:

1.5 ml buffer

0.1 ml plasma dilution

0.2 ml platelet substitute

0.05 ml calcium chloride

0.2 ml Factor Xa

Incubated for set period

at 37^{o}C

+ 0.25 ml Chromozym TH (1.5 mM)

ΔA/min followed at 405 nm

The assay was found to be 1) dependent on the presence of platelet substitute 2) dependent on the presence of a small amount of calcium, 3) more efficient in TRA buffer than buffer A, 4) limited by the amount of Factor Xa added.

The reaction rate using Factor Xa as activator, was about one tenth that of an equivalent assay using E. carinatus venom. This implied that the rate of generation of active thrombin was very slow in the Factor Xa assay. It is unlikely that thrombin generated by Factor Xa would have only one tenth the affinity for Chromozym TH that ecarin-produced thrombin has. Also, curvilinear chart recordings were observed with incubation times of up to 4 min. It was suggested that human Factor Xa did not recognise rat prothrombin sufficiently well. However, when human plasma was incorporated in the assay reaction rates were

still low.

The use of 5x concentrated Factor Xa produced a significant improvement in reaction rate but due to the vast amount of Factor Xa which would have been required for assays, this was not felt to be justified.

A2.2.3 Activation by Tiger Snake Venom

The prothrombin-activating activity of Tiger snake (Notechis scutatus scutatus) venom was characterised by Jobin and Esnouf (1966). Both platelet substitute and calcium are required for its activity.

The assay procedure chosen was as follows:

2.0 ml buffer
0.1 ml plasma dilution
0.1 ml platelet substitute
0.1 ml calcium chloride
0.1 ml Tiger snake venom (5 mg/ml)

Incubated for set period of time at $37^{o}C$

+ 0.25 ml Chromozym TH (1.5 mM)

A/min followed at 405 nm

With a 6 min incubation time the reaction rate was linear. Background hydrolysis of the chromogenic reagent by the snake

venom was very high. The pH of the reaction was varied in an attempt to reduce background hydrolysis without adversely affecting the assay itself. Buffer A, pH 7.8 proved to be the best reaction medium. Both calcium ions and platelet substitute were required for the assay.

When a calibration curve was constructed using a range of dilutions of plasma and plotting reaction rate versus prothrombin percentage, the points fitted a curve which did not pass through zero. (See Figure A2.2.2.)

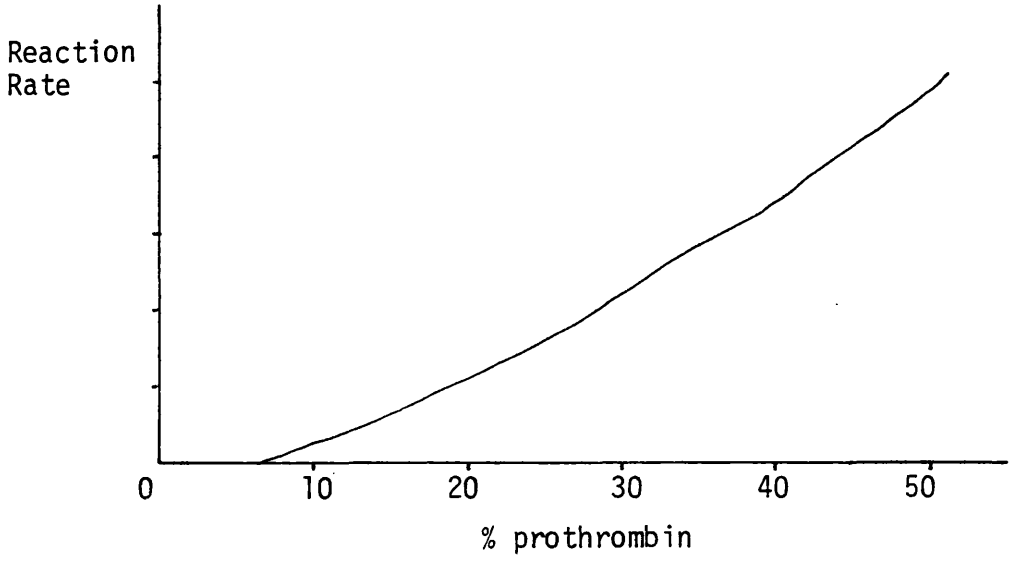


Figure A2.2.2 : The calibration curve of the Tiger snake chromogenic assay

According to Dixon and Webb (1979) this indicates the presence of an inhibitor in one of the components of the assay. To determine which component was inhibiting the reaction, each was put in the E. carinatus assay. 0.1 ml 0.05 M calcium chloride was found to cause slight inhibition, which was overcome when

the concentration was reduced to 10mM. The Tiger snake assay was therefore suitably amended.

The Tiger snake chromogenic assay was applied to plasma and liver samples isolated from warfarinised rats and the results compared with those of the E. carinatus assay. (See Table A2.2.2.)

GROUP	SAMPLE	PERCENTAGE PROTHROMBIN	
		E. CARINATUS ASSAY	TIGER SNAKE ASSAY
1	Plasma	111.8	108.9
2		51.5	45.6
3		18.8	10.4
4		20.6	8.0
1	Homogenate	76.0	31.5
3		108.4	25.3
1	Supernatant-1	64.7	27.1
3		95.2	16.9
1	Microsomes	38.2	9.8
3		78.4	12.4
1	Supernatant-2	22.9	-
3		3.4	-

Table A2.2.2 : Comparison of the prothrombin concentrations of plasma and liver fractions determined by the Tiger snake and E. carinatus chromogenic assays.
(Group 1, control; Group 2, 0.1 mg/kg; Group 3, 0.5 mg/kg; and Group 4, 1.0 mg /kg body weight)

If it were proposed that Tiger snake venom activates biologically complete prothrombin only, then one can say that the results in

Table A2.2.3 give the predicted trends in each class of samples.

A2.3 Discussion

Christensen (1980) summarised the requirements for a valid chromogenic assay applied to the determination of pro-enzymes. All the pro-enzyme must be converted to active enzyme and no irreversible reaction of the enzyme with inhibitors must occur. (For a discussion of the latter point, see Section 4.5.) To ensure that all the pro-enzyme was activated, an incubation time which yielded the maximum initial reaction rate was chosen.

The E. carinatus chromogenic assay measures both prothrombin and PIVKA. The survey of alternative activators did not reveal any activator which could be used to determine prothrombin alone.

Although the general activity of the Taipan assay was low, the main objection to this assay was its variable response to liver fractions. The Factor Xa assay generated low activity, and required large amounts of the activator. Its application to liver fractions was not therefore considered.

The Tiger snake assay showed some promise when applied to liver fractions. However, it was realised that the Tiger snake venom might be measuring not only biologically complete forms of prothrombin, but also those nearing completion (e.g. with 60% carboxylation). Esnouf and Prowse (1977), have shown that Tiger snake venom can activate bovine prothrombin containing only seven γ-carboxyglutamic acid

residues (as opposed to its normal complement of ten residues per molecule). Obviously, a lot more work would have to be done to characterise the action of the Tiger snake venom in this assay.

APPENDIX 3

DEVELOPMENT OF A VITAMIN K-DEPENDENT CARBOXYLATION ASSAY

Several published methods of vitamin K-dependent carboxylation were compared in an effort to discover which gave the maximum incorporation of radioactivity into TCA-precipitable protein.

A3.1 Induction of Precursor Prothrombin

The basic requirement for a vitamin K-dependent carboxylation assay is the presence of a large amount of substrate - namely the precursor prothrombin. This can be induced either by vitamin K absence or antagonists.

A3.1.1 Induction by Vitamin K Absence

The vitamin K-deficient diets of Esnouf (personal communication) and Mameesh and Johnson (1959) were prepared as follows:

DIET NO. 1 (from Dr Esnouf)

For 10 kg	Sucrose	6.63 kg
	Casein	2.40 kg
	Liquid parrafin	0.43 kg
	Linoleic acid	0.10 kg
	Salt mix	0.43 kg (see below)
	Vitamin mix	0.01 kg (see below)

DIET NO. 2 (from Mameesh and Johnson, 1959)

For 10 kg	Sucrose	6.65 kg
	Purified soy protein	2.00 kg

DL-methionine	0.05 kg
Salt mix	0.40 kg (see below)
Vitamin mix*	0.01 kg (see below)
Wheat germ oil	0.05 kg
Cod liver oil	0.15 kg
Glycerol	0.20 kg
* added with cerelose	0.50 kg

Vitamin Mix

Thiamine HCl	100 mg
Riboflavin	100 mg
D-pantothenic acid	500 mg
Pyridoxine HCl	50 mg
Nicotinic acid	200 mg
Folic acid	10 mg
D-biotin	1 mg
Cyanocobalamin	1 mg
Choline chloride	10 mg

Salt Mix

NaCl	104 g
$MgSO_4$	166 g
$NaHPO_4$	204 g
KH_2PO_4	272 g
$CaHPO_4$	324 g
Ca lactate	780 g
Ferric citrate	70 g
KI	200 g
$Cu\ SO_4$	2 g
$MnCl_2$	2 g

Drinking water contained 0.1% neomycin sulphate. The animals were allowed to eat and drink ad libidum. Rats were housed individually in cages with wide mesh floors to prevent coprophagy. As the rats became vitamin K-deficient, the condition of their coats became poor and they experienced difficulty in walking. Although stools were meant to become 'loose', the rats also showed signs of constipation. The diets were administered for 10 days. To assess vitamin K-deficiency, plasma samples were screened every 2 days using the Kaolin-cephalin time and the Prothrombin time tests.

The prolongation of clotting times indicated that the rats were becoming vitamin K-deficient. The degree of deficiency varied from one animal to another and was more extreme in the animals fed on diet No. 1. Therefore, after 8 days, microsomal fractions were prepared from rats on diet No. 1. The specific activity of "thrombin" as assessed by chromogenic activity was 50 mU per mg of protein.

A3.1.2 Induction by Vitamin K Antagonist

In Section 3.4 the specific activity of "thrombin" in microsomal fractions prepared from warfarin-treated rats was reported. At a warfarin dose level of greater than 2 mg per kg body weight, specific activity in excess of 100 mU per mg of protein was regularly achieved after only a single dose.

Since greater specific activity could be achieved by warfarin administration and the rats did not show signs of distress, this

method was chosen to induce precursor prothrombin.

A3.2 Methods of Vitamin K-Dependent Carboxylation

A3.2.1 Vitamin K-Dependent Carboxylation (Esnouf, personal communication)

Microsomes were prepared from rats on a vitamin K-deficient diet, showing plasma prothrombin concentrations of 20% or less. Livers were homogenised in buffer containing 25 mM Imidazole, pH7.5, 0.2 M sucrose and 80 mM KCl (3 ml buffer/g tissue.) The microsomal fractions were prepared as described in Section 2.8. For a solubilised system, the microsomal pellet was resuspended in homogenisation buffer containing 1% Triton X-100, and spun again at 105,000 g for 60 min. The supernatant was then incorporated into the assay.

The incubation mix was made up as follows:

1.00 ml microsomal fraction
0.02 ml 3 mM DTT, NADH 50 mg/ml
0.10 ml buffer (containing inhibitors if required)
0.02 ml $NaH^{14}CO_3$ 1 mCi/ml
0.01 ml vitamin K, 10 mg/ml

The reaction was triggered by the addition of the vitamin and incubated at 27°C for 30 min. The reaction mix was then added to 5 ml 10% TCA, the tube rinsed with 0.5 ml 0.9% NaCl containing 10 mg/ml BSA and 100 mM $NaHCO_3$, and the mixture allowed to stand at 4°C for 30 min.

The TCA-precipitated protein was collected by gentle centrifugation, redissolved in 2% $NaCO_3$ for 30 min, then reprecipitated with TCA. This cycle was repeated once more and the precipitate was finally redissolved in 1 ml NCS. 7 ml of aquasol were added to 0.4 ml of this mixture, 0.01 ml glacial acetic acid was added and the radioactivity determined.

A3.2.2 Vitamin K-Dependent Carboxylation (Bell, personal communication)

Microsomes were prepared as described in Section 2, except that 3 ml of buffer (0.25 M sucrose, 25 mM imidazole, 0.1 M KCl, 0.005 M Mg acetate, pH 7.2) were added for every 1 g of tissue. 1 ml of the microsomal suspension was added to 1 mg NADH and 1 mg DTT. The reaction was initiated with 5×10^7 dpm of $Na_2{}^{14}CO_3$ (Amersham, 59 mCi/nmole) and 50 µg of vitamin K_1 in less than 0.02 ml ethanol. The tubes were sealed and incubated for 30 min at 27°C. They were then placed on ice and 0.1 ml of 1 M $NaHCO_3$ was added. The incubation mix was centrifuged at 105,000 g for 1 hr and the supernatant was poured off. The microsomes were surface washed with buffer and then resuspended by adding 0.03 ml of a 10% Triton X-100 solution (final concentration = 0.3%) and 0.1 ml of 1 M $NaHCO_3$ to 1 ml of the buffer. The mixture was frozen at -20°C, thawed and spun on a vortex. The suspension was then centrifuged for 1 hr at 105,000 g. The supernatant was decanted off and 2 mg of albumin was added. This solution was precipitated twice with 10% TCA and the precipitate then dissolved in 0.5 ml NCS solubiliser, 10 ml of Biofluor (New England Nuclear) and 0.015 ml of glacial acetic acid was added and the radioactivity determined.

A3.2.3 The method used for vitamin K-dependent carboxylation was a modification of the methods described in Sections A3.2.1 and A3.2.2. Livers were fractionated according to the method of Sadowski et al (1976). (See Sections 2.8 and 2.9 for full details of the procedure.) Initially, vitamin K-dependent incorporation of ^{14}C was very low. This was eventually attributed to the harshness of the homogenisation procedure. When 4 passes of the teflon-glass homogeniser were used instead of 8, much greater incorporation was observed.

A second problem encountered was the rapid loss of activity of the radioactive source due to equilibration of $^{14}CO_2$ with atmospheric CO_2. This problem was overcome by re-sealing the $Na^{14}CO_3$ solution in soda glass ampoules which were only opened as required.

Initially, no dithiothreitol (DTT) was included in the incubation mix in imidazole buffer. Since this did not adversely affect the reaction is was concluded that DTT is not essential. Its function in the assay is probably to regenerate NADH from NAD.

APPENDIX 4

COMPARISON OF SEVERAL METHODS OF mRNA ISOLATION

mRNA was isolated from rat liver by several different methods, to discover which method gave the highest yield and the most active preparation.
In all methods, protein is denatured as soon as possible so that ribonuclease activity is inhibited. As a further precaution all solutions are pre-treated with diethyl pyrocarbonate.

A4.1 The Methods of Isolation

A4.1.1 Isolation of Polysomes Followed by Phenol Extraction of RNA (Nardacci et al, (1975))

Rat livers were excised, weighed and minced in 2.5 volumes of homogenisation buffer (0.25 M sucrose containing 50 mM Tris HCl, pH 7.7, 25 mM NaCl, 5mM $MgCl_2$ and 100 mg/l heparin). The mixture was homogenised using 3 passes of a teflon-glass mechanical homogeniser. The homogenate was centrifuged at 11,000 rpm (15000 g) for 10 min at 4^oC. One volume of 10% sodium deoxycholate-10% Triton X-100 solution was added to nine volumes of supernatant, and the mixture stirred for 30 min at 4^oC.

The mixture was layered over a discontinuous sucrose density gradient consisting of 10 ml of 2 M sucrose overlaid with 5 ml of 0.5 M sucrose, both containing 50 mM Tris, pH 7.7, 25 mM NaCl, 5mM $MgCl_2$ and 100 mg/l heparin. Polysomes were sedimented through the gradient at 38000 rpm (105,000 g) for

16 hr at 4°C. The pellet was suspended by gentle homogenisation in 5 ml 50 mM Na acetate, pH 5.0, 10 mM EDTA and 0.5% SDS. After adjusting the ribosome concentration to 2.5 mg/ml, the solution was extracted with a 1:1 mixture of phenol:chloroform by vortexing for about 5 min. The phases were separated by centrifugation at 5000 rpm for 10 min. The phenol phase was removed and re-extracted with buffer while the aqueous phase was re-extracted with phenol:chloroform. After thorough mixing, the phenol phases were discarded, the aqueous phases pooled and then re-extracted with phenol:chloroform and finally chloroform to remove all denatured protein. The aqueous phases were pooled, made up to 0.1 M with solid NaCl and the RNA was precipitated overnight with 2 volumes of ethanol. The RNA was collected using an MSE 18 at full speed for 15 min. The precipitate was washed twice in 95% ethanol and dissolved in a small volume of sterile distilled water. A solution of 10 mM Tris HCl, pH 7.5 containing 0.5 M KCl was added to give a final RNA concentration of 6 - 8 mg/ml. The crude preparation, which was milky and gelatinous, caused increasing inhibition of *in vitro* protein translation with increase in RNA addition. To further purify the mRNA, the crude preparation was passed through an oligo-dT column by the method of Scolnick *et al* (1973). The total RNA was applied to the column and washed through with the Tris-KCl high salt buffer. The optical density of the 1 ml fractions collected from the column, was followed at 260 nm. When the OD had dropped to the baseline level, the high salt was replaced with sterile water and 1 ml fractions

again collected. Tubes containing mRNA were pooled, made 0.3 M with LiCl, and precipitated overnight with 2 volumes of ethanol. Following centrifugation, the precipitate was redissolved in sterile water and the RNA concentration adjusted to 1 μg/μl.

On addition of the mRNA to a wheatgerm *in vitro* translation system, very little stimulation in incorporation was observed, even when K^+ and Mg^{2+} ion concentrations were optimised.

It was suggested that the long preparation time prior to phenol extraction might allow ribonuclease to attack the mRNA. This would prevent initiation of translation of the exogenous mRNA in the *in vitro* system.

A4.1.2 A high salt homogenisation buffer and shorter polysome-precipitating spin were used in an effort to overcome the problems encountered with Method 1. Livers were homogenised in high salt buffer (0.25 M sucrose, 0.225 M KCl, 0.025 M NaCl, 0.05 M Tris, pH 7.7, 5 mM $MgCl_2$, 100 mg/l heparin). Cell debris was brought down with a 10 min spin at 5000 rpm. The polysomes were precipitated at 30,000 g (16000 rpm) for 30 min. The pellet was resuspended in Na acetate, SDS, EDTA buffer and phenol extracted as described for Method 1 (A4.1.1).

The yield of mRNA by Method 2 was poor, indicating that the second centrifugation step had failed to bring down all the membraneous material. In the subsequent preparation, the

second centrifugation step was increased to 105,000 g for 60 min. Since the mRNA isolated by this method again failed to stimulate *in vitro* protein translation, it was desalted on a G-25 column as described by Astell and Ganoza (1974).

The mRNA was translated in three different wheatgerm preparations. In the first, radioactive incorporation was inhibited, in the second, there was no effect and in the third, there was slight stimulation. This indicates that the test system varied from one preparation to another.

A4.1.3 Direct Phenol Extraction of Homogenate and Centrifugation Through a Caesium Chloride Cushion

This method is described in Section 2.13. Initially, 30 ml of TLE buffer and PCI mix were used per 10 g of liver. In one preparation, a particularly large excess of denatured protein in the interface caused inadequate separation of the phases and this led to a small yield of mRNA. To prevent this from happening again, the amounts of TLE buffer and PCI mix were increased to 40 ml of each per 10 g of tissue in subsequent preparations.

Direct phenol extraction of the homogenate caused immediate denaturation of ribonuclease. Centrifugation of the aqueous phase on a CsCl cushion achieved separation of the RNA from DNA which banded above the cushion. Passage of the total RNA down two oligo-dT columns greatly enriched for mRNA. Scolnick *et al* (1973) reported that between 97 and 99% of

the RNA is not retained on the column. Therefore, a total RNA solution containing 1% mRNA applied to the column may be eluted as a solution containing 50% mRNA. The second column will enrich this still further.

A4.2 Comparison of the Yields

The yields of the three methods and the activity of the respective mRNA preparations in the *in vitro* translation assay are given in Table A4.2.1. All methods gave comparable yields of mRNA but only the mRNA from Method 3 was active.

METHOD	No. OF RATS	LIVER WEIGHT	TOTAL RNA (mg)	mRNA (µg)	% RECOVERY	ACTIVITY
1	2 ♀ 2 ♀ 2 ♂	12.0 13.0 19.0	14.8 12.7 23.0	250 257 254	1.7 2.0 1.1	Slight Slight Slight
2	2 ♂ 4 ♂	26.4 44.0	8.6 45.5	54.6 539	0.6 1.2	- Variable
3	2 ♂ 2 ♂	17.6 18.5	32.3 -	250 318	0.8 -	High High

Table A4.2.1 : Comparison of the yields and activity of the mRNA prepared by three different methods.

In all cases the mRNA recovered from the column represented about 1% of the total RNA applied to the column. Nardacci *et al* found that less than 0.1% of the total RNA, isolated by phenol extraction of liver homogenate, was recovered from an oligo-dT column. However, they made no attempt to separate RNA from DNA and this may have interfered with the binding to the column.

Since the mRNA fraction from Procedure 3 stimulated total protein synthesis in the rabbit reticulocyte lysate system, it was used for all subsequent experiments.

APPENDIX 5

COMPARISON OF SEVERAL METHODS OF IN VITRO PROTEIN TRANSLATION

Several *in vitro* protein translation systems were compared in an effort to discover which method showed the greatest stimulation of total protein synthesis on addition of exogenous mRNA, and to see which procedure gave the most reproducible results.

A5.1 The Methods of in vitro Protein Translation

A5.1.1 The Wheatgerm Cell-Free Extract

The wheatgerm cell-free extract was prepared by a modification of the methods of Roberts and Paterson (1973) and Alton and Lodish (1977).

6 g of wheatgerm were ground at $4^{o}C$ with 6 g of autoclaved sand and 28 ml of extraction buffer (20 mM Hepes, pH 7.2, 1 mM $CaCl_2$, 2 mM magnesium acetate, 100 mM potassium acetate, 1 mM dithiothreitol) for about 5 min using a pestle and mortar. Following centrifugation at 30,000 g for 10 min at $4^{o}C$, the supernatant was carefully decanted from the pellet, taking as little of the surface layer of fat as possible. The supernatant was adjusted to 3.5 mM magnesium acetate, 2 mM DTT, 40 g/ml creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 10 mM creatine phosphate. Following preincubation of the extract at $22^{o}C$ for 15 min, it was passed through a Sephadex G-25 column (2.5 x 60 cm) equilibrated with 20 mM Hepes, (pH 7.6), 120 mM potassium acetate, 5 mM magnesium acetate and 1 mM DTT. When the column eluate turned cloudy and a protein precipitate was observed in trichloracetic acid, the next 30 ml of eluate were collected.

The wheatgerm S-30 fraction was dispensed in small aliquots and stored under liquid nitrogen until required.

Standard protein synthesis assays were set up as follows:

1. Incubation cocktail -30µl
   (containing 10 mM ATP; 4 mM GTP; 100 mM creatine phosphate; creatine kinase (0.33 mg/ml) 8 mM DTT; 46.6 mM Hepes; 240 mM KOAc; spermidine (30µg/ml); 'minus Met' amino acid mix (20µM each); 16.6 µM methionine; ^{35}S-methionine (0.5µM))
2. Preincubated wheatgerm S-30 fraction -40µl
3. mRNA (varying concentration) -30µl

The reaction mixture was incubated at 25°C for 90 min and then 5 µl were removed for counting. The remaining mixture was made 10 mM with EDTA (pH 7.3) and 10 µg/ml with pancreatic ribonuclease A and was incubated at 37°C for 15 minutes. Immunoprecipitation was carried out as required.

The reaction conditions were optimised for Mg^{2+} and K^{+} ion concentrations. Radioactive incorporation was highest at a KCl concentration of between 85 mM and 95 mM. At 95 mM KCl, the Mg^{2+} optimum was fairly sharp with maximum incorporation at 3.0 mM. These salt optima agree broadly with those reported by Astell and Ganoza (1974).

As noted in Appendix 4, the response of the wheatgerm system to the addition of exogenous mRNA varied from one preparation

to another. Marcu and Dudock (1974) attributed lack of reproducibility to the harshness of the grinding and the length of the grinding time. They suggested that the wheatgerm be ground dry with an equal weight of powdered glass for only 60 sec prior to the addition of a small volume of extraction buffer. They also reported that the efficiency of protein synthesis varied with the source of the wheatgerm.

A5.1.2 The Rabbit Reticulocyte Lysate System

The rabbit reticulocyte lysate was prepared by the method of Pelham and Jackson (1976) at the ICI Joint Laboratory. It was nuclease-treated and supplemented with haemin prior to use.

For the standard protein synthesis assay, a basic (5 x) cocktail was constructed as follows:

amino acid mix excluding Met (1 mM of each)	- 200 µl
100 mM ATP	- 100 µl
10 mM GTP	- 200 µl
4 M KCl	- 188 µl
1 M Magnesium acetate	- 20 µl
500 mM Tris, pH 7.6	- 200 µl
200 mM glucose	- 100 µl
creatine phosphate	- 68 mg
H_2O	- 938 µl
TOTAL	2.0 ml

The standard incubation mixture consisted of:

5 x cocktail	- 10.0µl
tRNA (1 mg/ml)	- 2.5µl
^{35}S-methionine	- 4.0µl
mRNA (1 mg/ml) from rat liver	- 2.0µl
nuclease-treated lysate with creatine phosphokinase (0.1 mg/ml)	- 25.0µl
H_2O	- 6.5µl
TOTAL	50.0µl

The assay was incubated at 30°C for 90 min. 2µl were removed for counting and the remainder was used for immunoprecipitation. The K^+ optimum ion concentration was between 85 mM and 95 mM. Within this range, a sixfold stimulation of total protein synthesis was observed on addition of the exogenous mRNA.

A5.1.3 Commercially Prepared Rabbit Reticulocyte Lysate

This method is described in Section 2.14 Problems related to storage of the lysate were initially encountered. When stored under liquid nitrogen, the lysate rapidly lost its ability to translate exogenous mRNA. Loss of activity on storage at -70°C was attributed to the effect of acetone trapped in the sample tubes whilst the aliquots of lysate were being frozen. The acetone caused precipitation of the haemoglobin. Once the cause of these problems had been identified, no further difficulties were met.

The rabbit reticulocyte lysate was optimised for total protein

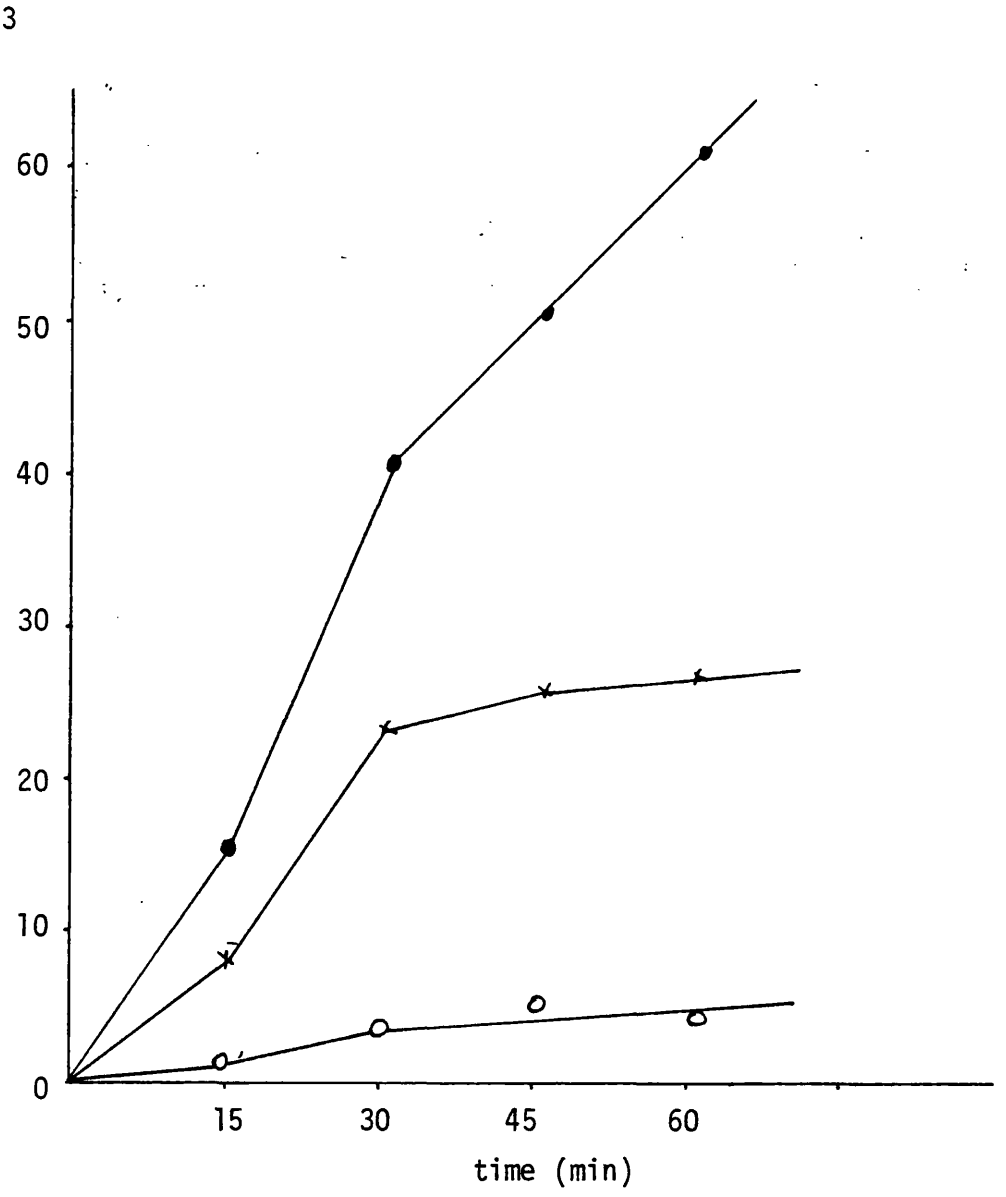


Figure A5.1.1 : Time course of the translation assay using rabbit reticulocyte lysate (Amersham) ((o—o) - no mRNA; (x—x) - rat liver mRNA; (●—●) - D. discoidium mRNA)

synthesis prior to distribution. K^+ ion concentration lay within the range of 85 mM and 95 mM and was not therefore altered. A mRNA optimum of 1 μg per 10 μl of incubation mix was found. Figure A5.1.1 shows the time course of typical translation assay using mRNA isolated from rat liver and the slime mould, Dictyostelium discoidium respectively. The degree of stimulation of total protein synthesis is limited by the ease with which the rabbit reticulocyte lysate translation system recognises exogenous mRNA. However, a tenfold stimulation of protein synthesis could regularly be achieved.

A5.2 Conclusion

The response of the wheatgerm system to the addition of exogenous mRNA varied considerably from one preparation to another. Also, the stimulation of total protein synthesis was not as great as that with the reticulocyte lysate systems.

Although the 'home-made' rabbit reticulocyte lysate system gave an acceptable level of protein synthesis on the addition of exogenous mRNA, the assay was less convenient than the commercially prepared system. Therefore the latter was used in the present studies.

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ABSTRACT

STUDIES ON PROTHROMBIN

by

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Clotting and chromogenic assays utilising Echis carinatus venom were set up to determine the total prothrombin-related material (PRM) in plasma and liver fractions. The chromogenic assay revealed an increase in the PRM of microsomes prepared from warfarin-treated rats, reflecting a build-up of prothrombin precursors. A clear relationship between warfarin dosage and the increase in PRM in the microsomal fraction was demonstrated.

The chromogenic assay was not only useful for the determination of PRM in turbid samples. It was also used to assess specific activity at each stage in the purification of prothrombin, in the kinetic analysis of ecarin-generated thrombin, and as a functional test for anti-prothrombin specificity of antisera, raised against the prothrombin concentrate.

It was hoped that the anticoagulant, PP888, could be used to investigate post-translational modification of prothrombin. However, PP888 was found to be much less active than warfarin in vivo and was inactive in the in vitro vitamin K-dependent carboxylation assay. This indicated that a metabolite of PP888 was the active anticoagulant. The structurally related compound, PP493, was found to inhibit vitamin K-dependent carboxylation in both solubilised and non-solubilised microsomes.

The processing of prothrombin precursors in the rat liver is both complicated (five different isoelectric forms of precursor have been identified) and poorly understood. It was decided that the best way to investigate post-translational modification was to isolate the primary, unmodified translation product. To accomplish this, messenger RNA, isolated from rat liver, was translated in vitro, using a rabbit reticulocyte translation system. Prothrombin-related translation products were then identified by immunoprecipitation, using antisera raised against the prothrombin concentrate.

Two prothrombin-related translation products were obtained in this way. They were characterised biochemically and their properties compared with those of prothrombin. Finally, attempts were made to modify the primary translation products and mimic in vivo processing. The potential of a system where proteins were translated in the presence of rat liver microsomes is discussed.