TICK (R. APPENDICULATUS) SALIVARY GLANDS BIOACTIVE MOLECULES WITH A BIOCHEMICAL STUDY OF THE ANTICOAGULANT IN RELATION TO VECTOR CONTROL.

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D E C L A R A T I O N

This thesis is my original work and has not been presented for a degree in any other University.

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List of symbols and abbreviations.

Abbreviation	Full name/meaning.
AT-III	antithrombin III.
BAEE	N- -benzoyl-L-arginine ethyl ester.
BTEE	N- -benzoyl-L-tyrosine ethyl ester.
Chromozym PL	Tosyl-glycyl-prolyl-lysine-4-nitroanilide acetate.
Chromozym TH	Tosyl-glycyl-prolyl-arginine-4-nitro anilide acetate.
DFP	diisopropylfluorophosphate.
DTT	dithiothreitol
EtOH	ethanol.
EDTA	ethylenediamine-tetra acetic acid.
g	number of times the gravitational force.
Gla	ɣ-Carboxyglutamic acid
h	hour or hours
HOAC	acetic acid
HPLC	High Performance liquid chromatography
kDa	Kilodaltons
mg	Milligram
min	Minutes
ml	Millilitre
Mr	Molecular weight
mM	Millimolar
M	Molar

nM	Nanometers
O.D.	optical density
PGE_2	Prostaglandin E_2
PBS	phosphate buffered saline
PMSF	phenylmethanesulfonylfluoride
PL	phospholipid or phospholipoprotein
PAGE	Polyacrylamide gel electrophoresis
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
S-2222 (Bz-Ile-Glu Gly-Arg-pNA)	N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginyl-paranitro anilide.
TSGE	Tick salivary gland extracts
Tos-Arg-OMe	Tosyl-L-arginine methyl ester
Temed	N,N,N',N'-tetramethylene diamine
TLCK	p-tosyl-L-Lysine chloromethyl-ketone
TPCK	L-tosyl-phenylethly chloromethyl-ketone
Tris	Tris (hydroxy-methyl) aminomethane
u	Units
ug	Microgram
ul	microlitre.

SUMMARY.

The ixodid tick, Rhipicephalus appendiculatus, the vector of the cattle disease East Coast Fever, an economically important disease in East and Central Africa, remains attached to hosts for several days thus providing a sustained antigenic stimulus to the host. In addition to transmission of Theileria sporozoites during feeding, the tick also releases, in saliva, enzymes and enzyme inhibitors, an anticoagulant and pharmacological agents which facilitate the tick attachment and feeding processes. That ticks feed on immunologically competent hosts, and do so with impunity, suggests the presence in the tick salivary secretions, of bioactive molecules used by the tick to prime the attachment site early in the course of feeding, and also to block, biochemically, host defense cascades which would induce detachment. Immunisation of rabbits with tick salivary gland extract (TSGE) or active sensitization by repeated tick feeding, often induces protection to subsequent tick challenge which is observed as a reduction in vector engorgement weights. Inhibition of the salivary bioactive molecules may account partly or entirely for the level of resistance induced by immunisation.

In an attempt to understand the mechanism by which immune hosts adversely affect tick feeding, functional screening of tick salivary gland extract and saliva for biologically active molecules was undertaken. The salivary glands extract and

saliva contain a mixture of both biochemically active and pharmacological agents with various effector functions including inhibition of host enzymes or inactivation of molecules important in mounting vector rejection. These molecules which have hitherto been unreported in the TSGE of R. appendiculatus were identified using different techniques and their significance discussed in terms of the complex host - parasite - vector interaction. Of the activities detected, the tick salivary anticoagulant was studied in detail because of its apparent importance to tick feeding and subsequent disease transmission.

The secretion of anticoagulant(s) by ticks has been known as far back as 1908. Nevertheless, knowledge of their chemical nature, mode of action and their possible role in induction of host immune resistance to tick infestation still remains unknown. It is crucial for the survival of the tick that it be able to arrest coagulation at the site of the tick bite, and to promote the flow of blood and tissue fluid in the canals of the mouthparts during feeding. Assays employing re-calcified citrated bovine plasma, purified fibrinogen and plasma deficient in various factors of the coagulation cascade have been used to investigate the anti-clotting activity.

The anticoagulant has been purified by a procedure employing Sephadex G-100 gel filtration, ion-exchange on DE-52 cellulose, aprotinin-Sepharose affinity chromatography and size-exclusion chromatography on High Performance Liquid

Chromatography. The molecule has pI in the range of 8.0 to 8.5 on chromatofocusing and an Mr of 35kDa. The molecule appears homogenous as judged by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE).

Further work was done to elucidate the nature and mechanism of action of the purified anticoagulant. These investigations showed that it exerts its anticoagulant action by inhibiting the prothrombinase complex which catalyses the conversion of prothrombin to thrombin. Factor Xa can be activated in situ by the intrinsic and extrinsic pathways of coagulation. The anticoagulant prolongs the factor Xa clotting time.

The anticoagulant activity is detectable in blood and serous fluids from the immediate vicinity of the tick attachment sites and in pilocarpine stimulated tick saliva. These results strongly suggest that this anticoagulant is secreted into the feed pool. By 'Western' blotting, it was shown that sera from animals immunized with crude TSGE recognized several proteins from TSGE whereas functionally intact and denatured anticoagulant protein was not recognised by any of these antibodies. Furher attempts to raise antibodies in rabbits have been unsuccesful. It is thus suggested that the tick has evolved mechanisms to circumvent or block host defense by having anticoagulant molecule(s) which are relatively poorly immunogenic as compared to other TSGE moieties.

Chapter I

1.1 OVERVIEW OF TICK SALIVARY GLAND MOLECULES IN RELATION TO TICK FEEDING AND DISEASE TRANSMISSION.

1.1.1 Introduction.

Among the diverse functions of the salivary glands of ixodid ticks is the synthesis and release of enzymes and a variety of other substances which may assist in the development of a feeding lesion and in increasing blood flow into the site of a tick bite as well as into feeding canals for successful tick engorgement (1). In many species, the salivary glands secrete a cement substance that attaches the tick to the host for several days (2, 3,). The males salivate during copulation suggesting a role for salivary secretions in reproductive physiology as well (4). Saliva is injected into the animal upon which ticks are feeding, causing an immunological response which may be detrimental to the ticks especially during their slow feeding developmental stages (5). Thus the salivary components produced and which appear necessary for successful tick feeding are considered attractive candidate molecules for immunological intervention. The disease causing organisms transmitted by the tick depend on salivary glandular cells for their development and perhaps on salivary secretions for their transmission.

In this section, the salivary gland secretions with biological activity involved in tick feeding, are reviewed taking into account their possible role in induction of host resistance to tick infestation.

1.1.2 Ticks and disease.

Ixodid ticks are the most important vectors of pathogens to animals and are second only to mosquitoes as vectors of transmissible agents to man (6). Economic losses in livestock production due to ticks and the diseases they transmit are immense (7). Control of tick-borne diseases would thus have enormous positive economic input in terms of improvement of animal health and increase in productivity which would be beneficial to humans living in areas where these diseases are endemic.

The dreaded East Coast Fever (ECF), a form of theileriosis, still remains one of the major constraints on the development of the cattle industry in East and Central Africa. Theileria parva parva is transmitted mainly by the brown ear tick, Rhipicephalus appendiculatus. ECF causes 90% mortality in fully susceptible cattle. Other diseases transmitted by ticks include those caused by protozoa such as anaplasmosis (gall sickness) and babesiosis (red water), and others caused by viruses, rickettsiae and bacteria including spirochaetes (8,9). Others such as "Q" fever (caused by Coxiella burneti) and Encephalitides are important because livestock may act as reservoirs of infection to human beings. In most cases, however, the relationship between ticks and disease in man is not well understood.

Ticks, including R. appendiculatus (10) cause tick paralysis or toxicosis. The salivary toxin(s) are introduced into the host in saliva during tick feeding. Anaemia and death of host animals due to tick burden have been reported. Riek

(11) noted that severity of anaemia in cattle heavily infested with Boophilus microplus adult females was roughly proportional to the level of infestation. The ticks cause loss of blood thus affecting the health of the animal host making it prone to other disease. Van Rensberg (12) described severe anaemia and death in cattle with large numbers of R. appendiculatus.

Little is known of the development of these pathogens in the tick salivary glands which may merely act as organs of transmission. However, a more complex relationship exists between the theileria parasites and its vector, R. appendiculatus (13, 14).

1.1.3 Existing and alternative methods of tick control.

Current methods of tick control, despite their many successes, are not without problems. The most widely used method involves the application of chemical acaricides to hosts in dips and sprays to control ticks. Unless cattle are dipped or sprayed regularly - as often as every three days, the cattle may become infested. This method has become increasingly costly due to lack of foreign exchange in developing countries and the development of resistance to acaricides has also led to serious problems hence the increased concern about the threat of the spread of tick - borne pathogens (15). The effectiveness of this approach is also limited because of concern for environmental pollution and the need to develop new acaricides which would also be expensive (16). The cost of developing new acaricides in relation to economic return expected from their use before resistance occurs has discouraged research leading to new compounds for tick

control. In addition, the presence of chemical acaricide residues in animal products have potential undesirable toxic effects on humans.

Nevertheless the use of systemic acaricides is currently under development (17). This involves the use of chemical compounds with low toxicity to mammals which can be administered by injection or by oral feeding so that there are sufficient levels of the agents in the body to kill ticks feeding on the animal. Curative drugs are also available. Again constraints to their general use relate to cost which is forbiddingly high for the average animal owner.

Chemosterilants include antimetabolites and antagonists of folic acid, glutamine, purines, pyrimidines which inhibit ovarian development, oviposition and hatching of eggs. Alkylating agents are also known to be effective in sterilizing male vectors but these are mutagenic in man and most likely in other mammals.

Farm management practices including pasture spelling is another method that has been used to control some ticks. This is also a problem for three host tick species like R. appendiculatus as they can be maintained by wild animals and rodents. Burning grass can contribute to tick control but this has serious environmental consequences. Irradiation, which makes the male sterile,can be used to decreasee tick population. There is also no immediate prospect of employing field chemosterilization techniques to control ticks.

Biological control should result in reduction in tick population as well. However, biological control using microbes

and parasite wasps (18, 19) has been attempted but neither have been efficacious. In view of the above factors, it is important that new approaches to tick control be developed in addition to improving the existing methods. An immunological approach to vaccine production against tick infestation has the advantage of being long term and environmentally non-toxic and may help to circumvent the above problems. Thus the utilization of host resistance to tick infestation by immunizing with antigen preparation of ticks is less risky and perhaps deserves more attention. Employing this method it may be possible to immunize without using pathogenic material.

1.1.4 Differences between feeding strategies of argasid and ixodid ticks.

The principal difference of the two families Argasidae (Soft ticks) and Ixodidae (hard ticks) are seen in their feeding strategies. This difference is also important in terms of disease transmission and control methods.

The argasid ticks, which are blood vessels feeders, feed rapidly and pass through several moults to reach the engorged adult stage. In most argasid species, nymphs and adults take their blood meal, within 1/2 hour to 2 hours, and drop off the host. However, some larvae of argasid feed more slowly and remain attached for periods up to several days. They do not appear to use cement to assist attachment. However, their chelicera are well developed stages, compared to those of ixodid ticks. The argasid ticks are provided with specialized heavier cutting digits which have more musculature and is a possible adaptation for more rapid penetration and feeding.

On the other hand, ixodid ticks remain attached to their vertebrate host for long periods and feed continously for days, even weeks. Most of the food taken is blood, but immature stages also ingest significant quantities of tissue fluids, especially during the initial periods of slow feeding. Their attachment is secured by large teeth on the stout hypostome and this is strengthened by the secretion of an attachment cement substance. The cuticle grows during the initial slow feeding phases and subsequently stretches during the final rapid ingestion period (20) to enable intake of large volumes of blood (an increase in weight of upto 200 times). The ticks feed from pools of blood and tissue products generated by the action of mechanical trauma and salivary enzymes.

An adaptation to feeding on blood by both families is characterised by the concentration of the blood meal by removal of excess water. Argasids concentrate their blood meal by use of the coxal organ on the legs to excrete excess water to the outside, while the ixodid ticks eliminate most excess water via the salivary glands back into the mammalian host. These glands have the capacity to secrete large volumes of water particularly during the later stages of feeding (21).

It is anticipated that differences in the mechanisms of host resistance to argasid and ixodid ticks probably exist in addition to that between host species themselves.

1.1.5 Life-cycle of the three host tick Rhipicephalus appendiculatus.

Theileria parva, the protozoan, which causes East

Coast Fever, is transmitted by one of the several species within the Ixodidae, called R. appendiculatus. Under optimal conditions of temperature (20-25°C) and high relative humidity (80-90%R.H.), about 110 to 130 days are necessary for completion of the life cycle of this tick vector.

Larvae normally feed on a naive host for about three to five days. The engorged larvae drop off the host to the ground where they moult into nymphs after 18-21 days. Nymphs attach to a new host and feed for about 5-6 days before dropping to the ground where they moult into adults in about 21 days. The males and females attach to a new host where copulation takes place during feeding. Fertilized engorged female ticks drop off after 7-8 days. Oviposition commences 5-6 days after dropping off from the host and is completed by 13-14 days under favourable environmental conditions. During this time, the female lays from 3000 to 5000 eggs. The eggs hatch into larvae after 21 days. Two to three weeks after hatching, the larvae become active and seek new hosts and thus the cycle continues.

1.1.6 Changes in the lesion during tick feeding.

Tick feeding, salivation and host immunity are very closely interrelated. Ticks penetrate the host skin with their cheliceral digits and many of the ticks are highly selective in their choice of site before penetration. R. appendiculatus has a complex anatomical and functional arrangement of the apparatus producing and dispensing the saliva. The cutting action of the cheliceral digits is confined to the epidermis and possibly cause some damage to the capillaries and tissues

adjacent to the site of penetration. There is destruction of host tissue as shown by histological studies of the feeding lesion which appears as a cavity in the dermis. Lesion formation is apparently a result of tissue damage induced by cytolytic action of tick salivary secretions and by the host's own inflammatory response caused by infiltrating and degranulating host neutrophils (23, 24). Fig 1.1 shows changes in the host lesion during feeding, as illustrated by the ixodid tick B. microplus feeding on cattle previously exposed to the tick. The size of the lesion is small during the slow feeding phase. This has been shown for the feeding lesion of larvae on previously unexposed hosts. Some authors have suggested that the feeding lesions of ticks is caused by extra-oral digestion through the action of cytolins in their salivary secretions (25). However, a study of the development of the feeding lesions caused by the ixodid tick B. microplus suggested that specific vascular damage results from the saliva of the tick while tissue damage is caused by the host responses (26, 27). This hypothesis is based upon the finding that collagen destruction beneath the mouthparts of the tick was preceded by an intense infiltration of polymorphonuclear (neutrophil) leucocytes.

It can be reasonably assumed that tick saliva secretes a variety of lytic agents, anticoagulant(s) and fibrinolysin(s), which could assist tick feeding. The tick could also take advantage of a lesion formed by host autolysis caused by an immunological response to obtain a blood meal.

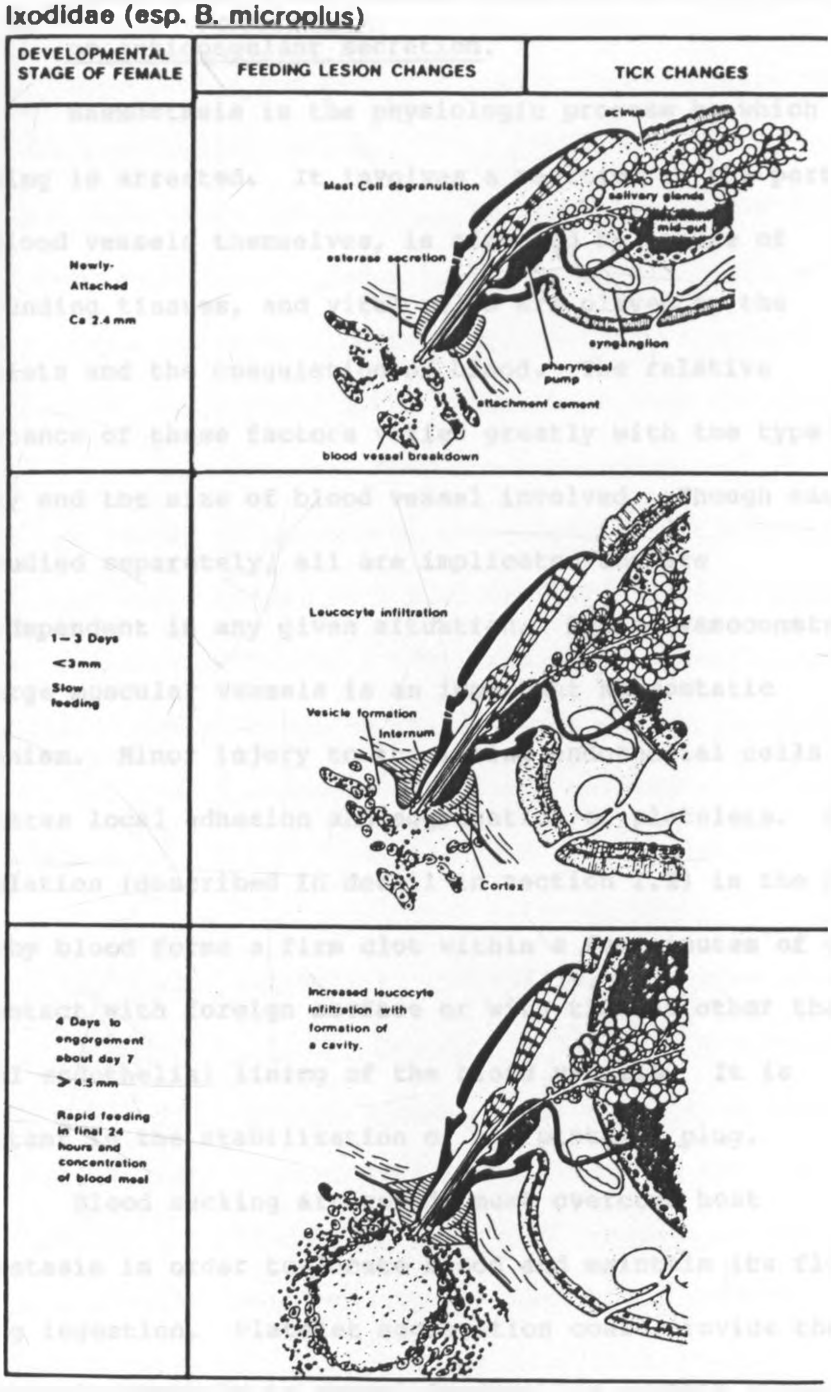

Fig 1.1 Diagrammatic representation of changes in the host lesion during feeding of B. microplus on cattle previously exposed to tick infestation (10).

1.1.7 Tick attachment and feeding: Role of salivary components.

1.1.7.1 Tick antihaemostatic components with special emphasis on anticoagulant secretion.

Haemostasis is the physiologic process by which bleeding is arrested. It involves a reaction on the part of the blood vessels themselves, is affected by nature of surrounding tissues, and vital roles are played by the platelets and the coagulation of blood. The relative importance of these factors varies greatly with the type of injury and the size of blood vessel involved. Though each can be studied separately, all are implicated and are interdependent in any given situation. Active vasoconstriction by large muscular vessels is an important haemostatic mechanism. Minor injury to the lining endothelial cells initiates local adhesion and aggregation of platelets. Blood coagulation (described in detail in section 1.2) is the process whereby blood forms a firm clot within a few minutes of coming in contact with foreign surface or with tissues other than normal endothelial lining of the blood vessels. It is important in the stabilization of the platelet plug.

Blood sucking arthropods must overcome host haemostasis in order to locate blood and maintain its flow during ingestion. Platelet aggregation could provide the main haemostatic obstacle in vessel feeders i.e argasid ticks. In the small blood vessels the initial process in haemostasis involves mainly the platelet-plug formation and the vasoconstriction of vessel smooth muscles with coagulation

playing a secondary role. The ixodid tick tears the host vessels forming a feed pool as illustrated in Fig 1.1. Thus one would anticipate that any antihaemostatic properties of R. appendiculatus focuses mainly on coagulation and secondarily on platelet aggregation and vascular constriction because of the nature of the feeding lesion.

The hard ticks including R. appendiculatus feed solely on blood, each engorgement generally extending over several days, thereby providing ample time for inflammation to promote hemostasis at the feeding site, while increasing the ticks need to antagonize this process. There are numerous demonstrations in old and recent literature of anticoagulant activity in extracts of whole ticks, tick guts or tick salivary glands and saliva. Aside from Tatchell (28), who found an anticoagulant in the gut but not in the salivary glands of B. microplus, a one host tick, most accounts agree that the saliva of ticks contains substances that inhibit blood coagulation to facilitate feeding. Anticoagulant activity has been detected in salivary glands extracts of the following species of ticks; Argas persicus (29), Ixodes holocyclus (30), Dermacentor sinicus (31), Ornithodoros moubata (32), and Ixodes ricinus (33). Hellmann and Hawkins (34), identified two types of anticoagulant activity in extracts of O. moubata salivary glands, and noted that activity was weak after feeding but increased with starvation. For example, in I. ricinus, extracts of 2.5 salivary glands from partially engorged females, delayed clotting in 2ml of sheep blood for at least 12 hr (33).

Anticoagulant activity is also found in the saliva of the ticks Hyalomma asiaticum, O. savignyi (35) and I. dammini (36). Howell (35) reported that 5ul of saliva from O. savignyi inhibited clotting of 10ml of sheep blood. An antithrombin was found in the salivary gland and other tissues of O. moubata (34). A thrombokinase inhibitor was demonstrated in the gut extracts and homogenates of whole I. ricinus (37). Willadsen and Riding (38) described a proteolytic enzyme inhibitor with anticoagulant properties in homogenates of B. microplus larvae. Both activated - partial thromboplastin (PTT) and prothrombin times (PT) were significantly prolonged. Balashov (8) showed that blood remains unclotted in the ixodid tick gut. It was suggested that the salivary anticoagulant was probably ingested to prevent blood clotting in the gut. Lester and Lloyd (39) showed that although blood sucking tsetse flies feed successfully when their salivary ducts were severed (anticoagulant is withheld from the lesion), their mouthparts were eventually blocked with clotted blood. Tsetse appear to feed successfully because of the short duration of vector - host contact unlike the ixodid ticks. It is also probable that tick saliva contain fibrinolysin(s) to aid in tick feeding.

Platelet anti-aggregating activity has previously been described for a blood sucking bug (40), tsetse flies (41) and mosquitoes (42). It has recently been reported for the first time, that the saliva of the tick I. dammini, (an argasid) inhibits platelet aggregation induced by adenosine-5'-diphosphate (ADP) and collagen, thus counteracting the main expected stimuli of platelet aggregation at the ticks feeding

site. However, this activity could be attributed at least in part to apyrase enzymes and PGE_2 that are present in saliva of the same species (36).

It thus appears that most tick species require antihaemostatic principles in their saliva for successful feeding. Anticoagulants shown to be present in the saliva are probably introduced to the host by regurgitation from the gut. All the above studies identified anticoagulant activity but show no evidence of anticoagulant secretion nor role in tick feeding. The substance(s) with anticoagulant activity in saliva or salivary glands and gut may also have some other roles such as complement inhibiton or proteolytic enzyme inhibition.

It is assumed that the physiological role of the anticoagulant and other antihaemostatic functions is to inhibit extravascular coagulation that would occur from procoagulants associated with tissue damage at the tick bite site, and also to promote the flow of tissue fluids and blood in the canals of the mouthparts while feeding.

The concept that forms the theme of this thesis, is that if protective antibodies can be raised against the above molecules, block the antihaemostatic mechanism, allow clot formation, the tick should succumb by starvation as the resistance response in the host skin cuts off the blood supply.

1.1.7.2 Cytolytic and Other Enzymes.

Enzymes such as aminopeptidases, esterases and phospholipases are probably important in facilitating

penetration of the ticks mouthparts (hypostome) into the host, as well as creating a conducive feeding environment by destroying cellular and tissue integrity. They may also have other unidentified physiological roles within the host as well as in digestive processes within the tick. Some proteases are known to have anticoagulant activity. For example, the anticlotting protease in the saliva of the leech H. ghilliani acts through a fibrinogenolytic mechanism (43). Enzymes secreted by ticks may also act as antigens which will facilitate studies of immune response of the host and its relationship to the mechanism determining the immune resistance of cattle to ticks. The enzymes of salivary secretions are thus of obvious interest for studies on feeding mechanism, pathogenicity to the host, and disease transmission.

Enzymes have been identified in tick salivary glands, but their functions remain unknown. The saliva of ixodid ticks contains enzymes which could digest tissues, a role, in nature, that would contribute to successful formation of a feeding lesion as well as ingestion of the blood meal. Esterases, aminopeptidase, and acid phosphatases are found in the saliva of B. microplus (3). In the salivary glands of B. microplus, Binnington also demonstrated histochemically the presence of acetycholine esterase. Other enzymes present in B. microplus salivary glands include a protease and monoamine oxidase (3). But strong hydrolytic enzymes that rapidly break down tissues such as hyaluronidase, phospholipase and proteinase were absent from B. microplus saliva (3). It is thought that too destructive enzymes would endanger tick attachment.

Argasid ticks may have more destructive cytolytic activity as demonstrated in preliminary studies on the proteolytic enzyme activity in the saliva of O. savignyi (44) and a similar identification of such an enzyme in the saliva of A. persicus (45). Haemorrhage and tissue destruction consistently occurs in tick attachment sites (46). However, there is no direct proof, as yet, that enzyme digestion of tissues or damage to blood vessels assists in feeding. There is always the problem of distinguishing between lytic salivary agents and autolysis for situations involving the longer feeding ixodid ticks. Tatchell and Moorhouse (23) reported that the immunological response of cattle to the attachment of B. microplus causes much more dramatic histolytic and phamacologic effects than those caused by the tick saliva. However, breakdown of blood vessels leading to haemorrhage beneath the mouthparts of the ixodid ticks has frequently been reported, and this occurs before leucocyte infiltration is observed in tissues (47, 8).

It is particularly interesting to know which enzymes are secreted in saliva for such proteins may be involved in sensitizing the host to subsequent tick feeding. Histochemical reactions for esterase, lipase and aminopeptidase were located in the dermis immediately adjacent to the mouthparts of B. microplus larvae and within the tick (48). In view of the roles played by tick enzymes, antibodies that bind to or drugs that cause inhibition of such enzymes could be useful in programmes directed at control of tick infestation. Currently

it is not known, however, whether inhibition of these enzymes is related, in any way, to tick immune resistance responses.

1.1.7.3 Enzyme inhibitors.

The mechanisms by which proteinase inhibitors function has received a great deal of attention in recent years due to their possible role or relationship with disease states. It is known, for example, that the complement,fibrinolytic and coagulation processes are intricate multimolecular enzyme systems found in the fluid phase of blood and other body fluids which must be regulated by inhibitors and activators.

Various types of cells such as basophils which have been shown to be involved in resistance to tick infestation contain chymotrypsin-like enzymes (49). During the feeding process, mast cells (which contain histamine, heparin and several proteinases) degranulation, eosinophil accumulation, and neutrophil leucocyte infilration occurs at the tick bite site in association with immediate and delayed hypersensitivity reactions (50). This is of interest because, during inflammatory process, pholymorphonuclear leucocytes release their granule contents, including elastase and cathepsin G, into the extracellular medium. Cathepsin G (Chymotripsin-like in most respects) has been reported to have a number of functions (51). For example, it has the ability to generate chemotactic activity from serum and C3 and C5 complement proteins, resulting in the production of biologically active principles involved in cytolysis, chemotaxis, immune adherence, phagocytic enhancement, and histamine release - some of these

products may be deleterious to the tick. It has also been shown that complement activation occurs at the site of the tick bite (24). The properties of cathepsin G are very similar to those of chymase I, the chymotrypsin - like proteinase of mast cells (52). Ticks probably secrete proteinase inhibitors to neutralise the undesirable effects of these hydrolytic enzymes.

Larval homogenates of the tick B. microplus contain a double-headed trypsin-chymotrypsin inhibitor which has been implicated in the successful establishment of a feeding lesion (53). There is a rapid fall in the concentration of the inhibitor after attachment. The proteolytic enzyme inhibitor, inhibits a range of proteolytic enzymes, prevents blood coagulation and inhibits complement dependent lysis of erythrocytes. Since complement participates in most antigen-antibody reactions, the blocking or minimization of the many biological activities of complement should be advantageous to the tick. Difficulty in ascribing function to the tick enzyme inhibitors is in no way unique to the tick situation: Such inhibitors have been studied in a variety of other situations, but their functions are still a matter of speculation. Thus the inhibitors could be involved internally in the control of the ticks own proteolytic enzymes or could be important in the interaction between the tick and the host if they are secreted into the host in sufficient concentration to stimulate host immune responses. It is, however, clear that the ability of the tick to feed on hosts requires that it be able to inhibit host enzymes and other agents activated or released by tissue damage or by inflammatory host reactions.

1.1.7.4 Pharmacological agents.

An oedematous reaction and/or dilation of skin capillary blood vessels at or adjacent to the tick attachment site is a common occurrence even in host animals that have had no previous exposure to ticks (26, 54, 55). An oedematous reaction on injection of salivary gland extract or saliva is not, therefore, sufficient proof of a pharmacologically active agent in tick saliva. However, it has been suggested that pharmacological agents would aid in tick feeding.

Chinery and Aitey-Smith (56) demonstrated a histamine-like activity and a histamine blocking agent in extracts of R. sanguineus salivary glands. The role of antihistamines in induction of host resistance is discussed in section 3.3.2.

Tatchell and Binnington (57) separated on a Sephadex column a fraction, from B. microplus pilocarpine stimulated saliva from engorged females and salivary gland extracts of partially fed females, which caused contraction of rat fundus preparation and also induced oedema in bovine skin. This fraction also produced an increase in permeability of bovine skin capillaries when injected intradermally. Due to its deactivation by 15-hydroxy- prostaglandin dehydrogenase, this vasoactive agent was subsequently identified in bioassay and chromatographic systems as a prostaglandin E_2 (PGE_2) (58). The greatest activity of PGE_2 was found in the salivary glands of females just before final rapid engorgement (59). In cattle, PGE_2 is more effective in promoting an increase in cutaneous blood flow, by increasing vascular permeability at

the tick attachment site or increasing vasodilation than in promoting cutaneous oedema (10).

It was also suggested by Dickinson et al (58) that prostaglandins may be normal components of tick haemolymph where they serve some function unrelated to tick feeding. PGE_2 produces a spectrum of effects that may hinder as well as help tick feeding. The helpful category includes erythema (increasing the flow of blood to the feeding tick), inhibition of mast cell degranulation (which helps minimize release of platelet aggregating, edema-promoting, and vaso constrictive factors) (60), and immunosuppresion (potentially preventing the production of antibodies against salivary antigens) (61). On the other hand, PGE_2 potentiates pain produced by bradykinin (62), as well as edema caused by substances that increase vascular permeability. The possibility of immunizing cattle against hormones produced by ticks has also been suggested. These hormones are active in moultings from one stage to the next in the life cycle. Galum et al (63) found that cattle can produce specific antibodies against hormones produced by the tick.

There is still lack of concrete information on when these pharmacological agents are secreted and on their necessity for tick feeding. Further evidence of a positive role in tick feeding is needed; for example, it may be possible to deplete prostaglandins in the tick or block the tick induced elevation in blood flow and observe effects on tick feeding. Those antibodies produced against the action of this molecules would be able to inhibit physiological actions either in vivo or in vitro.

1.1.7.5 Toxic Salivary Secretions.

The most important form of tick toxicosis is paralysis of the host and this has been reviewed by Murnaghan and O'Rourke (64) and Cothe et al (65). Forms of tick toxicosis other than paralysis also occur (66).

Tick toxicosis is caused by the injection of toxic substances by the certain instars of ticks, usually the adult female, sometimes nymphs. Approximately forty tick species in ten genera have been associated with tick paralysis. The severity of tick paralysis depends on the susceptibility of the host (man is highly susceptible and cattle less so) and the amount of toxin injected. In cattle, paralysis is often proportional to the size of tick infestation, whereas in man, the bite of one tick can have dramatic effects. Tick paralysis can affect herds of animals and cause severe losses if untreated.

The biological significance of tick toxicosis is not clear, since there is no apparent advantage to the tick in causing paralysis to the host. Extracts of salivary glands from female I. holocycles do cause paralysis, and their glands reach their peak of toxin content on the 5-6th day after commencement of feeding (67). A better understanding of the nature of the toxin substances may show that they have some important function in tick feeding. The increase in toxicity, and presumably toxic secretions, just before rapid feeding supports this view. The toxins may also be metabolic by-products excreted by the salivary glands. It is probable that ticks which live in harmony with their hosts largely do so due to a well-developed immunity to the paralysing toxin or the immunosuppressive effects of the toxins.

1.1.7.6 Secretion of attachment cement.

With the exception of Argas persicus larvae (68), argasids are not known to secrete cement. Nearly all species of ixodid ticks secrete cement from their salivary glands during the attachment phase (2) and probably during the feeding phase as well (69). The slow-feeding ixodid ticks have greater need for a stout hypostome and more secure attachment for a prolonged period of attachment on their host. Cement material is retained around the mouthparts during feeding to aid in anchoring the tick to the host. Unlike other salivary secretions that remain soluble, the cement solidifies when it comes in contact with host tissues.

There is considerable variation among the Ixodidae in the length of the hypostome, depth of penetration and presence of an attachment cement (70) as illustrated in Fig 1.2. Ixodid ticks with short mouthparts, for example R. appendiculatus, rely on the more copious secretion of cement cone which initially spreads over the skin and later extends into the lesion to secure the shallow attachment. Cement from B. microplus was found to be a glycoprotein-lipoprotein complex (71). Binnington et al (3) suggested that ixodid ticks which form a shallow attachment and secrete copious cement, excrete a more complex saliva than ticks that penetrate deeply and secrete no cement or relatively small volume of cement.

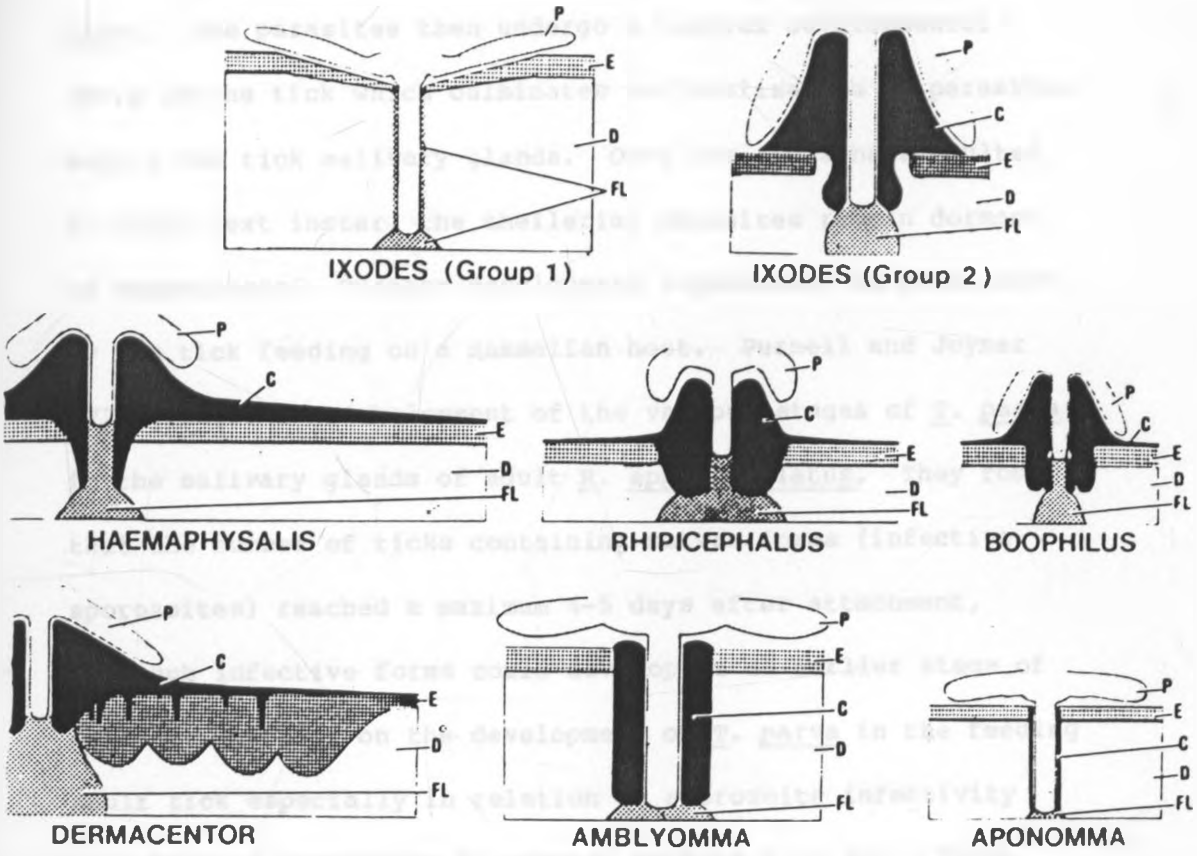

Fig 1.2 Schematic diagram of sections through host skin showing the pattern of attachment of female ixodid ticks of the named genera during the final stages of feeding. P, palps; C, cement; E, epidermis; D, dermis; FL, feeding lesion (70).

1.1.7.7 Tick salivary secretions and T. parva transmission.

Theileria parva, a protozoan parasite, causing East Coast Fever (ECF) in bovidae, is transmitted by the tick R. appendiculatus. Larval and nymphal ticks become infected by feeding on the blood of cattle containing piroplasms of T. parva. The parasites then undergo a complex developmental cycle in the tick which culminates in localization of parasites within the tick salivary glands. Once the ticks have moulted to their next instar, the theilerial parasites remain dormant as sporoblasts. Further development (sporogony) is stimulated by the tick feeding on a mammalian host. Purnell and Joyner (72) studied the development of the various stages of T. parva in the salivary glands of adult R. appendiculatus. They found that the number of ticks containing mature forms (infective sporozoites) reached a maximum 4-5 days after attachment, although infective forms could develop at an earlier stage of feeding. Studies on the development of T. parva in the feeding adult tick especially in relation to sporozoite infectivity were further undertaken by several workers (13, 14). These investigators concluded that infected R. appendiculatus ticks normally had to feed on their host for 3-4 days before parasites infective to the cattle were produced.

So far there is no information on stimuli for granular cell development or salivary secretions which are more relevant to T. parva development in the salivary glands. The biologically active molecules and other salivary components at the site of the tick bite, may act to counteract host immune responses against the sporozoites.

1.2 BLOOD CLOTTING CASCADES: BACKGROUND.

1.2.1 Introduction.

The cascade mechanism for blood coagulation involves the interaction of several components many of which lead to the formation of highly specific proteases, to eventually give rise to fibrin formation (for review see [73, 74]). Most of these proteins occur in plasma as zymogens. When the coagulation process is initiated, many of these precursor proteins are converted to active enzymes in a series of concerted reactions. Most of these enzymes have been identified as serine endopeptidases, which exert their effects on the coagulation process by a highly selective proteolysis of specific protein substrates. *In vivo*, these enzymes are shortlived since the activated clotting factors are diluted in flowing flood, or inactivated by proteolysis and protease inhibitors.

The blood coagulation model has evolved from one consisting of a linear sequence of activation of zymogens to one in which protein - protein and protein - lipid - Ca^{2+} interactions among proteinase, protein substrates, and protein cofactors result in descrete reaction complexes and reaction stages. At the present time, the majority of the steps in the intrinsic and extrinsic pathways of blood coagulation are known. At each stage zymogen activation rates are amplified (10^4-10^5 times) over that occurring with the zymogen and

proteinase alone as a result of the protein-protein and lipid-protein interaction.

When the vessel wall is ruptured, either by injury or by formation of a lesion, two main responses which lead to clot formation are set in action. One is a series of cellular interactions of blood platelets enabling them to aggregate and produce a primary haemostatic plug. The other response is blood coagulation.

1.2.2 Blood coagulation nomenclature.

The following nomenclature for the procoagulant substances in blood coagulation is used throughout this study. This was adopted by the International Committee on Blood Clotting factors established in 1954. The committee gave Roman numerals to the plasma components to serve as an equivalent to the diverse common names invented by several workers (Table 1.1).

The above system has been expanded to designate active enzymes as follows, Factor IIa (Thrombin), Factor lXa, Factor Xa (autoprothrombin C, thrombokinase), Factor Xla, Factor XIIa. etc.

Table 1.1 Glossary of blood coagulation components.

Roman numeral designation of factor	Synonyms and Common names
I	Fibrinogen.
II	Prothrombin.
III	Thromboplastin, tissue factor extract.
IV	Calcium ions.
V	Proaccelerin, labile factor accelerator
VII	Proconvertin, serum prothrombin conversion accelerator (SPCA), Cothromboplastin, stable factor.
VIII	Antihaemophilic factor or globulin (AHG), platelet Cofactor I, haemophilea A factor, thromboplastinogen.
IX	Antihaemophilia B factor, Autoprothrombin II, Christmas factor, platelet Cofactor II.
X	Autoprothrombin II, Auto-III, Stuart-Prower factor, Prothrombokinase.
XI	Plasma thromboplastin antecedent (PTA), antihaemophilic factor C.
XII	Hageman factor, glass factor, Contact factor.
XIII	Fibrin stabilizing factor (FSF), fibrinoligase, plasma transglutaminase
XIV	Protein C, autoprothrombin II-A

1.2.3 Activation processes in the blood coagulation system.

It is convenient to divide the blood coagulation system into three basic reactions as follows:

(1) The formation of factor Xa.

(2) The formation of Thrombin.

(3) The formation of fibrin.

1.2.3.1. Formation of factor Xa.

A critical step in the coagulation of blood is the proteolytic conversion of prothrombin to thrombin by factor Xa. Factor X (Stuart factor) is a plasma glycoprotein, that is common to both the intrinsic (fluid phase) and extrinsic (tissue) pathways of the blood coagulation cascade as illustrated in Fig 1.3. During the clotting process, factor X is converted from an inactive zymogen to an active protease (factor Xa) by limited proteolysis (75) under a variety of conditions.

In the initial step of the intrinsic coagulation scheme, factor XII in the presence of a negatively charged biological surface, such as exposed blood vessel endothelial collagen, or artificial surfaces such as glass or Kaolin will convert prekallikrein to Kallikrein in a reaction which is accelerated by High Molecular Weight (HMW) kininogen (kgn). The Kallikrein thus generated converts factor XII to factor

XIIa in the presence of a surface and HMW Kininogen. This reaction is in turn followed by the conversion of factor XI to factor XIa by factor XIIa and factor IX to factor IXa by factor XIa. At present, it is generally believed that in the intrinsic coagulation pathway, factor X is converted to the active form by a complex composed of factor lXa, factor VIIIa, phospholipid or phospholipoprotein (PL) and calcium ions (for review see [73, 74]). Factor lXa and Xa are highly specific serine proteases, whereas factor VIIIa appears to function as a cofactor, like factor Va. Factor VIII like factor V undergoes limited proteolysis by a protease, such as thrombin before they are active in these reactions. Under physiological conditions, the phospholipid or phospholipoprotein is provided by platelets. At the site of injury, the platelets make available binding sites for many of the coagulation proteins that interact during the coagulation process.

Factor X has been purified to homogeneity from bovine blood and consists of a light and a heavy chain linked by a disulfide bond (76). The light chain contains ɣ- carboxy glutamic acid residues, which function in the binding of calcium ions and a single residue of ß-hydroxy aspartic acid the function of which is unclear. The heavy chain contains the peptide bond that is cleaved during the activation of factor X and also contains the catalytic region that is essential for the protelytic activity factor Xa (75).

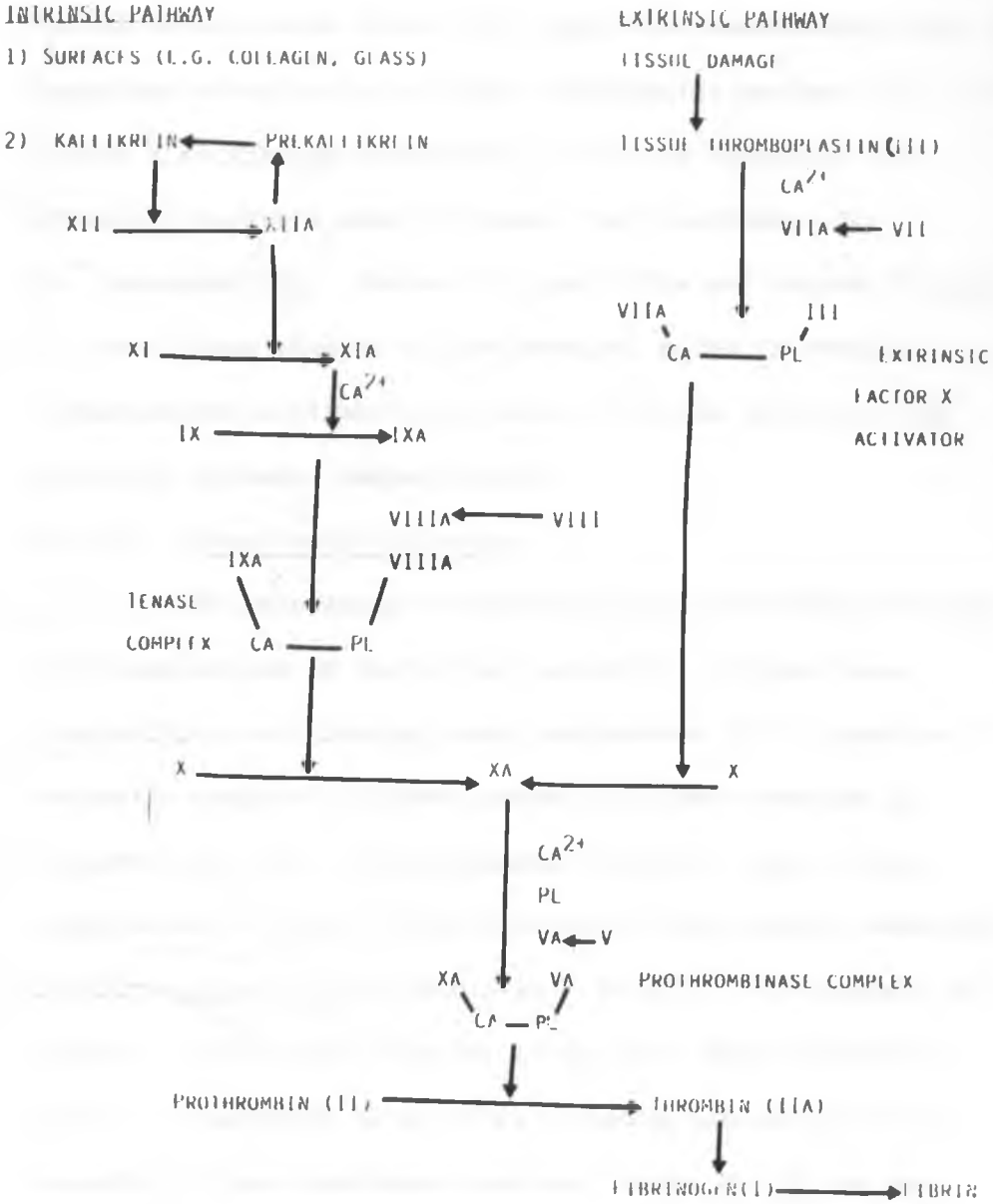

Fig 1.3 Extrinsic and intrinsic coagulation pathways in mammalian plasma (75).

The extrinsic pathway, is activated following tissue injury. Tissue thromboplastin activation (or release) occurs and this activation is closely related to the function of factor VII, a vitamin K-dependent plasma protein. Recently, other important functions of factor Xa have been reported. Factor Xa activates factor VII, and this reaction may play an important role in the extrinsic coagulation pathway (77). The factor X activating complexes in both the intrinsic and extrinsic pathways have in common the requirement for Ca^{++}-phospholipid. Factors XIa and VIIIa and factor VIIa and III would thus appear to play parallel roles in complex formation and activation of factor X in the intrinsic and extrinsic pathways respectively.

1.2.3.2 Formation of thrombin.

The generation of thrombin from prothrombin through the interactions of factor Xa, factor Va, calcium ions, phospholipids or platelets and prothrombin which comprise the enzymatic complex (Prothrombinase) have been studied and reported (73, 74). It represents a central event in the coagulation of blood. The formation of the complex enhances the activation of prothrombin at 1.39×10^{-6}M (0.1mg/ml) by a factor of 2.78×10^{5} relative to the rate with factor Xa alone. In addition to providing binding determinants for assembly of prothrombinase complex, factor Va, by as yet unidentified mechanism, enhances the V_{max} of the turnover rate of prothrombin conversion to thrombin by a factor of 3000 or more. Current evidence suggests that *in vivo*, platelets comprise the locus of assembly of the prothrombinase complex.

Factor Va augmentation of thrombin generation can be rationalized based on the ability of factor V to bind both phospholipid and factor Xa with high affinity, thereby promoting the formation of phospholipid-bound factor Va and factor Xa. Because of its Ca^{2+} and phospholipid binding properties, prothrombin can efficiently interact with the enzymatic complex. Studies of the binding of factor Xa to platelets, the binding of factor V and factor Va to platelets, the coordinate binding of both factor Xa and factor Va to platelets are consistent with the notion that factor Va constitutes a binding site for factor Xa on platelets. The prothrombinase assembly is depicted in Fig 4.8.

As shown schematically in Fig 1.4 bovine prothrombin is a linear polypeptide chain of 582 amino acids. During the process of activation, it can be cleaved at three positions which give rise to distinct functional domains of the molecule (78, 79). Prothrombin fragment 1 at the N-terminal side contains the binding sites which anchor prothrombin to a phospholipid surface via bridging with calcium. Prothrombin fragment 2 shows affinity for factor Va while prethrombin 2 is the direct precursor of thrombin. Prothrombin fragment 1.2 consists of fragments 1 and 2, and prethrombin 1 comprises prethrombin 2 plus prothrombin fragment 2. Upon activation, factor Xa splits prothrombin to produce prethrombin 2 and prothrombin fragment 1.2 but both polypeptides remain associated by non-covalent interactions. Factor Xa subsequently cleaves a peptide bond between a disulfide bridge of prethrombin 2 molecule resulting in the formation of

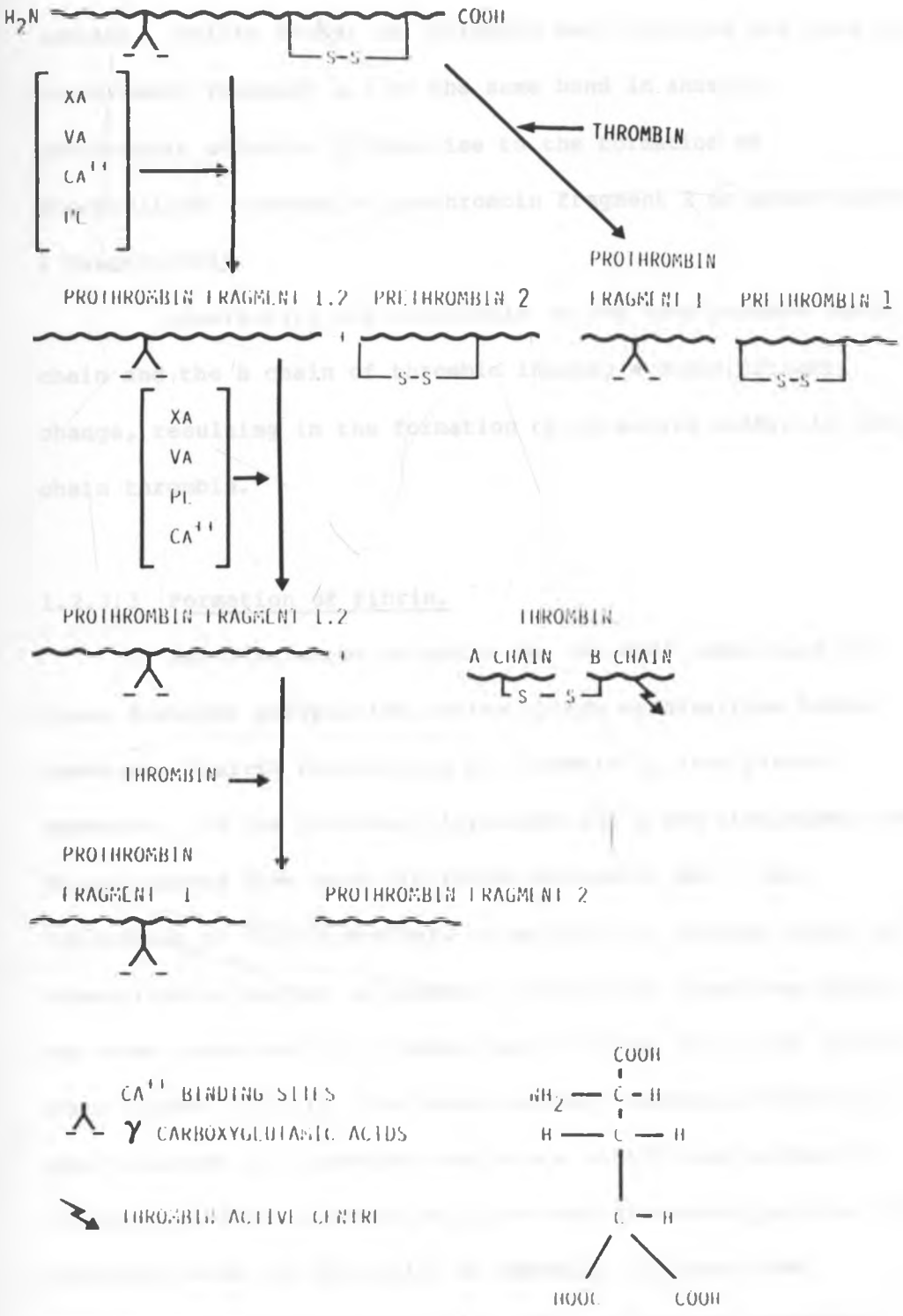

Fig 1.4 Activation pathway of bovine prothrombin by sequential bond cleavage by prothrombinase complex and thrombin (78, 79).

thrombin which no longer remains associated with prothrombin fragment 1.2 and, therefore, leaves the prothrombinase complex. Unlike factor Xa, thrombin can hydrolyze the bond in prothrombin fragment 1.2 or the same bond in another prothrombin molecule giving rise to the formation of phospholipid - uncoupled prothrombin fragment 2 or prethrombin 1 respectively.

Apparently, the hydrolysis of the bond between the A chain and the B chain of thrombin induces a conformational change, resulting in the formation of an active centre in the B chain thrombin.

1.2.3.3 Formation of fibrin.

The fibrinogen molecule (Mr 340,000) consisting of three distinct polypeptide chains linked by disulfide bonds, undergoes limited proteolysis by thrombin to form fibrin monomers. In the process, fibrinopeptide A and fibrinopeptide B are removed from each fibrinogen molecule (80). The conversion of fibrin monomers to polymers is brought about by a cross-linking enzyme in plasma. Factor XIII functions after it has been converted by thrombin and or factor Xa to its active form (factor XIIIa). The cross-linking reaction consists of the formation of a peptide bond via a schiff base mechanism involving lysine residues as donors and glutamine residues as acceptors with the formation of ammonia. Calcium ions accelerate the clotting of fibrinogen but are not necessary.

The fibrin clot is not permanent, but is eliminated as a result of its proteolytic degradation by plasmin (81).

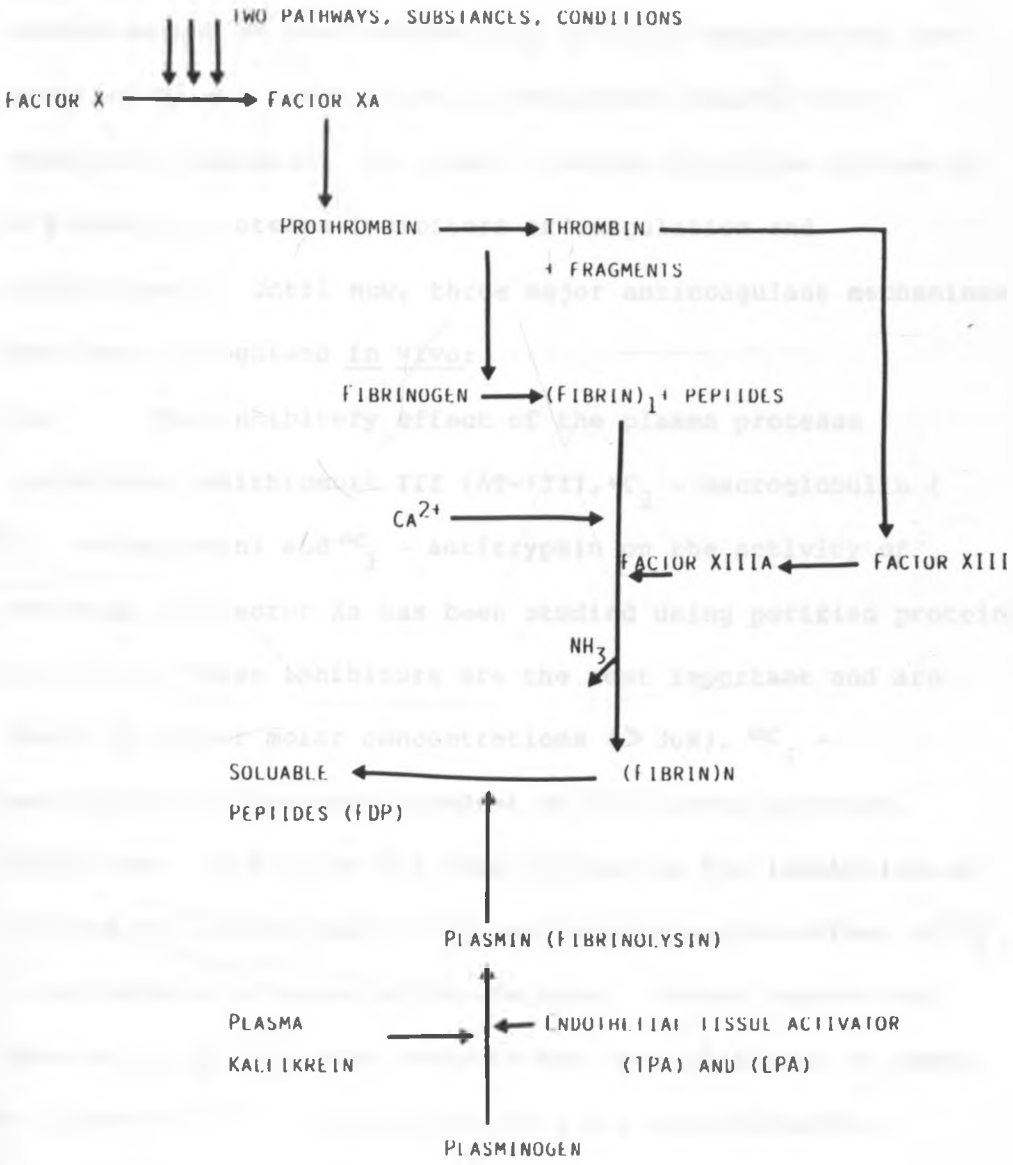

Fig. 1.5 Formation of fibrin (73, 74).

Plasmin, a serine protease, is formed from plasminogen by a variety of activators. The formation and degradation of fibrin is illustrated in Fig 1.5.

1.2.3.4 Natural plasma inhibitors of blood coagulation.

Although significant progress has been made in the understanding of the biochemistry of blood coagulation, the question of how coagulation is controlled remains only partilally answered. In blood, a mutual depletion system is effected by protease inhibitors of coagulation and fibrinolysis. Until now, three major anticoagulant mechanisms have been recognised in vivo:

(a) The inhibitory effect of the plasma protease inhibitors antithrombin III (AT-III), α_2 - macroglobulin (α_2 - antiplasmin) and α_1 - antitrypsin on the activity of thrombin and factor Xa has been studied using purified proteins (82, 83). These inhibitors are the most important and are found in higher molar concentrations ($>$ 3uM). α_1 - antitrypsin is the most abundant of the plasma protease inhibitors. Attention has been focused on the inhibition of trypsin and chymotrypsin with few reports on the effect of α_1 - antitrypsin on coagulattion factors. Recent reports by Downing et al (84) have demonstrated the inhibition of human α- thrombin by α_1 - antitrypsin in a 1:1 stoichiometric reaction resulting in the formation of a thrombin α_1-antitrypsin complex. It is also an effective inhibitor of factor Xla (85), but no reports of inhibition of factor Xa by antitrypsin. The inhibitory range of α_2 - macroglobulin is

less broad than $_1$ - antitrypsin. It interacts and inhibits plasma kallikrein, plasmin and thrombin and it has been suggested as the major plasmin inhibitor in the circulatory system. The mechanism of its action is unique in that it involves a proteolytic cleavage of a peptide bond on $_2$-macroglobulin molecule causing a conformational change probably followed by a physical entrapment of the coagulation enzyme within the $_2$-macroglobulin molecule (86). Inhibition of enzymatic activity results from steric hindrance of access of substrates to the protease-$_2$-macroglobulin complex. Alpha$_2$-macroglobulin neutralizes the clotting action of thrombin leaving the esterolytic function active. Antithrombin III has been shown to inhibit the action of factors IXa, XIIa, Xa and thrombin by the formation of a covalent complex involving a 1:1 stoichiometric reaction requiring the active site serine of the enzyme as well as the arginine residues of the inhibitor (82). The reaction between all these enzymes is accelerated by the presence of catalytic amounts of heparin. Both prothrombin activation and fibrin formation can thus be inhibited by the above plasma protease inhibitors.

(b) The recent discovery of protein C (87) has led to another anticoagulatory mechanism. Protein C, once activated, functions as an anticoagulant through selective proteolysis of the protein cofactors, factor Va (88) and factor VIIIa (89), thus inactivating prothombinase and factor X-converting enzymes.

(c) Plasmin cleaves fibrin 1 monomer, a product of the action of thrombin on fibrinogen, thereby preventing the formation of coagulable fibrin (81).

1.2.3.5. Snake venoms and blood coagulation.

Interference with blood clotting by snake venoms has been extensively studied (for review see [90]). Some venoms effect coagulant action by the activation of factor X and prothrombin and by thrombin-like activity, while others mediate anticoagulant activity through inactivation of thromboplastin, Ac-globulin, prothrombin and fibrinolysis. Other snake venoms, like Trimeresurus mucrosquamatus venom, in addition to having strong fibrinogenolytic activity also possess inhibitory activity on prothrombin activation. The inhibitor from Trimeresurus gramineus venom is a 20,000 molecular weight glycoprotein that inhibits the generation of thrombin in a system consisting of prothrombin + AC-globulin + phospholipid + calcium ions.

Factor X is activated by proteolytic enzymes such as pancreatic trypsin, cathepsin C, papain and a protease from Russel's viper venom (RVV-X). The RVV-X splits the same arginyl-isoleucine bond, as mammalian proteases, in the heavy chain of factor X thus leading to its activation. In addition, a factor V-activating enzyme (RVV-V) has been purified from Russel's viper venom. One of the widely used assay procedure is the one stage prothrombin activity. Procoagulants such as tissue thromboplastin are used. Some improvement in specificity for measuring prothrombin activity is achieved by using Taipan snake (Oxyuranus scutellatus scutellatus) venom as the procoagulant (91) which generates thrombin directly without the need of other coagulation factors except phospholipid.

Echis carinatus (Saw scaled viper) has a prothrombin activating enzyme. This enzyme also activates factor 1X and protein C. The process of activation of prothrombin by this venom activator differs significantly from that of factor Xa (78). It cleaves only a single arginiyl-isoleucyl bond linking the thrombin A and B chains in the zymogen molecule.

Snake venoms are thus useful reagents in various tests and analytical procedures of the coagulation cascade.

1.3 IMMMUNOLOGICAL BASIS OF HOST RESISTANCE TO TICKS.

1.3.1 Introduction.

Resistance to tick infestation has been known to occur in both laboratory and domestic animals (92). However, little is known of the molecular targets, among the many salivary antigen molecules presented to the host, of the protective response or of the relation of the target antigens to effects on the tick. It is evident that in the expression of such resistance antibody and cell-mediated immune responses are elicited by ticks, and it appears that biologically active mediators, such as complement components, vasoactive amines, and prostaglandins, play a role in the host response to infestation. The mechanisms involved in host resistance to ticks are discussed in this chapter.

1.3.2.1 Bovine and Laboratory animal resistance to tick infestation.

It has been known for sometime that animals become naturally resistant to ticks. Bovine resistance to tick infestation has been well documented and reported to consist of innate and acquired responsiveness (93). In East Africa

differences appear greatest when comparison is made between indigenous animals, Bos indicus (Zebu or Brahaman cattle), reared in tick infested areas and exotic animals, Bos taurus, meeting challenge for the first time. In the long association between B. microplus and Brahman cattle, the host has evolved an immunological response which is detrimental to the vector and on average 99% of the ticks are rejected, compared with 85% on the European breed. Acquisition of resistance to tick infestation by cattle has been reported by many investigators (92, 94, 95). These workers suggested the existence of an immunological basis for acquired resistance in a number of tick-host relationships; however, the actual mechanisms remain unknown. The use of cattle in attempts to characterize host resistance to ticks is not as practical as using laboratory animals as hosts. Information gathered from studies using laboratory animal-tick associations can be evaluated and promising approaches for characterization of tick-host relationship can be applied to important tick-host relationships. As is the case with cattle it has also been shown that laboratory animals acquire resistance to ixodid tick infestation (92, 94, 95). In these studies resistance was assessed by the number and/or weight of ticks engorging on subsequent infestations. There are also reports of prolongation of feeding time, reduction of egg laying and egg viability. These reports thus show evidence of physical interference with proper tick feeding, probably induced by irritation of tick attachment and inhibiton of salivary bioactive molecules required for feeding by host factors.

1.3.2.2. Induction of host resistance to tick infestation by artificial immunization with salivary gland molecules and other tick products.

It is clear that natural resistance to tick infestation can be triggered by components of tick saliva. As early as 1939, Trager (96) showed that it was possible to induce resistance to tick infestations by inoculation of D. variabilus extracts of digestive tract, cephalic and salivary glands; the salivary gland extract appeared the most potent. Several other workers have also attempted to elicit tick resistance by immunisation with salivary gland extracts (95, 97, 98) and with tick midgut extracts (99, 100). Some investigators have used whole tick extracts (92). Here the expressed resistance varies greatly, depending on the host and tick species used. The effects range from simple rejection of the tick, apparently with little or no damage, to interference with proper feeding, prolongation of feeding time, reduction in engorgement weight, inhibition of egg laying and decreased viability of eggs, to death of tick on the host. These observations that resistance to tick infestation could be induced by host immunization with salivary gland molecules lend support to the notion that an immunologic approach for the control of ticks through reactions directed towards vector antigens is feasible. Immunisation with crude antigen preparations however, does not appear to be an optimal approach to the induction of tick resistance. Indeed the immunisation methods reported thus far have not improved upon the resistance resulting from repeated tick infestation and, therefore, are

not practical for field situations. The search for efficient methods of artificial immunization should be significantly advanced by obtaining purified vector antigens and using these with different adjuvant systems.

1.3.3 Possible mechanisms of expression of tick resistance.

Despite numerous studies, it is still not known what mechanism(s) permit such stable, chronic associations of ticks and their hosts. Evidence to date suggests that humoral factors (presumably anti-tick antibodies), cell mediated immunity, and soluble mediators could be potentially involved in the expression of resistance. With highly adapted ticks, a combination of immunological effector mechanisms in the expression of net host resistance is predictable and well recognised. In the expression of resistance in guinea pigs to ticks, an involvement of T cells, reaginic antibodies and basophils (+ complement) has been implicated (92).

1.3.3.1 Role of antibodies in the resistance response.

A number of reports on antibody responsiveness to tick- infestation have appeared in the literature, although evidence that this is a causal factor in immunity is lacking. In one of the first laboratory studies of immunity to ticks, Trager (96) observed that guinea pigs acquired resistance to Dermacentor variabilis larvae after one infestation, and immunity was systemic. A degree of tick resistance appeared to be passively transferred with serum from tick resistant to

susceptible animals. Trager (96) subsequently demonstrated complement fixing antibodies to D. variabilis antigen in the sera of infested animals. Brossard (98) described bovine antibody responses against B. microplus salivary antigens, but a role for these antibodies in resistance could not be established. Specific and non-specific antibodies induced by infestation to B. microplus salivary antigens, along with an increase in total serum gamma globulin concentration after tick infestation was observed. Roberts et al., (99) administered intravenously massive volumes of plasma from B. microplus resistant calves to tick-naive recipients and were able to transfer a limited degree of tick resistance. However, the plasma components responsible for resistance were not characterized. Cyclophosphamide, an immunosuppressive drug (used in concentrations just sufficient to block B cells, 300 $mgKg^{-1}$), given to immune guinea pigs before and after infestation with ticks blocked the expression of resistance (100). The observed loss of resistance was attributed to reduced B cell-mediated responsiveness, with antibody being the effector mechanism.

There are very few reports on the development of antibodies to tick saliva bioactive molecules. In a recent study, antibodies against B. microplus-derived, phosphomonoesterases were found in tick-infested cattle (101). This enzyme is generally associated with ticks absorptive surfaces, such as its midgut, suggesting that the observed inability to feed normally on previously infested animals might be due to neutralisation of these enzyme or other biological

functions by host antibodies ingested during feeding. The above studies, among other reports, in the literature [reviewed by Willadsen (92)] indicate that host antibodies may mediate the resistance response. Resistance in guinea pigs was also passively transferred with viable lymph node cells resulting in recepients expressing resistance to a tick challenge (102).

1.3.3.2 Immune System Mediators i.e Complement and other soluble biological mediators.

Notwithstanding the importance of immune resistance responses in tick-host interactions, the resistance need not have an immunological basis. The events in the microenvironment of the tick bite site are modulated by chemical mediators which may contribute to the inflammatory process. This response could be beneficial or detrimental to the tick. Thus soluble mediators very likely play a role in the expression of resistance response (10) caused by immune-mediated inflammatory skin reactions. Histamine could play a role in the resistance response, possibly through action on the feeding tick or in regulation of cellular interactions in the resistance response. An immediate hypersensitivity response was reported by Riek (103), who observed elevated blood histamine levels 48 hours after tick resistant cattle were reinfested with B. microplus larvae. Significant levels of histamine at tick attachment sites on cattle and guinea pigs have been reported (104). Administration of the strong antihistamine, mepyramine maleate, reduced the expression of

tick resistance (105). All these observations lead to the conclusion that histamine may be one of the principal intervening agents in host resistance.

Prostaglandins (PGE_2) were found in the saliva of some ticks (58). The role of prostaglandins in regulation of immune responses is established. PGE_2 is immunosuppressive (61) but also potentiates pain produced by bradykinin (62). Thus edema, and pain caused by prostaglandins may affect the outcome of a ticks attempt to draw blood from the host. Grooming has been shown to be important in limiting tick infestation (106). Pain, would thus in addition increase the host's grooming behaviour. An allergen in B. microplus larvae which elicited a skin reaction of 30 min post-injection was shown to be a trypsin inhibitor with a Mr of 18.5 KDa (38). Thus immediate hypersensitivity reactions could play an important role in resistance to tick infestation. The complement system which interacts with the coagulation cascade and the fibrinolytic systems of the blood constitutes an important part of the immune response. Although activation can occur by way of both immunologically mediated and non-immunological mechanisms, the effect of complement on D. andersoni on guinea pigs would seem to be an immunological reaction and presumably antibody mediated. Cobra venom factor was used to deplete susceptible and resistant guinea pigs of complement factor C3 (107). Depletion of C3 did not alter the acquisition of resistance but resulted in a marked reduction of the expression of resistance. B. microplus larvae excrete into the host a protein which is capable of inhibiting bovine complement in vitro (53).

Histological characterisation of the cell types at tick attachment sites has shown eosinophils (103), eosinophils and basophils (54) and, eosinophils and degranulating mast cells (108). Skin reactions of guinea pigs resistant to D. andersoni were found to possess the characteristic of cutaneous basophil hypersensitivity response (55).

1.3.4 Host resistance to tick salivary pathogens as a result of the immune response to the tick.

The saliva of the tick provides an all important vehicle for transmission of pathogens to vertebrate hosts. Evidence indicates that resistance to tick infestation can alter transmission of pathogens such as Babesia (109) and Francisella tularensis (110). This is important and points out potential advantage of this method of tick control. Cattle resistant to infestations by B. microplus were significantly less likely to develop babesiosis than animals susceptible to tick infestation. It was also reported that animals resistant to tick infestation were significantly protected against the transmission of highly virulent tick borne bacteria (111). In addition to the decrease in disease transmission caused by reduced numbers of vectors, acquired resistance can thus directly interfere with the transmission of pathogenic organims.

Moreover, the intense immune response directed towards the feeding ticks might create a local environment hostile to the development and spread of the pathogen. These observations require further study, but they suggest that host immune

responses to the vector might protect against arthropod-borne pathogens. However, the salivary secretions injected by the tick may, in some instances promote invasion of the host by pathogens by preventing macrophage activation and neutrophil activity (112).

1.3.5 Overall aims of the Study.

The development in the salivary glands of sporozoites of Theileria parva infective for the mammalian host is related to the feeding process of the tick vector (13, 14). The infective sporozoites of the protozoan parasite are inoculated in saliva into the mammalian host a few days after the tick commences feeding. Despite all the work reported above, the exact target antigen(s) which on immunization will provoke a potent immune response against tick infestation are still unknown. The immunization methods so far tried with tick materials have not proved any better than the limited resistance attained naturally by animals in the field. None of these materials or immunisation protocols are practicable for field application. There are still difficulties in drawing valid conclusions from the demonstrated resistance to tick extracts. It is thought that following an immunological attack, some of the functional activities of the bioactive molecules of the saliva which are required for successful tick attachment and feeding are probably inhibited, which may be the indirect consequence of the tick inability to feed properly. The reduced engorgement weights, prolonged feeding times, reduced egg laying or reduced egg viability of ticks feeding on

resistant animals could solely be due to poor nutrition or some toxic effects on ticks. Antibodies have been implicated in potentiation of protective effects achieved by immunisation with crude tick products.

The accumulated evidence outlined above suggest that secretion of anticoagulant(s), hydrolytic enzymes and pharmacological agents, coupled with capillary blood pressure and some tissue damage by cheliceral teeth of the tick, all function cooperatively to liberate enough host blood for tick engorgement. It is possible that immunologically mediated inflammatory reactions within the host skin render the bite site unfavourable for the feeding process and interfere with the normal acquisition of the blood meal. No reports have been published prior to this study describing the isolation and characterisation of R.appendiculatus tick saliva bioactive molecules capable of inducing host immunity to ticks or of eliciting skin responses in animals previously exposed to ticks.

The following study was undertaken to address several problems. First, to identify tick (R. appendiculatus) salivary biologically active molecules which may mediate successful tick infestation in the complex host-ectoparasite relationships. Second, to purify the tick salivary anticoagulant and study some of it's biochemical properties and mode of action. Thirdly, this investigation was also carried to find out if rabbits can be protected against R. appendiculatus by artificial immunisation using crude TSGE or purified salivary anticoagulant.

The immunogens in TSGE were identified by employing anti-tick antibodies. With this information in hand, further questions concerning TSGE's ability to control tick infestation or use as a vaccine against East Coast fever can be addressed.

Chapter II

2. IDENTIFIFICATION OF BIOACTIVE MOLECULES AND THE PURIFICATION OF AN ANTICOAGULANT FROM TICK SALIVARY GLANDS.

2.1 Introduction.

The complex interactions which occur between ticks and their hosts are mediated by a variety of molecules with specific biochemical functions which must be simultaneously necessary for the ticks success and a target for immune responses by the host. This part of the thesis is concerned with the identification of biologically active molecules in salivary gland extracts and saliva of R. appendiculatus tick which may reduce or enhance the transmission of theileria sporozoites. These activities which have hitherto been unreported, have been identified using different functional assays. Their significance is discussed in terms of the complex parasite-vector-host interaction.

Because of the complicated biochemistry (mixture of pharmacologically and biochemically active agents), the role in tick feeding played by these molecules, can only be studied individually; there is an obvious complex interaction between the host and tick salivary molecules. Reactions which could assist tick feeding can be inferred from reactions demonstrated in vitro.

Of the activities detected, the salivary anticoagulant principle is of major interest. It was studied in detail because it is important to tick feeding and because other anticoagulant molecules have recently been shown not only to keep blood anticoagulated but to serve some other functions such as complement inhibition or proteolytic enzyme inhibition (52). Heparin, a widely used anticoagulant is known to be a modulator of various reactions of coagulation and vascular inflammation involving neutralisation by direct binding, of various vasoactive mediators, plasma proteins and cationic substances. It also has anticomplementary activity (113).

The purification procedures of the anticoagulant employed are described in this chapter. A partial characterization of the anticoagulant molecular properties is reported in chapter V.

2.2 Materials.

2.2.1 Tick breeding.

The ILRAD colony of R. appendiculatus, originating from Muguga, were used throughout this work. Rearing procedures used were those described by Irvin and Brocklesby (114). Larvae were fed on ears of rabbits and nymphs on infected cattle. Adults were placed on the ears of rabbits or on cattle. Engorged stages were kept at 28 $\pm$ 0.5^{o}C and approximately 90 -95% relative humidity for moulting and oviposition. When moulting or hatching was completed, the ticks were transferred to 20 $\pm$ 0.5^{o}C and 80% relative humidity. Nymphs were put on cattle infected by inoculation of tick derived stabilate at such a time

that their dropping coincided with the anticipated peak of parasitemia. About 6 to 8 weeks after their moult to adults, the ticks were fed on rabbits for 5 days and then their salivary glands dissected into Tris-NaCl buffer as described in section 2.3.2.

2.2.2 Bovine Plasma.

Coagulation tests were performed on bovine plasma prepared from blood collected from the jugular vein of healthy animals. Blood was collected into plastic beakers containing 0.1M sodium citrate dihydrate solution (9 parts of blood to 1 part of 3.8% sodium citrate, v/v). The blood was stirred by gentle shaking, and if there was any sign of coagulation, the blood was discarded. Blood was centrifuged at 3000 rpm for 15 min to obtain citrated bovine plasma. Platelet poor plasma was obtained by further centrifugation at 5000 rpm. If the plasma was not to be used immediately, it was stored dispensed into 5ml plastic tubes and frozen at $-20^{o}C$ until needed.

2.2.3 Products and Chemicals.

Polyacrylamide gel chemicals employed in this study: Acrylamide, N,N^{3}-methylene bis acrylamide, ammonium persulfate and Temed were obtained from Bio-rad Laboratories Ltd, Watford, U.K. SDS was from BDH Chemicals Ltd, Parkstone, England. Sephadex G-100, activated CH-sepharose 4B and electrophoresis molecular weight standards were products from Pharmacia Fine Chemicals, Uppsala, Sweden. Pilocarpine hydrochloride, bradykinin, histamine, TPCK, TLCK, DFP, DTT, hippuryl-L-arginine, arginine ß napthylamide, hippyryl-L-phenylalanine, thrombin were purchased from Sigma London

Chemical Co. Ltd, U.K. Trypsin and alpha-chymotrypsin were from Millipore Corporation Massachusetts, U.S.A. whereas BAEE, Tos-Arg-Ome and N-BTEE were supplied by Serva Feinbiochemica Heidelberg-1, Germany. Leucine aminopeptidase, carboxypeptidase A and carboxypeptidase B, chromozym PL and aprotinin were obtained from Boehringer Mannheim, West Germany. DEAE - Cellulose was from Whatman Maidstone Kent, England and Spectraphor 3 dialysis membranes from Spectrum Medical Industries, Inc. Los Angeles, U.S.A. Ultrafiltration membranes were from Amicon Scientific Systems Division Lexington, Massachusetts, U.S.A. All other reagents and solvents used were of analytical purity grade or better.

2.3 Methods.

2.3.1 Pilocarpine - Stimulated Saliva Collection from engorged ticks.

Pilocarpine, a secretogogue, was used to induce ticks to salivate according to the method of Tatchell with some modifications (115). R. appendiculatus infected or uninfected female ticks were allowed to engorge for five to eight days on the ears of rabbits after which they were removed by traction, legs removed and immobilized to double sided adhesive tape on glass slides. To collect saliva, 1 ul of 5% (wt/v) pilocarpine hydrochloride dissolved in 0.7 M NaCl was injected, by syringe through a 27 gauge hypodermic needle under the cuticle in the shoulder area to the side of the mouthparts, to avoid piercing the gut. Care was taken to prevent excessive trauma. On removal of the needle, the puncture seals itself off with some small extrusion, although some hemolymph and fluid may escape.

Alternatively a 5 to 10 ul of 10% (wt/v) pilocarpine solution was applied on the dorsal surface from which it diffused into the tick and stimulated salivary secretion.

Saliva was obtained by allowing microcapillary tubes (internal diameter of 0.25 mm at the tip) cut in half to compress the cheliceral of the tick. The tubes placed in a downward slanting position and held in place with plasticine, filled by capillarity and gravity. Greater concentration of saliva were obtained with strong saline (0.7M NaCl) than with normal solutions. A maximum of about 30 ul saliva were obtained per tick. For optimum production of saliva, recently detached engorged adult females that had just started the final period of 12 hr rapid engorgement before detaching were used. Fully engorged ticks produced more saliva but this material was less proteinaceous. The production of secretion was enhanced if the ticks were allowed to salivate for about 8 to 12 hours after injection in an incubator at 37^{o}C and 80% relative humidity. Saliva samples from several ticks were pooled and stored at 80^{o}C prior to assay.

2.3.2 Tick dissection.

Unfed, and partially fed adult R. appendiculatus infected with Theileria parva and normal controls were removed from rabbits after feeding for various days; the feeding period lasting from one to five days. For salivary gland dissection, living ticks, (ventral side down on a small paraffin wax half filled petri-dish) were held firmly pinned in rows. The ticks

were immersed in 10 mM phosphate buffered saline (0.7 mM NaH_2PO_4, 4mM K_2HPO_4, 0.15 M NaCl), PH 7.4 at 4°C. Thus, viewing through a dissecting microscope, the dorsal surface of the tick was separated and removed by cutting the lateral edge of the cuticle with a scalpel blade and dissecting needles. The exposed salivary glands were cleared of the surrounding tissues and then dissected out using a fine tipped watch makers forceps. Following dissection, salivary glands were placed into vials containing 50 mM Tris-HCl, PH 8.0, 0.2 M NaCl in an ice-bath at 0°C with or without protease inhibitors. The vials were kept on ice or frozen until dissection was completed. The remaining part consisting of the guts were removed free of the salivary glands and placed in the same buffer in an ice-bath.

2.3.3. Preparation of tick salivary gland and gut extracts, and larval nymphal homogenates.

R. appendiculatus females after feeding for various periods of time, served as the source of salivary glands and guts. The dissection was done as described above (section 2.3.2). Salivary glands were homogenized by freeze-thawing in liquid nitrogen at -196°C and in a water bath at 37°C six cycles. The homogenates were centrifuged at 15,000 xg, for 15 min at 4°C. The supernatant of the centrifugation at 15000 xg was stored at -80°C and thereafter referred to as crude tick salivary gland extract (TSGE). In addition, the salivary gland wash or soluble buffer extract was obtained by spinning down the glands at 15,000 xg for 15 min. The supernatant was decanted

decanted and stored at -80°C. Extracts from tick guts were similarly obtained and stored at -80°C. Supernatants of the centrifugation from larvae and nymphs were prepared in the same manner from ticks that had been allowed to engorge on rabbits for four days. Approximately 1000 Larvae and 1000 nymphs were thoroughly washed in 0.15M NaCl and homogenized separately in a glass tissue grinder with 2 mls 0.01M phosphate-buffered saline, pH 7.2 in an ice bath. The resulting larval and nymphal suspensions were Centrifuged at 15000 xg, for 30 min to separate soluble and particulate fractions. The opague supernatant was removed and stored frozen at -80°C.

2.3.4 Absorbance differential spectroscopy.

The measurement of optical density was done at appropriate wavelengths in 1 cm path length cuvettes in a model 260 Gilford spectrophotometer (Gilford Instruments Oberlin, Ohio, U.S.A).

2.3.5 Protein determination.

Total protein concentrations were estimated by the Folin-Ciocalteau method of Lowry et al (116). Before actual determination was carried out a standard curve was prepared using bovine serum albumin (BSA) as the standard. Eight test tubes each of 4ml volume were arranged in a rack in duplicate. Into each duplicate pair of tubes increasing dilutions of BSA were put in the order: blank, 2, 4, 5, 10, 20, 50, 100 ul from a stock solution containing 1mg/ml BSA. This was followed by addition of distilled water to a final volume of 100ul in each tube and then 2.0 mls of Lowry reagent A (0.5 ml of 1% w/v

cupric sulphate, 0.5ml of 2% w/v sodium carbonate in 0.1N Sodium hydroxide). The contents in each tube were thoroughly mixed with a vortex mixer, and then hydrolysis allowed to proceed. Two hundred ul of Lowry reagent B(2N Folin - Ciocalteau diluted one part phenol to one part triple distilled water) was added to all tubes. After standing for 30 min at room temperature to allow colour development to take place, the optical density of the reaction mixture was determined at 700 nm (Tungsten lamp) against the distilled water blank. The results obtained were plotted on a graph paper with optical density as the ordinate and protein concentration as the abscissa.

Protein determination was performed using the same procedure as for the standard curve preparation. Several dilutions of the test sample were done and using the average optical density obtained from the sample, its protein concentration was determined from the standard curve.

2.3.6 Polyacrylamide gel electrophoresis.

The various preparations in this study were analyzed on polyacrylamide slab gels prepared by using the discontinuous gel system described by Laemmli (117). The acrylamide solution used for all gels contained acrylamide: bis acrylamide in the ratio of 30:0.8 (acrylamide:bis). The mixtures were degassed and polymerization initiated by the addition of 0.8% ammonium persulfate. A flat surface on top of the separating gel was obtained by overlaying the freshly poured gel mixture with 0.1% SDS in water or water. A 5% stacking gel was cast on top of the

5-20% acrylamide gradient resolving gel two to three hours before use. Sample wells were obtained by the use of a comb made of plexi glass. The electrophoresis buffer contained 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS, PH 8.3.

Non denaturing alkaline gel electrophoresis was performed using a 5-20 percent acrylamide gradient slabs. The resolving gel (0.375 M Tris, PH 8.8) was overlayed with a 3.0 percent acrylamide stacking gel (0.125 M Tris-HCl, PH 6.8). The electrode buffer consisted of 25 mM Tris, 192 mM glycine, PH 8.3.

For reduced samples, experimental samples and size markers were heated at 100^{o}C for 5 min in 0.0925 M Tris-HCl Sample buffer, PH 6.8, containing 2.5% SDS, 5% 2 mercapto-ethanol, 20% glycerol and 0.01% bromophenol blue. Electrophoresis was performed at a constant current of 30 mA per gel for about 4 hours or until the bromophenol blue tracking dye reached the bottom of the gel on the vertical plate - gel system. The molecular weights of the protein bands on the gels were determined by comparing the relative mobilities of the proteins with known reference protein standards purchased from Pharmacia Fine Chemicals. The following proteins were used as standards: Phosphorylase b (Mr 94,000), albumin (Mr 67,000), Ovalbumin (Mr 43,000), Carbonic anhydrase (Mr 30,000), trypsin inhibitor (Mr 20,100) and α-lactalbumin (Mr 14,400). Following electrophoresis, the gels were removed from the glass plates. The protein bands were visualized by staining either with coomassie blue R-250 or silver staining as described below.

Table:2.1. Staining Procedure for the Silver-based gel electrophoresis colour development system.

Steps		Solutions	Duration of agitation.
1.	Fix	50% EtOH, 10% HOAC	2 hr or more
2.	Wash	50% EtOH, 10% HOAC	2 hr
		25% EtOH, 10% HOAC	1 hr 2x
		10% EtOH, 0.5% HOAC	1 hr 2x
3.	Equilibrate gel	$AgNO_3$ (1.9g/l)	2 hr or less
4.	Rinse	H_2O	10-20 sec.
5.	Reduce Silver	$NaBH_4$ (87.5mg/l)	10 min or less
		H_2CO (7.5 ml/l) in 0.75N NaOH	
6.	Enhance Colour	Na_2CO_3 (7.5g/l)	1 hr, store

Coomassie blue Staining Procedure.

The gels were stained by gentle shaking with a solution containing 25% (v:v) Methanol, 10% (v:v) acetic acid and 0.025% (w:v) coomassie brilliant blue R-250 in distilled water for several hours, and later destained by shaking with several changes of solutions comprising 7.5% acetic acid, 25% methanol in distilled water.

Silver Staining Procedure.

The staining procedure described here are modifications of a method developed by Sammons et al (118). The basis of the process involves the complexing of silver with proteins reactive centres in polyacrylamide gels. This technique is about fifty fold more sensitive than are staining techniques using Coomassie blue. The electrophoretic plates were washed thoroughly in detergent, rinsed with water, finally rinsed with 96% ethanol, and air-dried. Gloves were worn throughout the preparations. The steps, all carried out at room temperature on a shaker to ensure thorough mixing taken following removal of gel from the electrophoretic plates are summarized in Table 2.1. Fairly extensive washing in Pyrex trays was done to remove excess SDS.

The gel, previously equilibrated with silver nitrate solution, is rinsed briefly to remove excess surface silver and placed into a reducing solution that contains sodium hydroxide and formaldehyde. The formaldehyde should be added to the reducing solution immediately prior to submersion of the gel. After an appropriate time in the reducing solution, the gel is equilibrated through two changes of enhancing solution that

contains sodium carbonate. The sodium carbonate is necessary for optimal colour development and prevention of excessive swelling of the gel. Sodium borohydride in the solution also enhances the colour slightly but is not absolutely necessary. The gel is stored in 7.5% sodium carbonate solution or in 10% acetic acid.

2.3.7 Coupling ligands to activated CH-Sepharose 4B.

Coupling of various ligands used in this study to activated CH-Sepharose (Pharmacia Fine Chemicals) was done following instructions provided by the manufacturer. Briefly, freeze dried activated CH-Sepharose 4B powder was weighed, suspended and allowed to swell in 10^{-3}M HCl (2g of freeze dried powder per 200ml 0.001 N HCl). The additives were washed with an additional 10^{-3}M HCl (200 ml, 10^{-3}M HCl per gramme dry powder) on a medium pore glass sintered filter funnel. The appropriate ligand was dissolved in coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M Nacl, 5 ml per gram powder) and stirred slowly with the gel in a stoppered vessel. This was followed by rotating the mixture gently end-over-end for 1 hr. at room temperature or for 12 hr at 4^{o}C. The beads were collected on a sintered glass funnel and washed with several portions of bicarbonate buffer to remove excess ligand. Ethanolamine (1M, pH 9.0) was stirred with the beads at room temperature to block the remaining active groups. Three cycles of alternating pH were used to wash the product on a sintered glass funnel. Each cycle consisted of a wash at pH 4.0 (0.1 M acetate buffer containing 1M NaCl) followed by a wash at pH 8.0 (0.1M Tris-HCl buffer containing 1M NaCl).

2.3.8 Technique's used in the determination of biologically active molecules in tick salivary gland extracts and saliva.

2.3.8.1 Assay for anticoagulant activity

Recalcification time was routinely determined by incubating 0.1 ml of the test sample in diluent buffer with 0.1 of normal citrated bovine plasma in 4ml plastic tubes for 3 min. at 37°C. A stop watch was started upon the addition of 0.1 ml of 25 mM $CaCl_2$, and the time for the formation of fibrin strands in the solution was determined; saline *in lieu* of test sample was used as the control. The glass test tube was gently tilted every 5 seconds until a firm clot forms;

2.3.8.2 Enzyme and enzyme inhibitors Assays

The enzyme activities in TSGE and saliva were assayed by continuous recording in a Gilford Spectrophotometer on the following substrates given below. Enzyme activities inhibition was measured by preincubating TSGE or saliva test sample, and measuring the decrement in activity. Analysis of the activities in the gut extracts were done to determine any contamination of salivary glands.

Trypsin	-	BAEE at 50mM Tris-HCl, 0.2 m NaCl, pH 8.0 at 253 nm.
Chymotrypsin	-	BTEE, in 1.5 ml 0.08 M Tris-HCl, 0.1 M $CaCl_2$ pH 7.8 plus 1.4 ml 0.00107 M. BTEE in 50% methanol at 256 nm.

Thrombin	-	Chromozym PL at 405 nm in 50 mM Tris-HCl, 90 mm NaCl, PEG (6.5 mg/ml) at pH 8.0.
Plasmin	-	Chromozym PL in 50mM Tris-HCl pH 8.0 at 405 nm.
Carboxypeptidase A	-	Hippuryl-L-Phenyalanine in 0.05M Tris-HCl pH 8.0 at 254 nm.
Carboxypeptidase B	-	Hippuryl-L-arginine in 0.05M Tris-HCl pH 8.0 at 254 nm.
Aminopeptidases	-	Arginine B naphthylamide, diarginine B napthylamide and leucine B naphthylamide using the N-1- napthyl ethylene diamine end point method.

2.3.8.3 Bioassay detection of histaminase and bradykininase.

Isolated smooth muscle preparations were obtained from random bred adult guinea pigs of either sex. The isolated smooth muscle (about 3 cm) were suspended in a water-jacketed organ bath at 37^{o}C. Longitudinal contractions of isolated guinea pig terminal ileum was recorded isotonically using ADAPS Automatic Bioassay Controller (smooth muscle tranducer connected to a recorder). Tyrode solution at 37^{o}C were used for the assays according to the method of Webster (119). The tissue preparations were allowed to stabilize for at least 30 minutes before tests were done.

The response of the isolated guinea pig ileum to aliquots of commercial pharmacological agents bradykinin and histamine before and after incubation at 37°C for 10 min were compared to those from a control incubation containing no TSGE.

2.3.9 Determination of the time course variation of anticoagulant in tick salivary gland and gut extracts during different stages of feeding and starvation.

Adult ticks were fed on rabbits and removed from day 0 to 4 after attachment (Day 0 refers to viable unfed ticks 30 days post repletion) for TSGE and gut homogenate preparation. For each day TSGE and guts were assayed for anticoagulant activity. The clot time was determined by incubating the known amount of TSGE and gut from each day with 500 ml of whole bovine blood diluted twice with 500 ml PBS in 4 ml plastic tubes. A stop watch was started upon the addition of fresh bovine blood, and the time for formation of a firm clot was determined. The control was 500 ml whole bovine blood diluted twice with PBS. Triplicate analysis were performed.

The ticks which were fed for four days and starved during appropriate periods were dissected for TSGE and gut homogenate preparation. The assay of anti-clotting activity was performed as described above. The influence of tick salivary gland extract on the bovine plasma clotting time was determined as described in section 2.3.8.1.

2.4 Results and discussion.

2.4.1. Biologically active molecules in saliva and tick salivary gland extracts.

That Theileria parva infected R. appendiculatus remain attached to hosts for several days, feed successfully on such hosts and transmit disease has been interprated as a manifestation or failure of host defense and suggest that ticks might have evolved mechanisms to circumvent host defense. In the work described below, an atttempt was made to identify some classes of molecules in tick salivary gland extract and saliva specifically looking for host enzyme inhibitors and for enzymes with the capacity to inactivate host peptides, biogenic amines and other molecules the host usually releases in the process of host defense at sites of inflammation.

The possible biological importance of these molecules to tick feeding has received little attention. Most investigators have been concerned with disease transmission, tick-induced paralysis, host immunity or tick control by other means.

Analysis of these activities in both uninfected and infected (T. parva muguga) TSGE showed (Table 2.2) that they had these activities in common (probably uninfluenced by the immune status of the host). Thus sporozoite contribution to these functions appears minimal.

Infected and uninfected saliva of R. appendiculatus have a histamine inactivating enzyme. It was not present in TSGE which may mean it was destroyed during the extraction procedure. Commercial histamine (50 ng) was deactivated to

Table 2.2

BIOLOGICALLY ACTIVE MOLECULES OF TICK SALIVARY GLAND EXTRACTS AND SALIVA.

Principle	INF. TSGE	UNINF. TSGE	INF. SALIVA	UNIF. SALIVA	GUT
Smooth Muscle contractor	-	-	-	-	-
Bradykininase	++	++	-	-	-
Histaminase	-	-	+	+	ND
Carboxypeptidase A	++	++	-	-	-
Carboxypeptidase B	++	++	-	-	-
Leucine aminopeptidase	+++	+++	-	-	+++
Monoarginine amino peptidase	++	++	-	-	++
Diarginine amino peptidase	++	++	-	-	++
BTEE esterase	++	++	-	-	+
Esterase (BAEE)	+	+	-	-	+
Amidase (Bz-ile-Glu-Gly-Arg-PNA)	++	++	ND	ND	+
Chymotrypsin inhibitor	+++	+++	++	++	++
Plasmin inhibitor	++	++	ND	ND	+
Blood Coagulation inhibitor	+++	+++	+	+	+

Activity: NIL, -; Slight, +; Moderate, ++; Considerable, +++; Not determined, ND.

about 25% of the activity shown by the controls by 10ul saliva. An histamine antagonist has also been reported in the salivary glands of R. sanguineus (56). This molecule is important because it has been reported that cattle resistant to B. microplus tick infestations have elevations of plasma histamine levels 24-48 hr after tick challenge. Willadsen et al., (105) also showed that the amount of histamine extractible from tick attachment sites correlates directly with the degree of resistance of cattle to B. microplus and its presence beneath the attachment sites of young tick larvae causes them to detach.

Prostaglandin has been identified in the saliva and salivary glands of B. microplus (58). Prostaglandins produce effects which may hinder or facilitate feeding. It is known that PGE_2 potentiates pain produced by bradykinin (62) and the possibility is there that the salivary bradykininase would act to nullify that effect by destroying bradykinin. Twenty five ug crude TSGE deactivated 100 ng commercial bradykinin to about 40% over a 5 min period of incubation. Thus the presence of bradykininase in TSGE and other pharmacological agents are of major interest in host resistance to ticks.

Although the guinea pig bioassay system was unable to detect complement anaphylatoxins in TSGE or saliva, Berenberg et al., (24) showed that complement activation was demonstrable at the site of a tick bite. In addition, Wikel and Allen (107) provided evidence that complement plays a role in tick resistance response. The complement system is the principal

humoral effector mechanism responsible for the elimination of foreign antigens. The anaphylatoxins C3a and C5a are two of the biologically active peptides generated following the activation of the complement system (120). C3a and C5a complement components induce degranulation of mast cells and basophils, causing the release of histamine and in some species, 5-hydroxytryptamine, both into the tissues and the circulation. It is noteworthy that resistance in ticks has been associated with cutaneous basophil hypersensitivity reactions (55).

Carboxypeptidases and aminopeptidases identified in TSGE, which have the capacity to inactivate host mediators during the inflammatory process are presumably introduced into the host during feeding and contribute to the mechanism(s) employed by the tick to elude the defenses of the host. The carboxypeptidase B would cleave C-terminal arginine or lysine from important host peptides such as anaphylatoxins C3a, C5a and C4a of the complement pathway. All the complement anaphylatoxins are rapidly destroyed in serum by endopeptidases and carboxypeptidases B and N, which also removes the carboxyterminal arginine shared by all the three molecules. The loss of the C-terminal arginine destroys the anaphylatoxin activity of all the three molecules (121). Carboxypeptidase B also inactivates bradykinin. Aminopeptidases remove leucine, arginine and diarginine from N-termini of host peptides. Carboxypeptidase A inactivates peptides generated by chymotrypsin - like specificities. Furthermore, other types of cells such as basophils which have been shown to be involved in

resistance to tick infestation (55), contain a high concentration of chymotrypsin like enzymes (49). TSGE also contain a strong inhibitor of bovine chymotrypsin that could be involved in neutralisation of key host enzymes involved in the inflammatory processes as discussed in section 1.7.3. Larval extracts of B. microplus contain a double-headed trypsin-chymotrypsin inhibitor which has been implicated in successful establishment of tick feeding (53).

The enzymes detected in TSGE (esterases, amidase and aminopeptidase) in addition to serving other functions, are certainly involved in cellular and tissue destruction, a role, in nature, that would contribute to successful formation and maintenance of the feeding lesion. Tick derived esterases have been shown to elicit skin lesions in B. microplus sensitized cattle (122).

Other molecular effector pathways appear to be activated at the tick attachment site. The tick saliva and TSGE contain an anticoagulant that is thought to prevent clot formation and also promote the flow of blood and tissue fluid during feeding. The role of the plasmin inhibitor detected in TSGE is not clear. It may be involved in some function unrelated to tick feeding The gut extracts had some of the above activities that are probably ingested with the blood meal during feeding.

Few of the activities present in TSGE's were secreted in detectable amounts in pilocarpine induced saliva. This may be because copious salivation occurs with engorged ticks, returning excess water from the blood meal to the host thus

diluting the saliva. The bioactive molecules could thus be present but not in detectable amounts. However, there is no information on whether this type of secretion is equivalent to physiological saliva. The possibility exists that other secretory activities of the salivary gland require different stimulation of the relevant cells to secrete. This is certain for the components of attachment cement (white appearance, rapidily solidify) which are not secreted following pilocarpine stimulation. Thus caution is warranted in interpretation of such data because the question whether these salivary activities are actually secreted is crucial before relating them to feeding and disease transmission.

In addition to the complex anatomical and functional arrangement of the mouthparts producing and dispensing the salivary secretions (10), it thus appears from the results shown in Table 2.2 that for successful feeding the ixodid tick require a cocktail of biologically active agents, and possibly other compounds not functionally screened, to exert synergistic effects beneficial to successful feeding during its prolonged period of attachment to the host. However, immune resistance induced by a vaccine need not cover the spectrum of these functional molecules. The immune effects against one critical molecule may tip the balance in favour of the host and render the tick more susceptile to attack by other soluble mediators.

The above findings and other reports support the idea that immediate-type hypersensitivity reactions may be important in host resistance to tick infestation. These observations also suggest that for successful tick feeding and subsequent

sporozoite transmission, the tick must have evolved effective mechanisms for circumvention of host defense. This can be done most effectively if the tick secretes inhibitors of key enzymes in the effector pathways recruited for host defense or enzymes which can inactivate important peptides or other bioactive principles released in these pathways.

2.4.2 Effect of tick salivary gland extract on calcium clotting time of bovine plasma.

Figure 2.1 shows that very small amounts of TSGE cause significant prolongation of the plasma clotting time. Maximum effect was achieved with about 17 ug TSGE at which concentration coagulation for all practical purposes was destroyed.

2.4.3 The time course variation of anticoagulant activity in TSGE and gut during different stages of feeding.

In order to obtain material for anticoagulant molecule purification, the changes in activity of anticoagulant factor was followed during feeding to establish the day at which the activity reached its highest level. Anti-clotting activity levels are present in small amounts during attachment and the slow feeding first few days as depicted in Fig.2.2. This suggests that this molecule is important in the initial establishment of the tick on its host. The glands reach their peak of anticoagulant concentration on day 4-5 following attachment just prior to final engorgement. This is in parallel with the development of the stage of theileria sporozoites infective for the host in the salivary glands during feeding and with the period of intense feeding (and

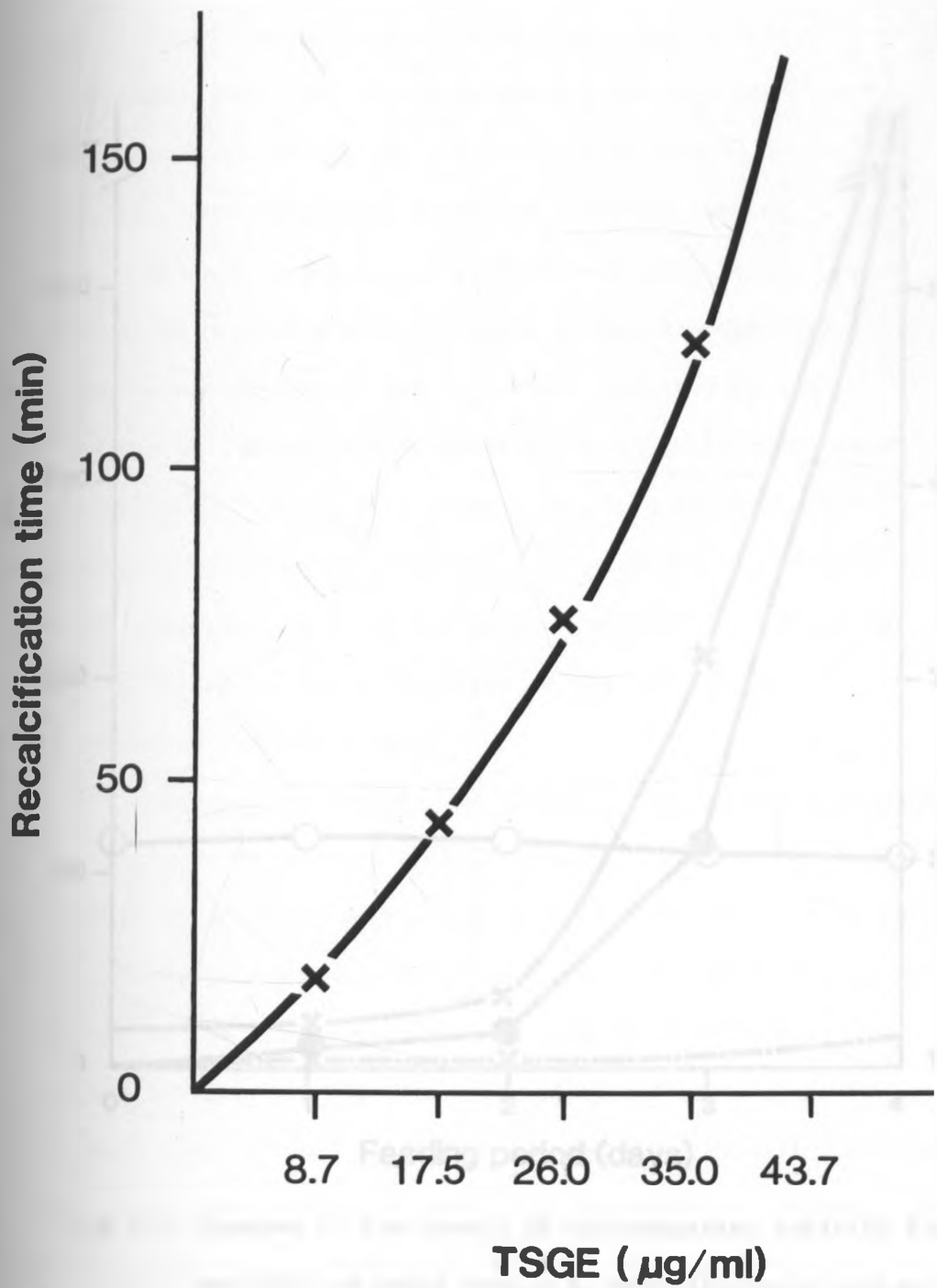

Fig 2.1 The influence of crude tick salivary gland extract on the plasma clotting time. The clotting assay was performed as described in Section 2.3.8.1.

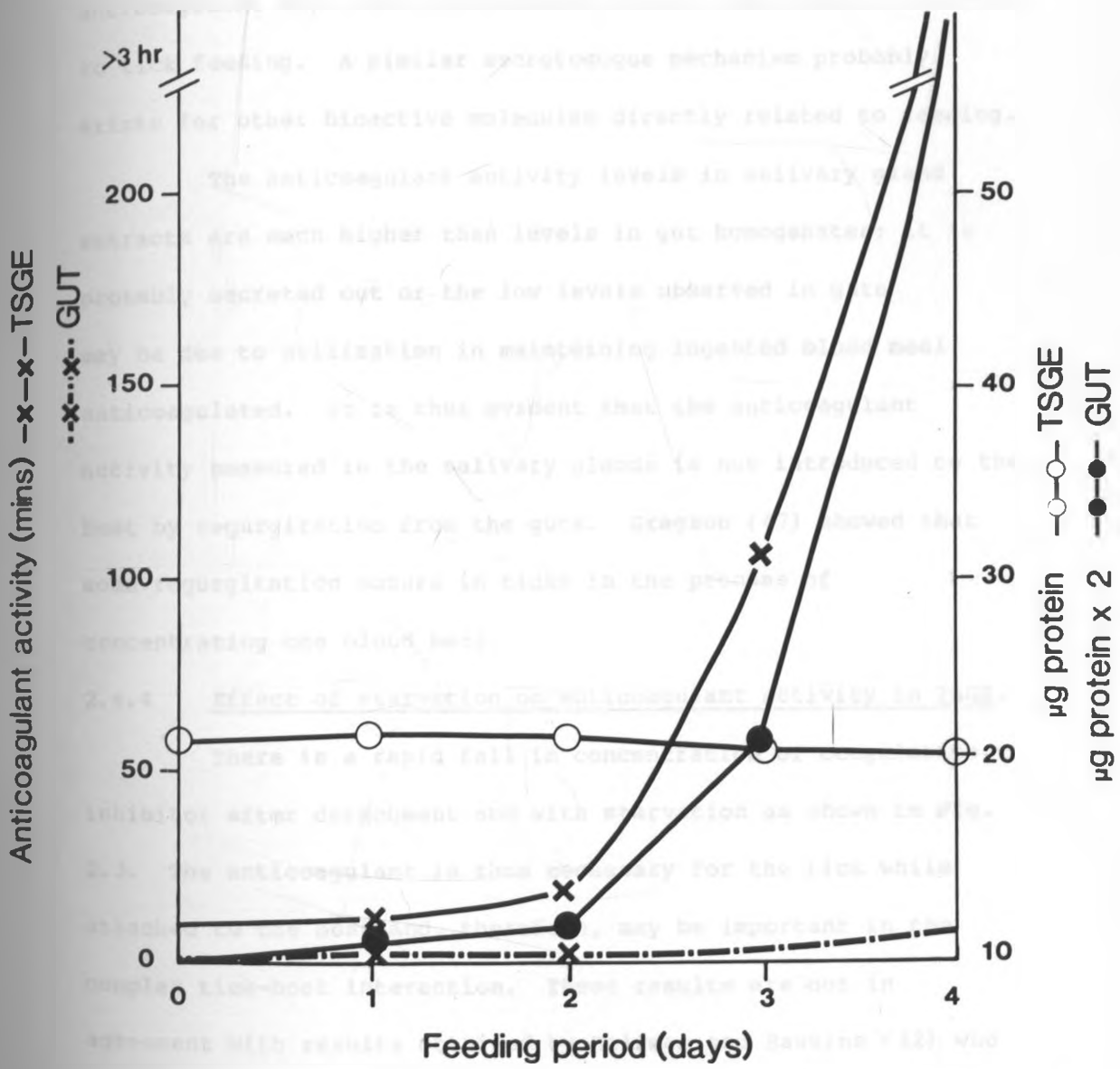

Fig 2.2. Changes in the levels of anticoagulant activity (TSGE and GUT) of adult female R. appendiculatus during different stages of feeding.

probably salivation). The fact that the anticoagulant is secreted in large quantities just prior to the short period of rapid engorgement suggests that components of the blood meal may serve as a secretagogue controlling production of the anticoagulant and that this molecule serves some function related to tick feeding. A similar secretogogue mechanism probably exists for other bioactive molecules directly related to feeding.

The anticoagulant activity levels in salivary gland extracts are much higher than levels in gut homogenates; it is probably secreted out or the low levels observed in guts may be due to utilization in maintaining ingested blood meal anticoagulated. It is thus evident that the anticoagulant activity measured in the salivary glands is not introduced to the host by regurgitation from the guts. Gregson (47) showed that some regurgitation occurs in ticks in the process of concentrating the blood meal.

2.4.4 Effect of starvation on anticoagulant activity in TSGE.

There is a rapid fall in concentration of coagulation inhibitor after detachment and with starvation as shown in Fig. 2.3. The anticoagulant is thus necessary for the tick while attached to the host and, therefore, may be important in the complex tick-host interaction. These results are not in agreement with results obtained by Hellman and Hawkins (32) who noted that anticoagulant activity in extracts of O. moubata salivary glands was weak after feeding, but increased with starvation. Their suggestion that feeding is a stimulus for secretion of anticoagulant is however, in agreement with observation reported in this work.

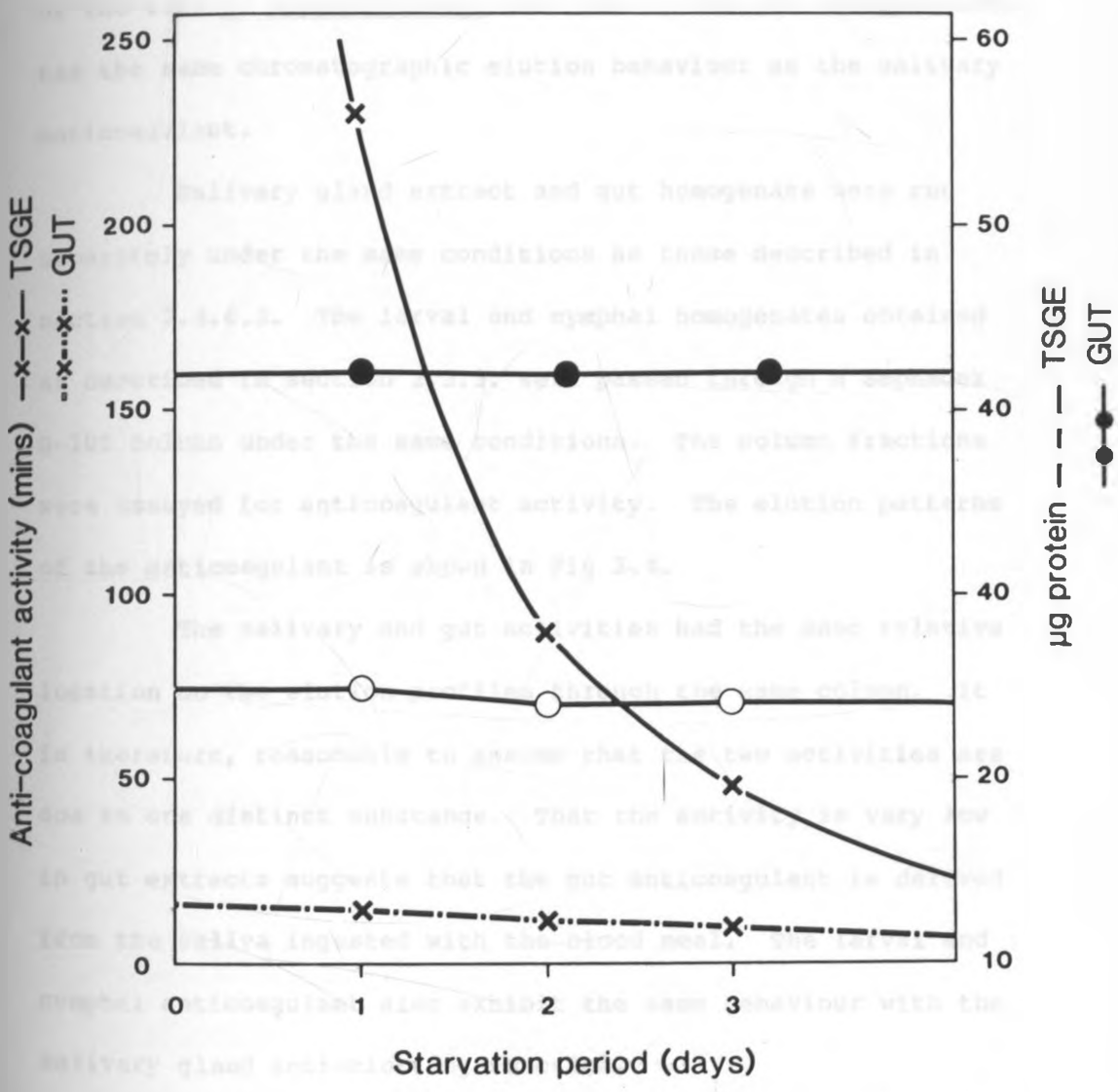

Fig. 2.3 Effect of starvation on anticoagulant activity (TSGE and Gut) of adult female R. appendiculatus.

2.4.5 Profiles of adult female gut, larval and nymphal anticoagulant on Sephadex G-100.

These experiments were performed to find out if the anticoagulant molecule is shared by all the life-cycle stages of the tick R. appendiculatus and also if the gut anticoagulant has the same chromatographic elution behaviour as the salivary anticoagulant.

Salivary gland extract and gut homogenate were run separately under the same conditions as those described in section 2.4.6.2. The larval and nymphal homogenates obtained as described in section 2.3.3. were passed through a Sephadex G-100 column under the same conditions. The column fractions were assayed for anticoagulant activity. The elution patterns of the anticoagulant is shown in Fig 2.4.

The salivary and gut activities had the same relative location on the elution profiles through the same column. It is therefore, reasonable to assume that the two activities are due to one distinct substance. That the activity is very low in gut extracts suggests that the gut anticoagulant is derived from the saliva ingested with the blood meal. The larval and nymphal anticoagulant also exhibit the same behaviour with the salivary gland anti-clotting function.

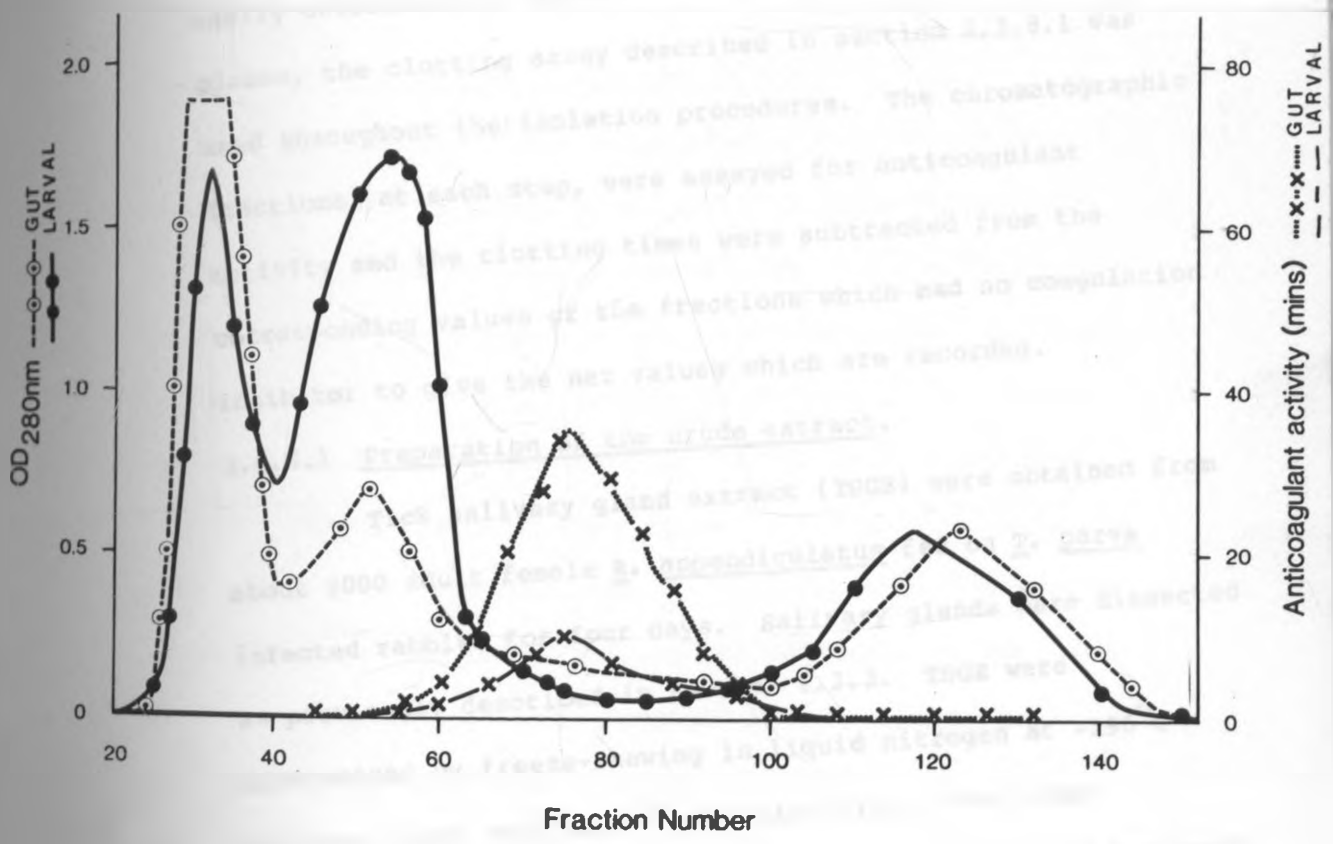

Fig.2.4 Chromatographic elution of larval (same as nymph) and gut anticoagulant on Sephadex G-100. Details of the procedure are described in the text (section 2.4.6.2).

2.4.6 Purification procedures for a coagulation inhibitor from TSGE.

General Methodology.

All experiments were carried out at 4°C unless otherwise stated. Because the anticoagulant principle was easily detectable by measuring the calcium clotting time of plasma, the clotting assay described in section 2.3.8.1 was used throughout the isolation procedures. The chromatographic fractions, at each step, were assayed for anticoagulant activity and the clotting times were subtracted from the corresponding values of the fractions which had no coagulation inhibitor to give the net values which are recorded.

2.4.6.1 Preparation of the crude extract.

Tick salivary gland extract (TSGE) were obtained from about 2000 adult female R. appendiculatus fed on T. parva infected rabbits for four days. Salivary glands were dissected as previously described in Section 2.3.2. TSGE were homogenized by freeze-thawing in liquid nitrogen at -196°C and in a water bath at 37°C for six cyles. The clear supernatant of the centrifugation at 15,000g (15 min) was used as anticoagulant source.

The pilocarpine saliva and gland wash had comparable protein components as the TSGE on SDS-PAGE. In addition, it was difficult to obtain sufficient quantities of pilocarpine stimulated saliva for anticoagulant activity purification. The gland wash contained about 80% of the total anticoagulant activity obtained from the Freeze-thawed homogenate. This was

interpreted to mean that the bulk of the anticoagulant activity is located in the periplasmic space or cell wall from which it is released in the medium. It appears that most of the proteins are not membrane associated. Therefore it was convenient to use the Freeze-thaw homogenate as the starting material and this was referred to as the crude extract.

2.4.6.2 Gel filtration on Sephadex G-100.

The Sephadex G-100 supplied as a dry powder was allowed to swell in 50mM Tris HCl, pH 8.0, 0.2m NaCl, 0.01% NaN_3 (buffer A) by boiling in a water bath for 5 hr according to the manufacturers instructions. Fine particles were removed by decantation and the gel suspension kept at $4^{o}C$ to reach the temperature of column operation before packing was begun.

The concentrated supernatant TSGE (final volume=6ml) obtained from the previous step was pre-treateted with serine protease inhibitors (DFP,. PMSF, TLCK, TPCK each at 5mM final concentration) and sulfydryl reagents (Iodoacetamide and chloromercuriphenyl-sulfonic acid at 5mM final concentrations). The sample was applied to 2.6 x 90 cm column of Sephadex G-100 equilibrated with buffer A and run in the same buffer at a flow rate of 22.5 mls/hr using a peristalic pump. The eluates (4.0ml fractions) were analysed for protein by absorhance (OD_{280nm}) and clotting time as shown in Fig. 2.5. The fractions containing anti-clotting activity were combined and concentrated using a PM 10 ultrafiltration amicon cell.

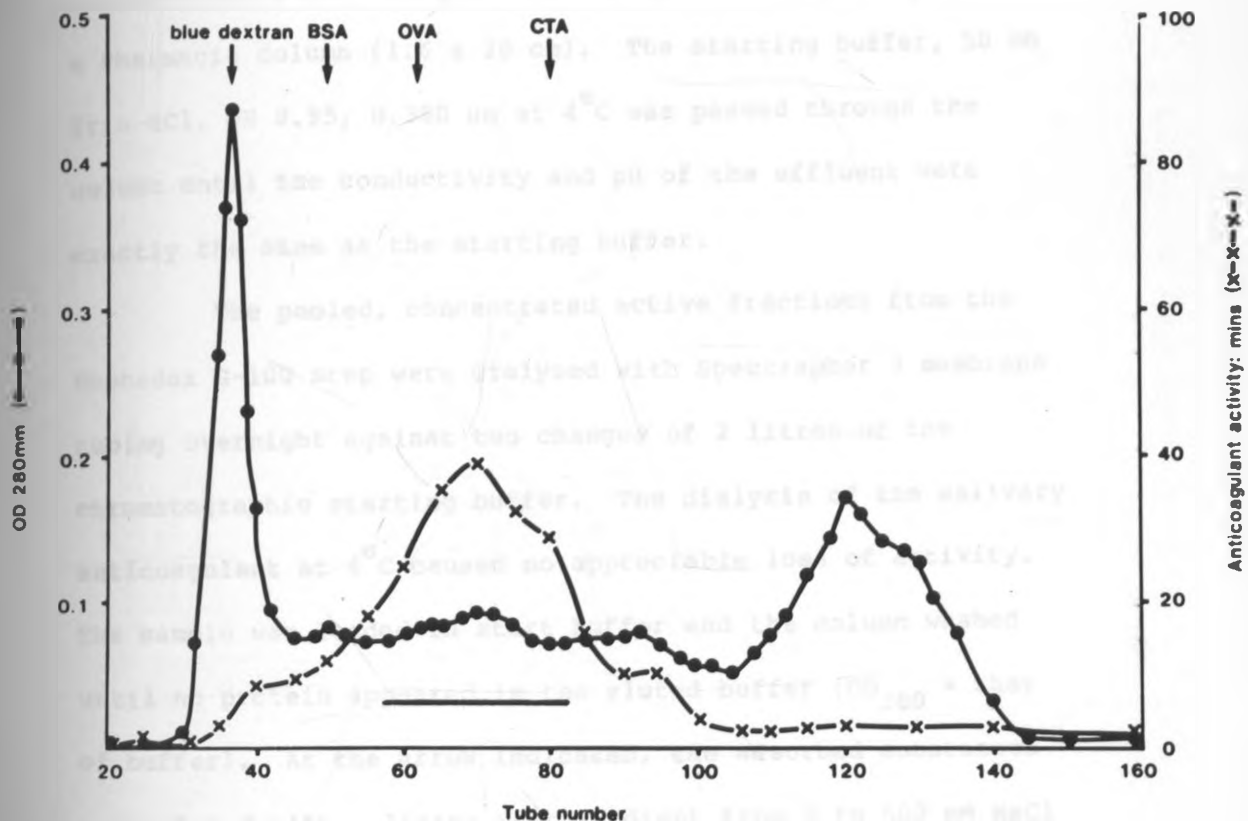

Fig 2.5 Gel filtration chromatography of crude TSGE on Sephadex G-100. Blue Dextran (Mr $\sim 2 \times 10^6$); Bovine serum albumin (BSA, Mr 67,000); ovalbumin (OVA, Mr 43,000); chymotrypsinogen A (CTA, Mr 25,000) were eluted at the positions indicated in the figure. The horizontal bar show the fractions that were pooled.

2.4.6.3 Ion-exchange chromatography on DEAE - cellulose:

Pre-swollen ion-exchange DEAE-cellulose was gently stirred with 50mM Tris-HCl pH 8.95 (about 6 ml per gramme of wet ion exchanger). The pH of the buffer/ion exchanger slurry was adjusted, while stirring to about pH 8.95, $4^{o}C$ with concentrated HCl. Fines were removed and the slurry packed in a Pharmacia column (1.6 x 20 cm). The starting buffer, 50 mM Tris-HCl, pH 8.95, 0.380 um at $4^{o}C$ was passed through the column until the conductivity and pH of the effluent were exactly the same as the starting buffer.

The pooled, concentrated active fractions from the Sephadex G-100 step were dialysed with Spectraphor 3 membrane tubing overnight against two changes of 2 litres of the chromatographic starting buffer. The dialysis of the salivary anticoagulant at $4^{o}C$ caused no appreciable loss of activity. The sample was loaded in start buffer and the column washed until no protein appeared in the eluted buffer (OD_{280} = that of buffer). At the arrow indicated, the adsorbed substances were eluted with a linear salt gradient from 0 to 500 mM NaCl in the equilibration buffer and the same buffer. Fractions of 2 ml were collected at a flow rate of 15 mls/hr. The active fractions shown in Fig. 2.6 with inhibitory activity as indicated by the horizontal bar were pooled and similarly concentrated with Amicon Ultrafiltration apparatus with a PM10 membrane.

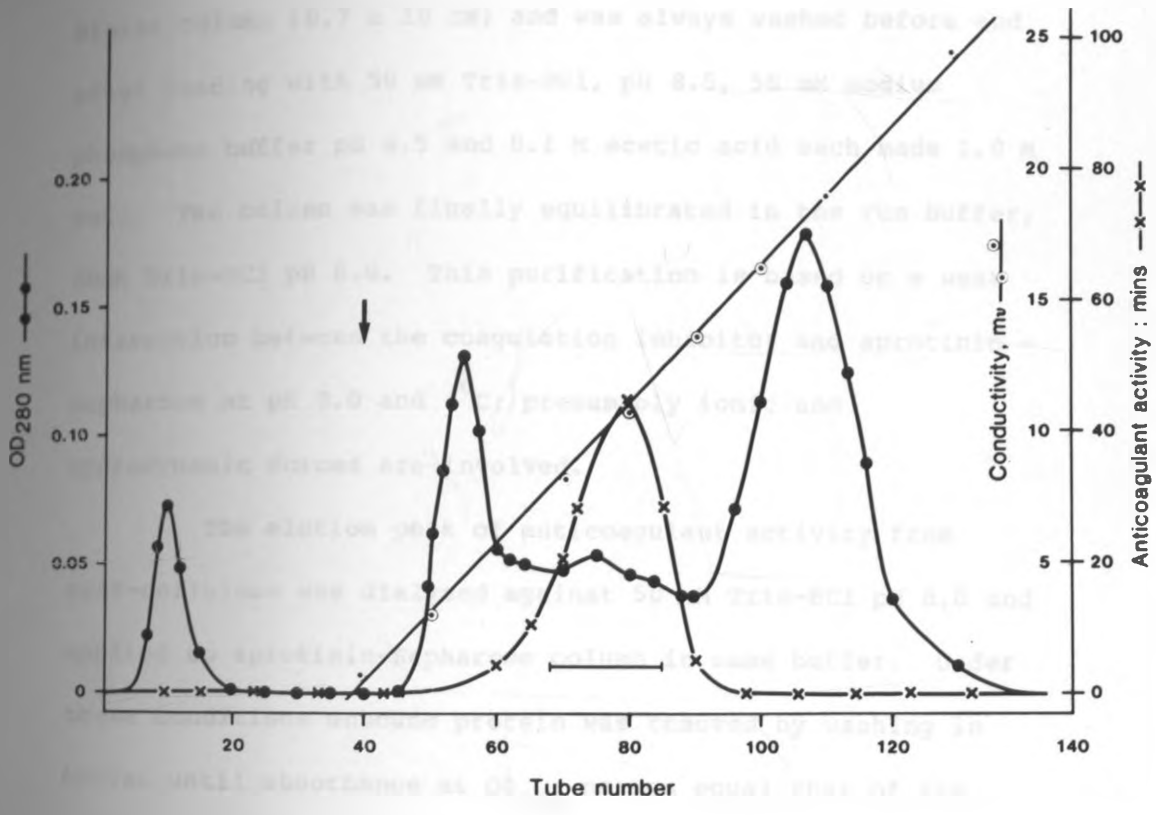

Fig 2.6 Active fractions from the Sephadex G-100 step on Ion-exchange chromatography on DEAE - Cellulose

2.4.6.4 Aprotinin - Sepharose affinity Chromatography.

Aprotinin is coupled to activated CH-Sepharose 4B as described in section 2.3.7. Aprotinin (200 mg) was dissolved in buffer and coupled to gel formed from 5 gm activated CH-Sepharose. The aprotinin - Sepharose gel was poured into a Biorad column (0.7 x 20 cm) and was always washed before and after loading with 50 mM Tris-HCl, pH 8.5, 50 mM sodium phosphate buffer pH 6.5 and 0.1 M acetic acid each made 1.0 M NaCl. The column was finally equilibrated in the run buffer, 50mM Tris-HCl pH 8.0. This purification is based on a weak interaction between the coagulation inhibitor and aprotinin - Sepharose at pH 8.0 and 4^{o}C; presumably ionic and hydrodynamic forces are involved.

The elution peak of anticoagulant activity from DEAE-cellulose was dialysed against 50 mM Tris-HCl pH 8.0 and applied to aprotinin-Sepharose column in same buffer. Under these conditions unbound protein was removed by washing in buffer until absorbance at OD_{280} nm was equal that of the buffer. All the anticoagulant remained bound and was subsequently eluted with the same buffer containing 0.2M NaCl. Fractions of 2 mls were collected at a flow rate of 10 mls/hr and were analyzed for protein and anticlotting activity as shown in Fig. 2.7.

2.4.6.5 High Pressure Size exclusion Chromatography:

This final purification step employed high performance gel filtration chromatography performed using a modified model

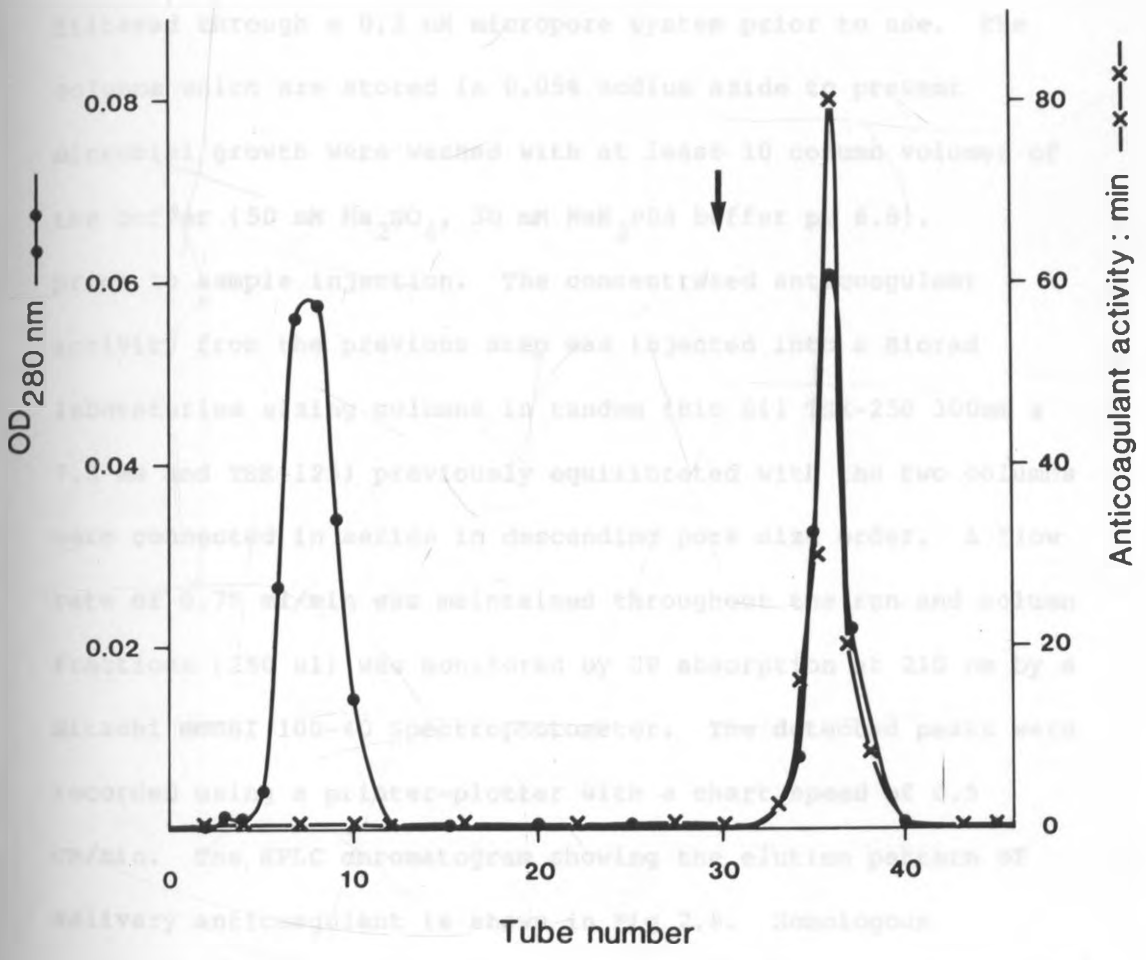

Fig 2.7 Concentrated fractions with inhibitory activity from Sephadex G-100, DEAE - Cellulose on aprotinin Sepharose affinity chromatography.

324 Beckman HPLC system. It consists of a syringe-loaded injector, fitted with an interchangeable sample loop, a column, two solvent metering pumps (Model 100A) and a variable wavelength detector. Separation was carried out at ambient temperature (Ca 25^{o}C). All solvents were degassed and filtered through a 0.2 uM micropore system prior to use. The columns which are stored in 0.05% sodium azide to prevent microbial growth were washed with at least 10 column volumes of the buffer (50 mM Na_2SO_4, 30 mM NaH_2P04 buffer pH 6.8). prior to sample injection. The concentrated anticoagulant activity from the previous step was injected into a Biorad laboratories sizing columns in tandem (Bio Sil TSK-250 300mm x 7.5 mm and TSK-125) previously equilibrated with the two columns were connected in series in descending pore size order. A flow rate of 0.75 ml/min was maintained throughout the run and column fractions (250 ul) was monitored by UV absorption at 210 nm by a Hitachi model 100-40 Spectrophotometer. The detected peaks were recorded using a printer-plotter with a chart speed of 0.5 cm/min. The HPLC chromatogram showing the elution pattern of salivary anticoagulant is shown in Fig 2.8. Homologous fractions from several runs each 200ul were concentrated using a PM 10 Amicon UItrafiltration cell, dialysed against distilled water and lyophilized. The dried sample was reconstituted in 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl and stored as aliquots at -80^{o}C. Standard proteins run under the same conditions gave the following retention times: Thyroglobulin (12.59), bovine gamma globulin (16.25); chicken ovalbumin (19.22); bovine myoglobulin (23.12); cyanocobalamin (29.05).

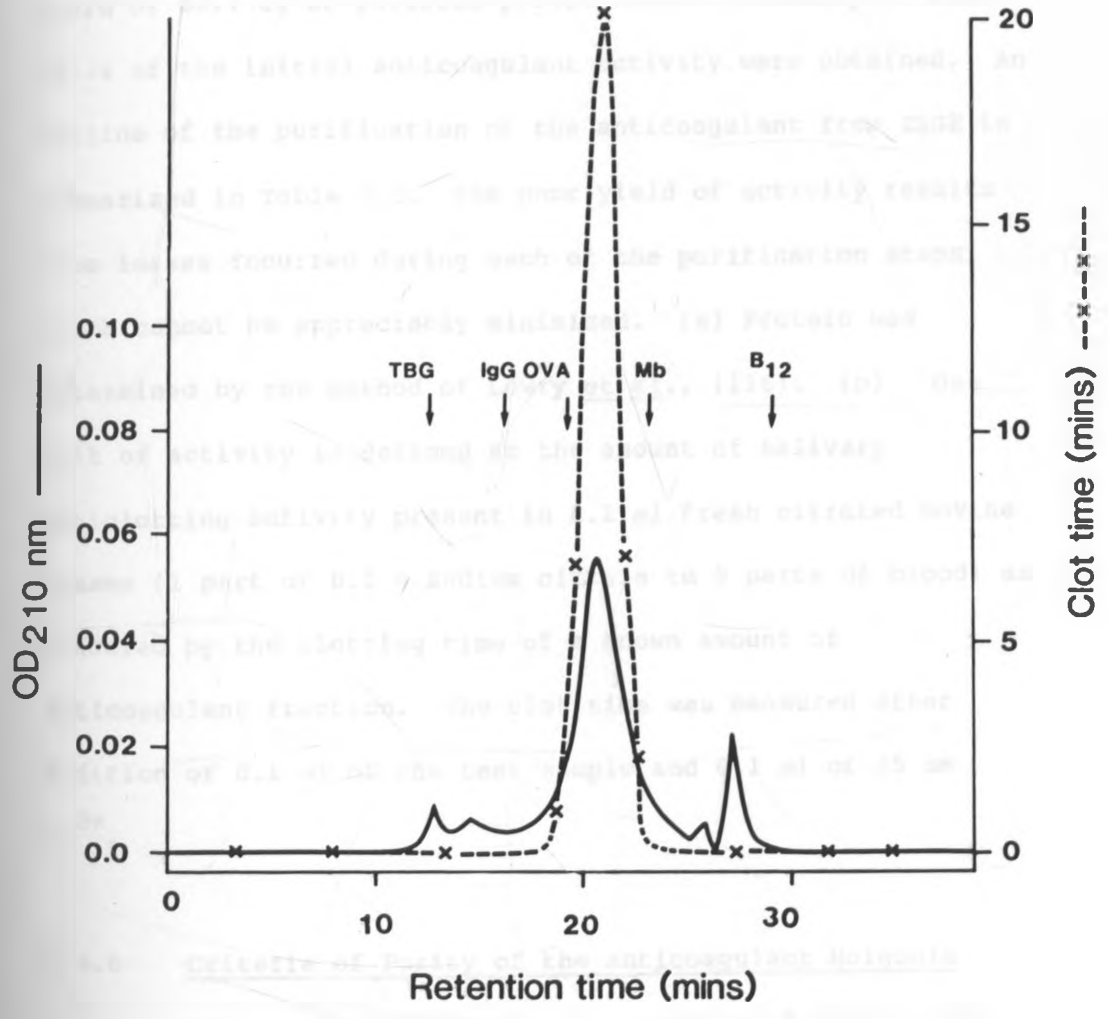

Fig 2.8 HPLC chromatogram showing the elution pattern of tick salivary anticoagulant from aprotinin-Sepharose chromatography. Thyroglobulin (TGB); bovine gamma globulin (IgG); chicken ovalbumin (OVA); bovine myoglobulin (Mb); cyanocobalamin (B_{12}) were eluted at the positions indicated in the figure.)

2.4.7 A summary of the purification procedure of the anticoagulant molecule.

The anticoagulant was purified from 88.8 mg of crude TSGE recovered from salivary glands of 2000 adult ticks. A yield of 80.7 ug of purified protein with a recovery of about 10.1% of the initial anticoagulant activity were obtained. An outline of the purification of the anticoagulant from TSGE is summarized in Table 2.3. The poor yield of activity results from losses incurred during each of the purification steps which cannot be appreciably minimized. (a) Protein was determined by the method of Lowry et al., (116). (b) One unit of activity is defined as the amount of salivary anticlotting activity present in 0.1 ml fresh citrated bovine plasma (1 part of 0.1 M sodium citrate to 9 parts of blood) as measured by the clotting time of a known amount of anticoagulant fraction. The clot time was measured after addition of 0.1 ml of the test sample and 0.1 ml of 25 mM Ca^{2+}.

2.4.8 Criteria of Purity of the anticoagulant Molecule.

The samples obtained during each purification step were subjected to 5-20% SDS-PAGE under non reducing conditions following the procedure described in section 2.3.6. The silver stain of the purified molecule revealed a single band on SDS-PAGE as shown in Fig 2.9. A low molecular-weight electrophoresis calibration kit (Pharmacia Fine chemicals Uppsala) was used for molecular weight standards. The apparent

Table 2.3 Summary of the purification procedure of tick anticoagulant from TSGE.

Purification step	Volume (ml)	Total Protein (ug)	Total activity (Units)	Specific activity Units/ug protein	Yield (%)	Purification factor
Supernatant of crude extract	6.0	88800.0	333000.0	3.75	100	1
Sephadex G-100	5.0	1953.6	214830.0	110.0	64.5	29.3
DE-52 Cellulose	4.5	210	52500	250.0	15.8	66.7
Aprotinin-Sepharose	2.0	115	43257	376.1	13.0	100.3
HPLC	2.0	80.7	33636	416.8	10.1	111.1

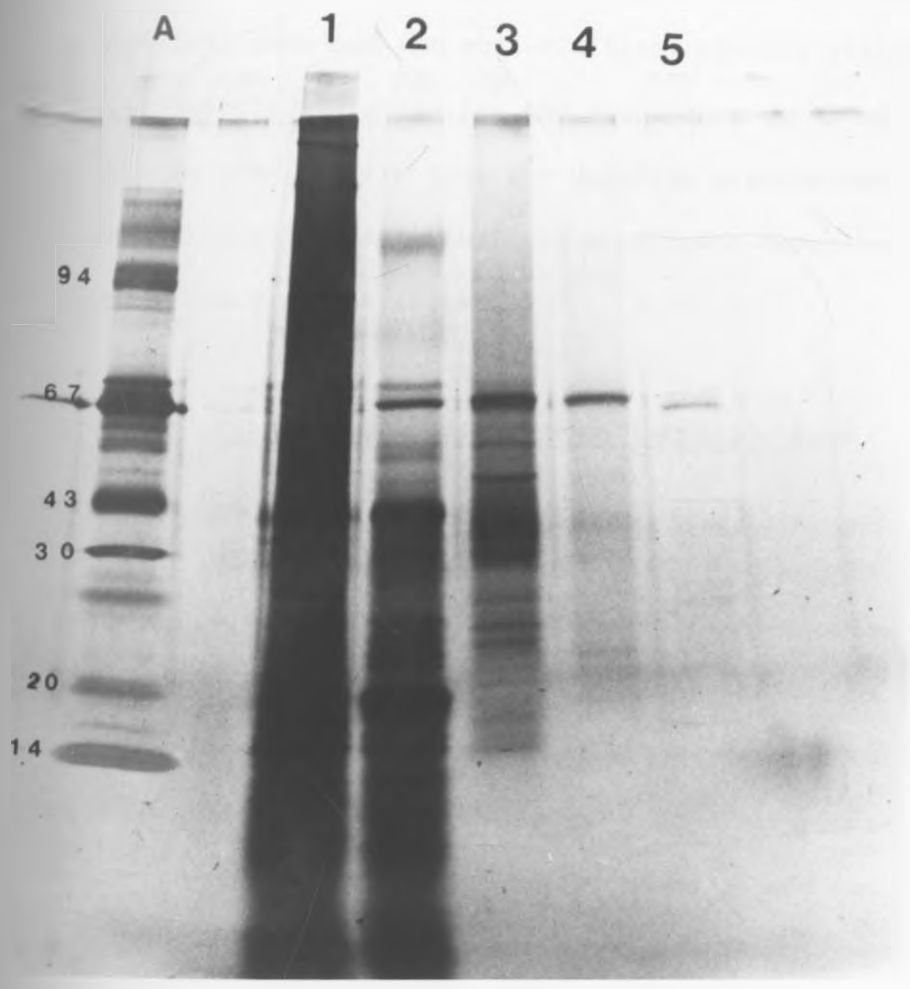

Fig.2.9 SDS-PAGE (5-20%) of tick salivary anticoagulant following each step of purification.

(A) Size markers; the numbers are Mr expressed in kilodaltons.

(1) Crude extract (25ug)

(2) after Sephadex G-100 column (20ug)

(3) after DEAE - cellulose (15ug)

(4) after aprotining-Sepharose (10ug)

(5) after HPLC (7ug).

The proteins on the gel were silver stained as described in 2.3.5.

molecular weight of the purified anticoagulant was 65KDa. The homogenity of the protein was also confirmed by HPLC. The elution profile of the inhibitory factor gave a single symmetrical peak and the anti-clotting activity coincided with a peak detected by measuring the absorbance at 210nm (Fig. 2.8). To also confirm that the purified protein was associated with anticoagulant activity, the experiment described in section 2.4.9 was performed.

2.4.9 Extraction of Coagulation inhibitor from polyacrylamide gels.

Non-denaturing gels were prepared as described in section 2.3.6. Duplicate samples were electrophoresed and the gel was cut into two; One half with molecular weight standards and the purified anticoagulant was silver stained until the band of interest could be detected. The other portion of unstained gel was washed in PBS for about 15 min and cut into slices of one cm each. The corresponding slices were crushed in 4 ml glass tubes containing 50 mM Tris-HCl, pH 8.0, 0.2M NaCl. To elute the activity the mixture was left equilibrating overnight at $4^{o}C$. The next day they were crushed again and left for 10 min at $24^{o}C$. The supernatant was obtained by passing each mixture through coarse Sephadex G-25 in a pasteur pipette plugged with a glass wool to remove polyacrylamide. The eluate was assayed for anticoagulant activity as described in methods (Section 2.3.8.1). Control experiment showed that anticoagulant activity can be kept at $4^{o}C$ overnight without

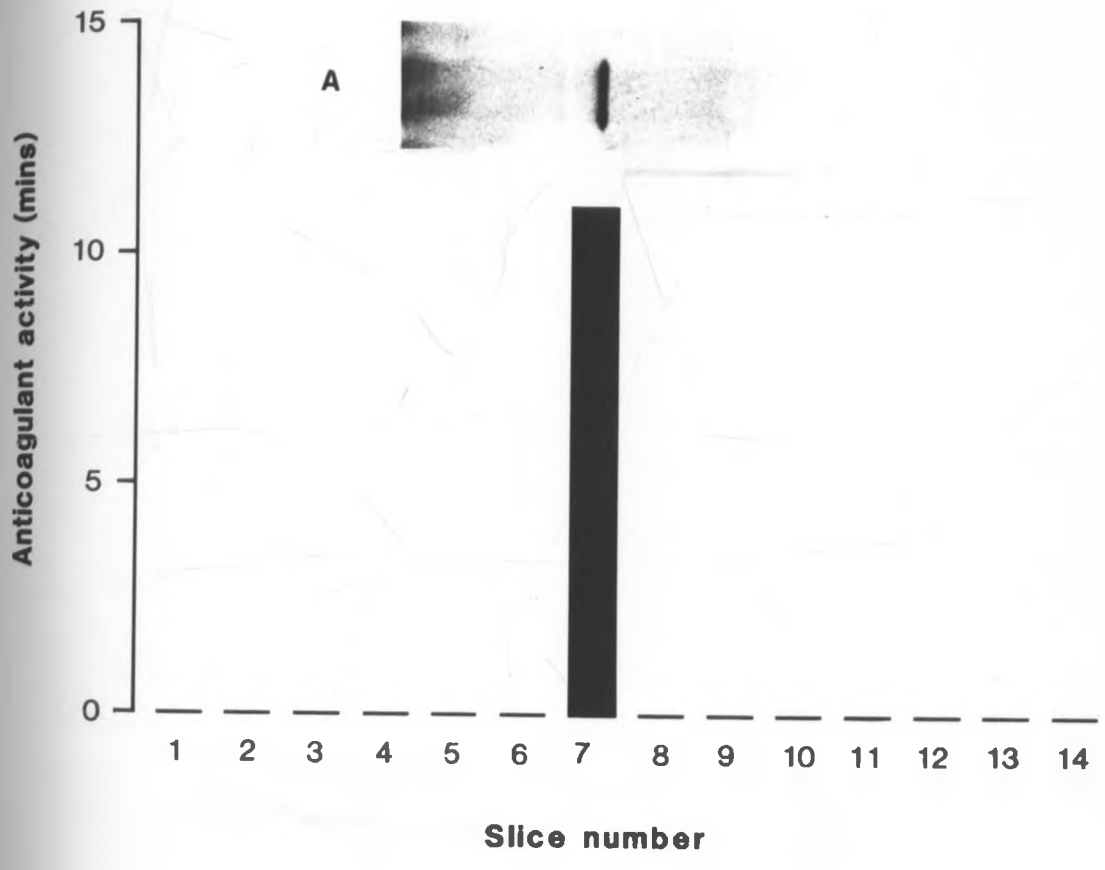

Fig.2.10 (A) Polyacrylamide gel (Native) electrophoresis of purified salivary anticoagulant (10 ug) which was silver stained.

A second identical gel was frozen, sliced in one cm sections and each section assayed for anticoagulant activity (results are shown above)

The PAGE of anticoagulant is shown in the insert.

significant loss of activity. As indicated in Fig. 2.10. the purified sample had its activity retained throughout electrophoresis and was detectable from the slice of the gel. The position of the anticoagulant activity in the sliced gel corresponded to that of the protein band in the stained gel. This suggests that the activity is associated with the protein band and not due to some trace substance in the preparation.

Chapter III

3. SOME PROPERTIES OF THE TICK SALIVARY ANTICOAGULANT MOLECULE.

3.1. Materials.

The protease inhibitors (DFP, PMSF, TLCK, TPCK, iodoacetamide, chloromercuriphenyl sulfonic acid), protamine sulfate, cephalin, toluidine blue, mixed glycosidases, heparin were purchased from Sigma. Pepsin and trypsin were obtained from Boehringer Mannheim. The gel filtration molecular weight standards, Sephadex G-100, Pharmacia PBE and polybuffer 9.6 were supplied by Pharmacia Fine Chemicals. Spectraphor 3 membrane tubing was from Spectrum Medical Industries and ultrafiltration membranes were from Amicon Scientific Systems division. All other chemicals were of the highest quality available.

3.2. Methods.

3.2.1 Determination of the effect of pH on the anticoagulant activity.

The anticoagulant activity was incubated in different buffers of varying pH for 15 min at 25^{o}C and the anticoagulant activity assayed as previously described. The buffers and solutions were 100ul each of 0.001 N HCl pH 3.0; 0.1M sodium phosphate at pH 6.0; 0.1M Tris-HCl at pH 8.0 and 0.001N NaOH at pH 11.0. After incubation 0.001N HCl was neutralized with an equal amount of 0.001 N NaOH and vice versa before assay. Parallel control experiments for each buffer system without the anticoagulant were carried out.

3.2.2 Determination of the effect of heat on the activity of the anticoagulant.

The purified anticoagulant molecule in buffer was aliquoted into several equal parts (about 5 ug) in glass tubes. Each aliquot was preincubated for 15 minutes in 50 mM Tris-HCl, 0.15M NaCl pH 8.0 exposed to the various temperatures (25, 35, 40, 45, 50, 60, 80, 89^{o}C) in a water bath.

After preincubation, the solutions were immediately cooled in ice and the remaining anticoagulant activity determined for each sample using the method already described (section 2.3.8.1). Triplicate determinations at each temperature were carried out. The anticoagulant activity was expressed as the percent activity, where 100% was the presence of anticoagulant activity in 50 mM Tris-HCl, 0.15 M NaCl pH 8.0 used as a control and assayed similarly at room temperature.

3.2.3 Measurement of the effect of different compounds on the anti-clotting activity.

The anticoagulant activity in 50mM Tris-HCl, 0.15M NaCl, pH 8.0 was aliquoted into several glass tubes and incubated at room temperature for 30 min with serine protease inhibitors (DFP, PMSF, TLCK, TPCK) each at final concentration of 5 mM, and sulfydryl reagents (iodoacetamide, chloromercuri-phenyl-sulfonic acid) each at a final concentration of 10mM; 5mM EDTA; Metal ions Mg^{++}, Zn^{++}, Hg^{++}, Mn^{++} each at 5mM final concentration; 5mM dibromoacetophenone, 5 mM DDT plus 5 mM iodoacetamide; After incubation, the mixtures were extensively dialyzed using spectraphor 3 membrane in 50 mM

Tris-HCl, 0.15 M NaCl, pH 8.0 to remove the above chemical reagents. The parallel control experiments were done without chemical treatment. The remaining anticoagulant activity was assayed as previously described.

Pepsin was dissolved in 1 mM HCl (pH 3.0) at a concentration of 1mg/ml. The anticoagulant solution was added to the enzyme solution to give enzyme to anticoagulant ratio of 10:1. The mixture was incubated at 25^{o}C for 15 min. After the incubation, the solution was mixed with 1/5 volume of 1M Tris-HCl (pH 8.0) to adjust the pH to about 7.5 and assayed. The anticoagulant was incubated without pepsin and post-treated in precisely the same manner in order to see whether the anticoagulant activity can resist the conditions of incubation. An aliquot of trypsin solution (100 ug) in 5 mM Tris-HCl (pH 8.0), was incubated with the anticoagulant activity in the same ratio 10:1 and passed through a Pasteur pipette small column of aprotinin-Sepharose equilibrated in 50 mM Tris-HCl pH 8.0. The trypsin was bound tightly to the column, but the anticoagulant was eluted with 0.2 M NaCl. A control experiment where the anticoagulant was applied to the column without prior incubation with trypsin was also carried out. Trypsin solution was heated in a boiling water bath for 10 min to be used as "heated trypsin". The 'heated trypsin' was incubated with the anticoagulant and assayed similarly. This control ascertains the absence of interference of products, generated during incubation.

To 10ug of purified anticoagulant in 100 ul of 10 mM sodium phosphate pH 6.0 was added 0.05U/ml of neuraminidase in

0.1 ml of 1mM NaCl, and the mixture was incubated at 37°C for 2 hr or 4 hr. After incubation, 0.1 ml of 0.1M Tris-HCl, pH 8.0, were added to the solution and assayed. 'Mixed glycosidases' in 0.1 ml 1mM NaCl were added to the anticoagulant. After incubation at 37°C for 2 or 4 hr, the solution was mixed with 0.1 ml of 0.1 M Tris-HCl pH 8.0 and assayed for anticoagulant activity.

To 10 ug of anticoagulant were added 10 ug of protamine sulfate. The mixture was assayed for anticoagulant activity. In a control experiment 1 mg of protamine sulphate neutralized about 1 mg (100 U) of heparin. Toluidine blue was added to the anticoagulant to observe for any metachromatic reaction. A control experiment with toluidine blue added to heparin was performed. The procoagulant effect of cephalin, which is mainly attributable to provision of phospholipids were tested. Cephalin of various dilutions (1, 1/2, 1/8, 1/256) of stock solutions was added to platelet poor plasma with or without the anticoagulant.

3.2.4 Measurement of isoelectric point of the molecule On Chromatofocusing.

All buffers used were degassed before use. The purified anticoagulant molecule (approximately 80 ug in diluent buffer) was dialyzed in 0.025 M ethanolamine - CH_3COOH buffer (pH 9.4) and applied to a Biorad column (0.7 x 20 cm) of Pharmacia PBE-94 equilibrated with that buffer. The column has reached equilibrium when the pH and conductivity of eluent match that of the starting buffer. Chromatofocusing was

Chromatofocusing was performed according to the manufacturers instructions. The experiment was performed in the 7.5 pH range.

The column was eluted with 1:10 dilution of Pharmacia Poly buffer 9.6 adjusted to pH 7.5. Elution was carried out at a flow rate of 22 mls/hr. The 2 mls fractions collected were assayed, at the running temperature ($4^{o}C$), for pH soon after collection, for protein at 280 nm and for anticoagulant activity.

3.2.5 Studies on molecular weight of the anticoagulant.

A column (2.6 x 90 cm) was packed with Sephadex G-100 and equilibrated in 50 mM Tris-HCl, 0.2 M NaCl pH 8.0 (buffer A). The Blue dextran (Mr 2 $x10^{3}$ kDa) was used to determine the void volume and the column was calibrated for molecular weights using standards obtained from Pharmacia: Bovine serum albumin (Mr 67kDa, 40mg); Ovalbumin (Mr 43 kDa, 39mg); Chymotrypsinogen (Mr 25kDa, 15.8 mg); and ribonuclease (Mr13 kDa, 35 mg). The standard proteins, dissolved in 5 ml buffer A plus 1.0 ml of 5 mg/ml Blue dextran, were passed through Sephadex G-100 under the same conditions described in section 2.4.6.2. The elution profile of TSGE anticoagulant (Fig.2.5) was used to estimate the apparent molecular weight. The elution profile of anticoagulant activity on HPLC (Fig.2.8) and it's mobility on SDS-PAGE (Fig.2.9) were also used to estimate the molecular weight of the anticoagulant.

3.3 Results and discussion.

3.3.1 Stability of the anticoagulant activity.

The stability of the anticoagulant is high over a wide pH range. Activity was retained when incubated with pH ranging from pH 3.0 - 10.0. Alkaline treatment at pH above 8.0 did not cause loss of activity. It is also relatively stable to acidification. It is, therefore, unlikely to be heparin or a heparin-like compound, because heparin hydrolyzes at low pH below 5.0 and in strong alkali solutions.

The anticoagulant activity is not thermostable. The different activities plotted against temperature are shown in Fig 3.1. Each point on the curve represents an average of three determinations. The anticoagulant withstands temperatures up to $50^{o}C$ and further heating diminished it's capacity to inhibit clotting. The anticoagulant in crude preparations is relatively stable when compared to the purified form. The purified anticoagulant is stable when stored frozen at $-80^{o}C$ in solutions for several weeks. However, freezing and thawing several times cause appreciable loss of activity.

3.3.2 Effect of different Compounds on the anti-clotting function.

A summary of the effect of different substances on the anticoagulant activity is given in Table 3.1. The anticoagulant activity is insensitive to DFP, PMSF, TLCK and TPCK each at a final concentration of 5 mM, implying that it does not function enzymatically by proteolytic inactivation of the coagulation factors. Activity was also retained with

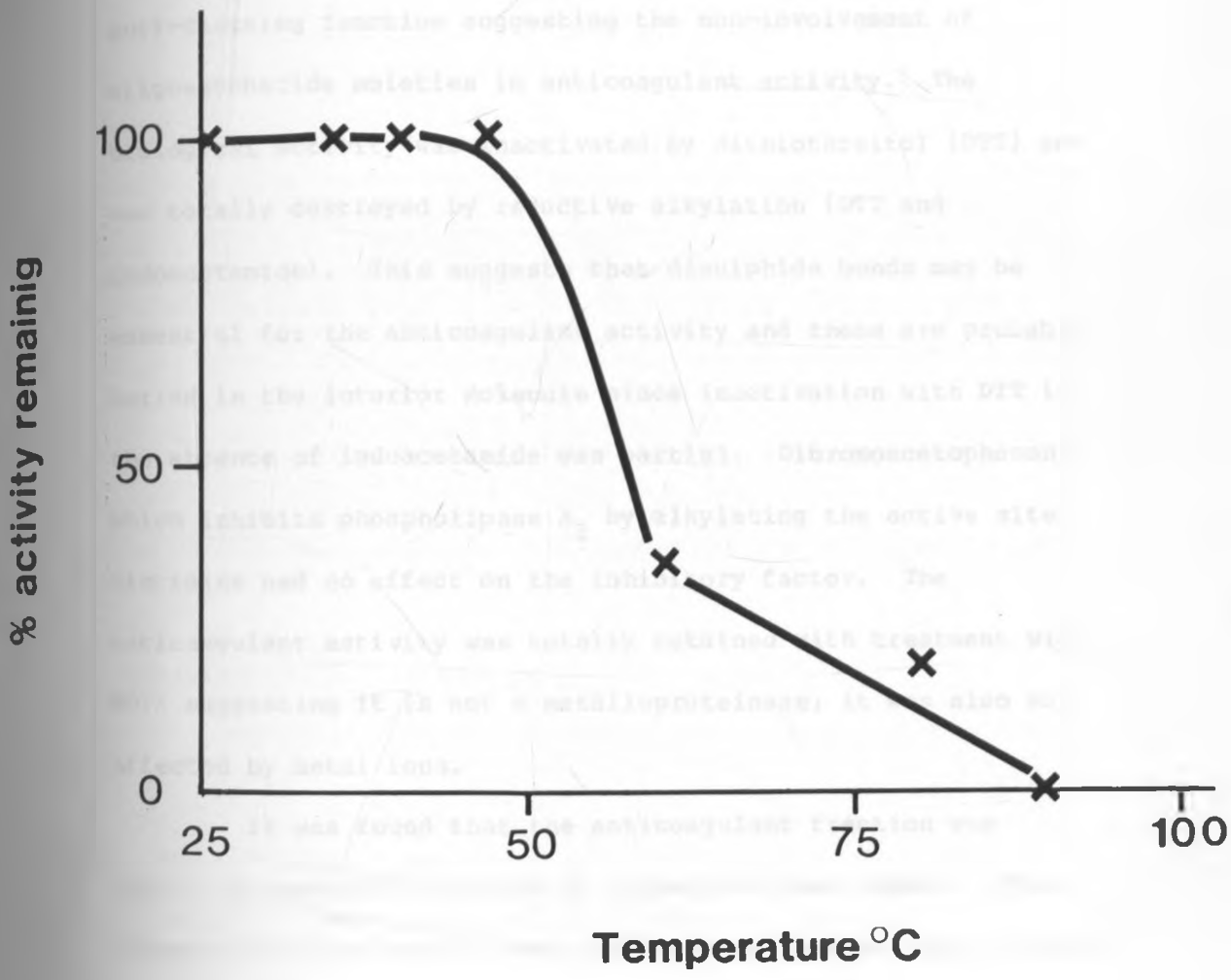

Fig. 3.1 Effect of heat treatment on anticoagulant activity. The anticoagulant was dissolved in 50 mM Tris-HCl, 0.15 M NaCl pH 7.4 at a concentration of 50ug/ml.

treatment with sulfhydryl reagents (Iodoacetamide and chloromercuri phenyl-sulfonic acid each at 10 mM final concentration) indicating that sulfhydryl groups are not necessary for anticoagulant activity. Treatment with neuraminidase and glycosidases had no effect on the anti-clotting function suggesting the non-involvement of oligosaccharide moieties in anticoagulant activity. The biological activity was inactivated by dithiothreitol (DTT) and was totally destroyed by reductive alkylation (DTT and iodoacetamide). This suggests that disulphide bonds may be essential for the anticoagulant activity and these are probably buried in the interior molecule since inactivation with DTT in the absence of iodoacetamide was partial. Dibromoacetophenone which inhibits phospholipase A_2 by alkylating the active site histidine had no effect on the inhibitory factor. The anticoagulant activity was totally retained with treatment with EDTA suggesting it is not a metalloproteinase; it was also not affected by metal ions.

It was found that the anticoagulant fraction was labile to proteolytic action by trypsin but not pepsin. This suggests that the active fraction possesses lysine and arginine residues that are acessible to trypsin. However, further exopeptidase treatment with carboxypeptidases B was not carried out to prove this point. Pepsin specifically catalyzes hydrolysis of peptide bonds without esterase and amidase activity.

Table 3.1 Efffect of inhibitors, enzymes and other substances on the anticoagulant activity.

Substance	Effect or Percent inhibition
Serine Protease Inhibitors (DFP, PMSF, TLCK, TPCK)	(-)
Sulfhydryl reagents (iodoacetamide chloromercuriphenyl-sulfonic acid)	(-)
DTT	68%
DTT + iodoacetamide	100%
Dibromoacetophenone	(-)
Trypsin	100%
Pepsin	(-)
Glycosidases, neuraminidase	(-)
EDTA	(-)
METAL IONS Mg^{++}, Zn^{++}, Hg^{++}, Mn^{++}	(-)
Protamine sulphate	(-)
Toluidine blue	No metachromatic reaction
Cephalin	(-)

The above considerations indicate that the tick salivary anticoagulant is of a protein nature. Balashov (8) suggested, but without proof, that tick anticoagulants were glycoproteins, mucoproteins or some protein-carbohydrate complex originating from the granular salivary cells. Chinery (2) suggested that sulphated mucopolysaccharides in the salivary gland of H. Spinigera ticks may act as anticoagulants and that the carbohydrate and protein precursors of the anticoagulants may be located in the salivary gland of A. persicus. The granules were positive for basic glycoprotein and were stained metachromatically with toluidine blue.

There was no colour change when toluidine blue was added to the salivary anticoagulant activity in 50mM Tris-Hcl, pH 8.0. In control experiment, a metachromatic reaction with toluidine blue was observed with heparin, with a colour change from blue to purple/violet. This suggests that the anticoagulant molecule is not a highly sulphated molecule. A number of other sulphated polysaccharides, grouped under the heading 'heparinoids' have been shown to have anticoagulant properties in vitro and in vivo (123). This characteristic of these compounds is perhaps due to high sulphate content (up to 15%), rather than their anionic carbohydrates. In addition, the anticoagulant effect of heparin was immediately neutralized with protamine sulphate (antidote), a strong basic substance which combines with and inactivates heparin. Protamine sulphate had no effect on the activity of the tick salivary anticoagulant as measured by the clotting assay.

The procoagulant action of cephalin was weak on the platelet rich plasma which was maintained incoagulable by the salivary anticoagulant. When suspensions of cephalin were added to platelet poor plasma, the anticoagulant action was not decreased. Since, the procoagulant effect of cephalin were mainly from provision of phospholipids, it can be concluded that the anticoagulant action is not due to inhibition of the action of phospholipids in the clotting system.

Because the purified anticoagulant molecule was available in limited quantities, the amino acid sequence and carbohydrate content were not determined. However, the molecule did not stain in the periodic acid-shiff reaction for glycoprotein nor bind to Concanavalin A-Sepharose. The above chemical studies, and the fact that other anticoagulants from lower organisms including hirudin (124), the anti-clotting factor of hook worms (125), are all peptides gave rationale for further characterisation of the tick anticoagulant with the assumption that it is a protein. Awaiting complete structural determination, the anticlotting principle of TSGE has been provisionally termed "Rephicephalin", a name derived from the species in conformity with nomenclature for enzyme inhibitors and anticoagulants isolated from other species.

3.3.3. Isoelectric point of the anticoagulant molecule:

The profile of the anticoagulant activity from the chromatofocusing column is shown in Fig. 3.2. The molecule has pI in the range 8.0 - 8.5. It is thus a basic protein. After chromatofocusing the activity was concentrated on PM10 Amicon cell, and polybuffer was removed from the molecule by gel filtration on HPLC as described in section 2.4.6.5. (Fig.3.3).

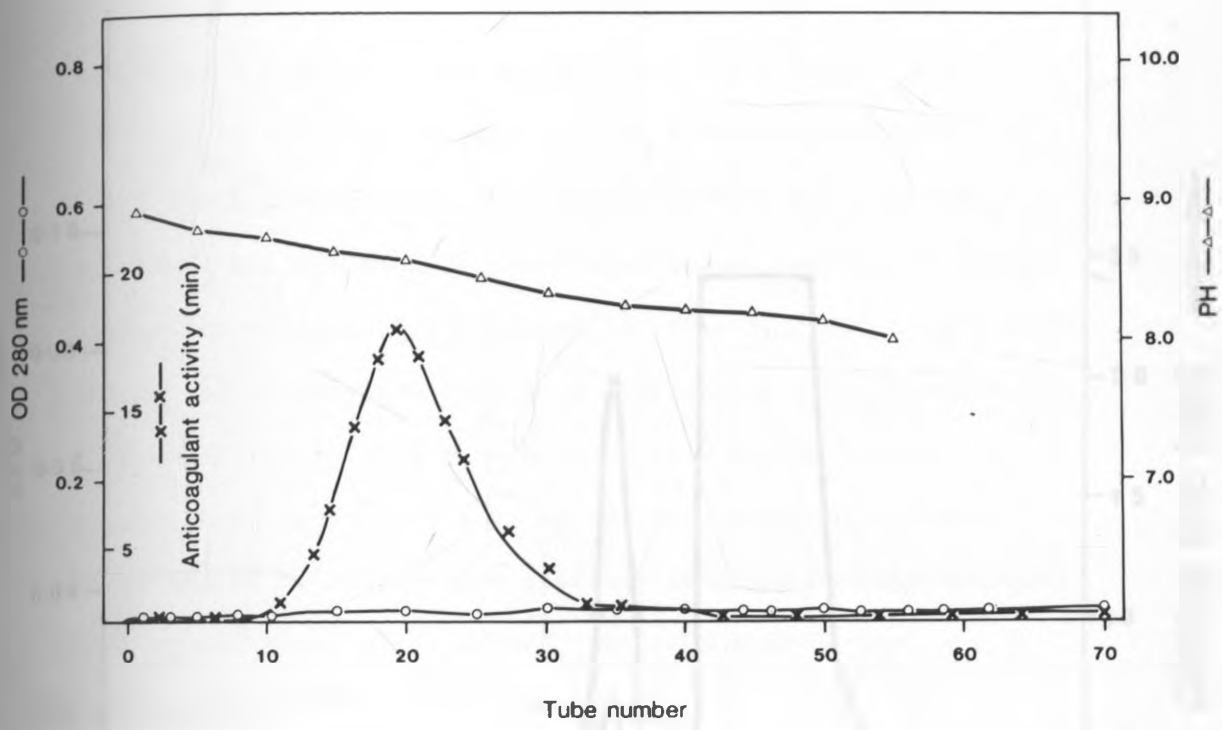

Fig. 3.2 Elution of the anticoagulant activity on chromatofocusing as described in section 3.2.4. Clotting System: 0.1 ml plasma + 0.1 ml anticoagulant + 0.1 ml 25 mM $CaCl_2$.

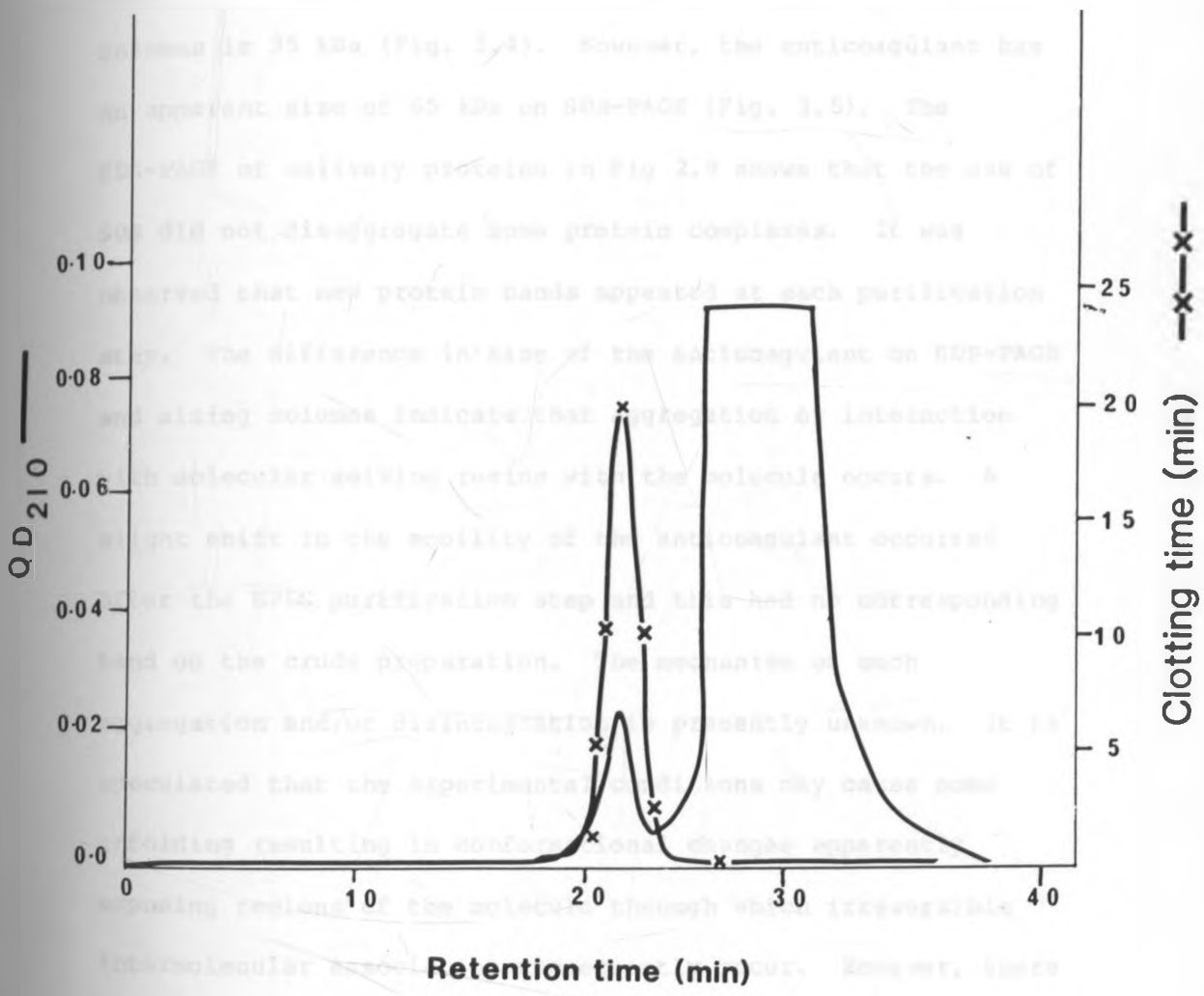

Fig 3.3 Concentrated fraction from chromatofocusing step applied to HPLC TSK-250: TSK-125 in tandem columns as described in section 2.4.6.5. Clotting System: 0.1 ml plasma + 0.1 anticoagulant in buffer + 0.1 ml 25 mM $CaCl_2$.

3.3.4 Molecular weight of the tick anticoagulant.

The molecular weight of the active fraction was determined by gel filtration on sizing columns and SDS-PAGE. A calibrated Sephadex G-100 gave a broad peak in the range of Mr of 30-70 kDa. The molecular weight obtained from HPLC sizing columns is 35 kDa (Fig. 3.4). However, the anticoagulant has an apparent size of 65 kDa on SDS-PAGE (Fig. 3.5). The SDS-PAGE of salivary proteins in Fig 2.9 shows that the use of SDS did not disaggregate some protein complexes. It was observed that new protein bands appeared at each purification step. The difference in size of the anticoagulant on SDS-PAGE and sizing columns indicate that aggregation or interaction with molecular seiving resins with the molecule occurs. A slight shift in the mobility of the anticoagulant occurred after the HPLC purification step and this had no corresponding band on the crude preparation. The mechanism of such aggregation and/or disintegration is presently unknown. It is speculated that the experimental conditions may cause some unfolding resulting in conformational changes apparently exposing regions of the molecule through which irreversible intermolecular association subsequently occur. However, there is no apparent loss of the anticoagulant activity caused by such macromolecular association. Willadsen and McKenna (126) purified proteinase inhibitors from the eggs of the cattle tick, B. microplus which exhibited polymerization behaviour and binding non-specifically to proteins, chromatographic and electrophoretic materials giving bands of variable size on SDS-gel electrophoresis. Similar phenomena are invoked

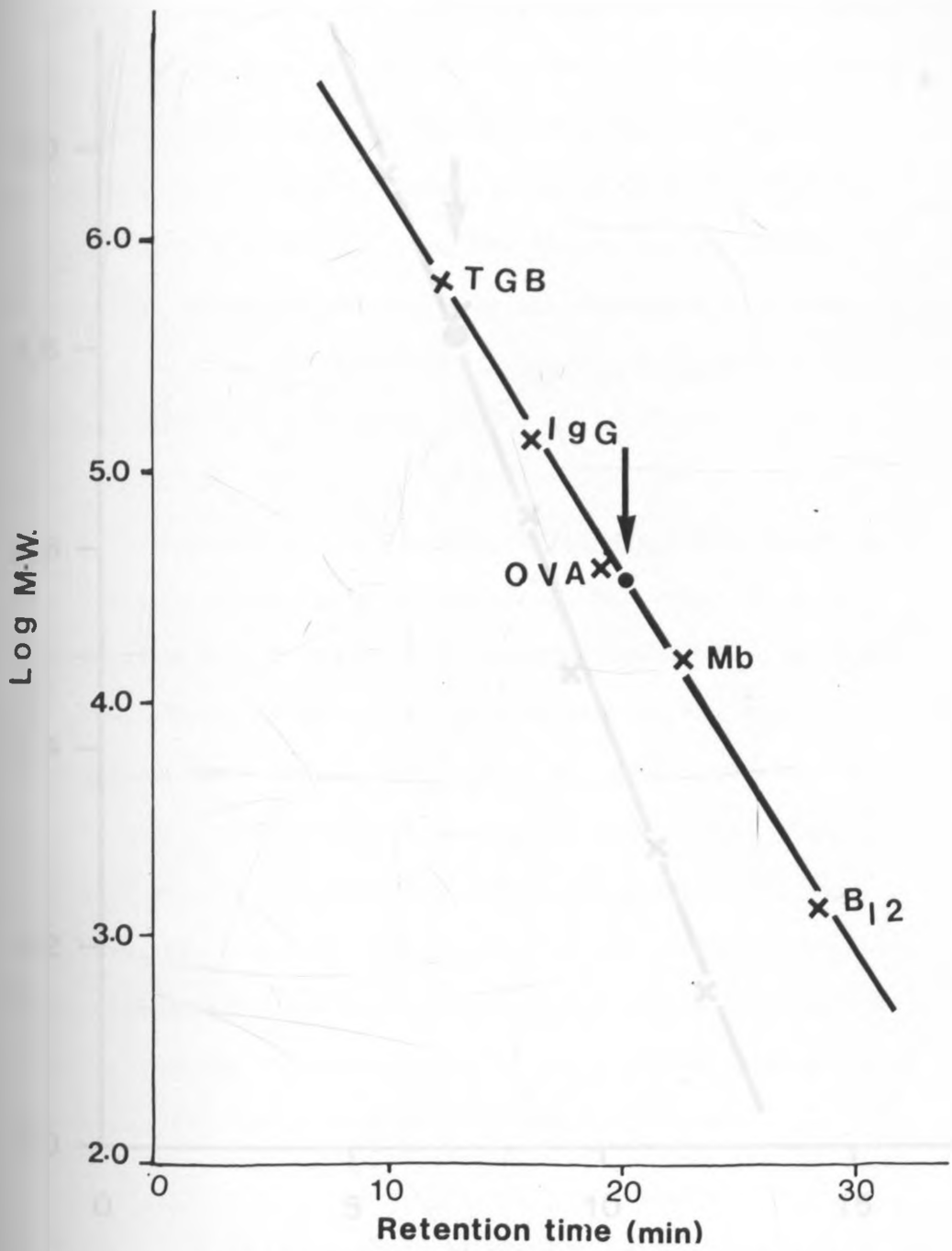

Fig 3.4 Molecular weight determination of coagulation inhibitor on HPLC (TSK-250: TSK-125) in tandem sizing columns. The retention times of the indicated marker proteins were determined as described in section 2.4.6.5.

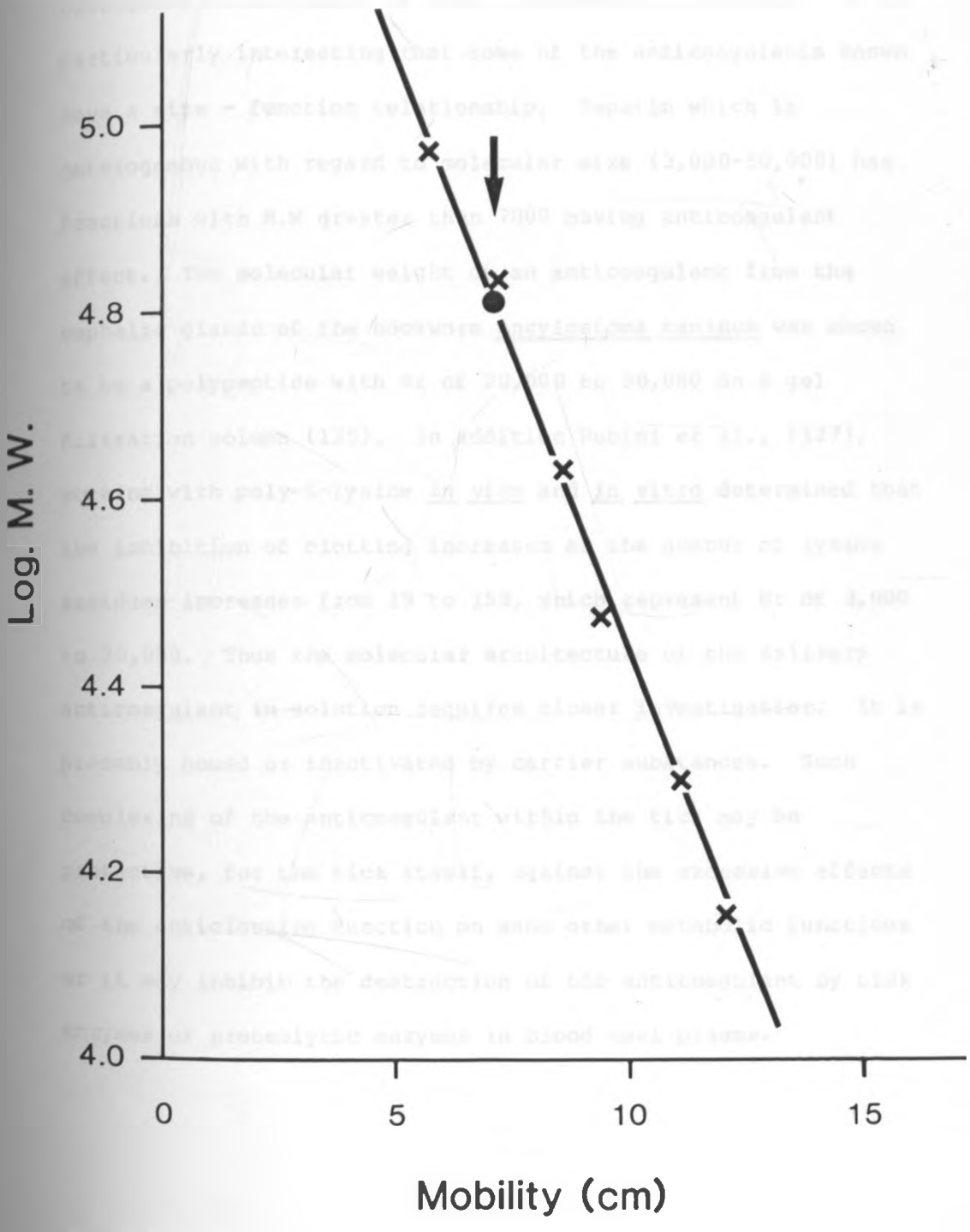

Fig 3.5 Molecular weight determination of coagulation inhibitor on SDS-PAGE. The gel mobilities of the reference protein standards from Pharmacia are shown.

to account for the anomalous behaviour of the tick salivary anticoagulant in sizing columns. It particularly elutes as a broad peak on Sephadex G-100 and DEAE-cellulose. The biological significance of such a phenomena is unclear. It is particularly interesting that some of the anticoagulants known have a size - function relationship. Heparin which is heterogenous with regard to molecular size (3,000-50,000) has fractions with M.W greater than 7000 having anticoagulant effect. The molecular weight of an anticoagulant from the cephalic glands of the hookworm Ancylostoma caninum was shown to be a polypeptide with Mr of 20,000 to 50,000 on a gel filtration column (125). In addition Rubini et al., (127), working with poly-L-lysine in vivo and in vitro determined that the inhibition of clotting increases as the number of lysine residues increases from 19 to 158, which represent Mr of 3,000 to 30,000. Thus the molecular architecture of the salivary anticoagulant in solution requires closer investigation. It is probably bound or inactivated by carrier substances. Such complexing of the anticoagulant within the tick may be protective, for the tick itself, against the excessive effects of the anticlotting function on some other metabolic functions or it may inhibit the destruction of the anticoagulant by tick enzymes or proteolytic enzymes in blood meal plasma.

Chapter VI

4. STUDIES ON THE MODE OF ACTION OF THE TICK ANTICOAGULANT.

4.1 Introduction.

Haematophagous organisms, such as ticks, tsetse flies, mosquitoes, leeches and bats contain anticoagulants in their saliva that prevent the host blood from clotting during feeding and after ingestion. These anticoagulant substances fall into two groups: One group consists of inactivators of clotting factors in the coagulation cascade. The most widely known are substances that inhibit thrombin catalyzed conversion of fibrinogen to fibrin (128). The other group consists of promoters of fibrinogenolysis (129), whose action directly or indirectly results in the cleavage of fibrinogen, rendering it unclottable. The mechanism of action of some anticoagulants in snake venoms (84) is direct fibrinogenolysis (130). The saliva of the vampire bat, Desmodus rotundus contains an anticoagulant that is an activator of plasminogen (131). The recent interest in the mechanism of action of anticoagulants stems from their use as specific tools for the investigation of blood coagulation. Snake venoms are widely used in this respect. Their potential use as therapeutic agents in thrombotic disorders or their use for therapeutic dissolution of blood clots has attracted some interest in these anticoagulant substances. R. appendiculatus ixodid ticks are blood-sucking ectoparasites which take a continuous blood meal over a period of several days. During this period the tick engorges with

whole blood and passes salivary secretions, excess water and some gut contents by regurgitation at intervals back into the host (47).

The long duration distinguishes the ixodid ticks from other blood sucking ectoparasites such as mosquitoes, tsetse flies and argasid ticks which take a rapid blood meal. To date the biochemical mechanism by which R. appendiculatus prevent coagulation while feeding remains unexplained. Previous studies have only shown that extracts of ticks contain an anticoagulant that prolongs the clotting time of whole blood or plasma (Chapter I, section 1.7.1.). The goal of the present investigation was to determine the specificity of purified TSGE anticoagulant activity which would account for the anti-clotting function observed. The mechanism of inhibitor action was evaluated using various assays including the alteration of bovine plasma clotting time. The identification of possible modes of the anticoagulant effect was carried out in the light of current concepts of mechanisms by which other known anticoagulants exert their effects.

4.2. Materials.

DEAE-Sephadex A-50 and Sephadex G-50 fine were obtained from Pharmacia Fine Chemicals. Barium chloride from B.D.H. was of a quality suitable for X-ray diagnosis. Benzamidine, factor X activating enzyme from Russel's viper venom, Taipan snake venom, factor V activating enzyme from Russels viper venom, fibrinogen, fibrin, plasminogen, streptokinase, hirudin, heparin, antithrombin III, thrombin,

prothrombin, tissue thromboplastin (extracted from rabbit brain acetone powder), purified bovine factor IX and Factor Xa, Factor V-, Factor VIII-, Factor VII- and Factor X and VII-deficient plasmas were all products purchased from Sigma (London) Chemical Co. Hydroxylapatite was a product from Bio-Rad. Bz-Ile-Glu- Gly-Arg-pNA was a donation from Kabi Vitrum Peptide Research, Stockholm, Sweden. L-Lysophatidyl choline 1-(1-^{14}C) palmitoyl, L-α- phosphatidyl choline B(1-^{14}C) palmitoyl-palmitoyl and ^{125}I-Labelled Bolton-Hunter reagent were from Amersham International PLC,. Chemicals for photography, Kodak-X-omat R film were purchased from Kodak.

4.3 Methods.

4.3.1 Preparation of vitamin K-dependent protein concentrate from bovine plasma.

Starting material: Fresh bovine blood was collected from the jugular vein in polyethylene bottles and mixed with one-tenth volume of 0.11M sodium citrate containing benzamidine - HCl (1g per liter). The blood was kept on ice and transported to the laboratory within 30 min and divided into smaller portions in plastic buckets and centrifuged at 4,000 rpm for 20 min to obtain the plasma. The plasma was either used at once or stored frozen at -20^{o}C. All subsequent procedures were performed in plastic containers at 4^{o}C unless otherwise stated.

Barium citrate absorption.

Barium citrate absorption was performed by a procedure similar to that of Moore et al (132). One-tenth volume of 1.0M $BaCl_2$ was added dropwise over 1 hr with gentle stirring to 7 liters plasma at 4^0C. The suspension was centrifuged at 4,000 rpm for 10 min and the supernatant discarded. The precipitate was resuspended in 0.1M NaCl containing 0.01M barium chloride, 1mM benzamidine -HCl and 0.02% NaN_3 and then again collected by centrifugation. The washing procedure was repeated twice.

Ammonium Sulfate elution.

The washed barium citrate precipitate was suspended in 500ml of 40% saturated ammonium sulphate solution. DFP was added to the solution to give a final concentration of 1mM in a fume hood in 20mM Tris-HCL using ammonium hydroxide to adjust the pH 7.0.

The suspension was stirred at 4^oC overnight. The supernatant was collected after centrifugation at 5,000 rpm for 30 min. This was fractionated by adding saturated ammonium sulphate solution to give 67% saturation. The precipitate was collected by centrifugation at 5000 rpm for 30 min.

The partially purified mixture was eluted from the precipitate by dissolving in 50-80 ml of 0.05M sodium phosphate buffer, pH 6.0, containing 0.2M NaCl, 1mM DFP and 1mM benzamidine-HCl, and dialyzed overnight against 10 litres of the same buffer. The insoluble material was removed by centrifugation at 2000 rpm and the clear supernatant used as the source of factor II and factor X.

4.3.2 Isolation of prothrombin and bovine factor X on DEAE - Sephadex A-50 chromatography.

Before use, DEAE-Sephadex A-50 was swollen in 1M NaCl. The batch-wise washings and equilibrations were performed on a sintered-glass funnel by stirring the DEAE-Sephadex for 10 mins followed by filtration using a filter pump. The equilibrated DEAE - Sephadex A-50 was then deaerated at the water pump for about 10 min. The vitamin K-depedent protein concentrate obtained above was applied to a column (3.5 x 22cm) of DEAE-Sephadex A-50 equilibrated with 0.05M sodium phosphate buffer, pH 6.0, containing 0.2M NaCl and 1mM benzamidine - HCl. After washing the column with 1.5 litres of the equilibration buffer, linear salt gradient elution was performed with 1 litre each of the equilibration buffer and the same buffer containing 0.6M NaCl. The flow rate was 90ml/hr and 14ml fractions were collected. The elution profile is shown in Fig. 4.1. These fractions were pooled as indicated by solid bars and sample subjected to SDS-PAGE for assesment of purity. The overall yield of factor X was about 35mg and prothrombin 700 mg from 7 liters of bovine plasma.

4.3.3 Preparation of factor Xa.

A solution of factor X (100 ml) containing 1 M $CaCl_2$ was incubated at 37^0C with the coagulant fraction from Russell's viper-venom (50 ul of a solution containing 0.5 mg Protein/ml) until the reaction was completed. The increase in factor Xa was monitored spectrophotometrically at 405 nm, by observing the increase in amidase activity towards S-2222.

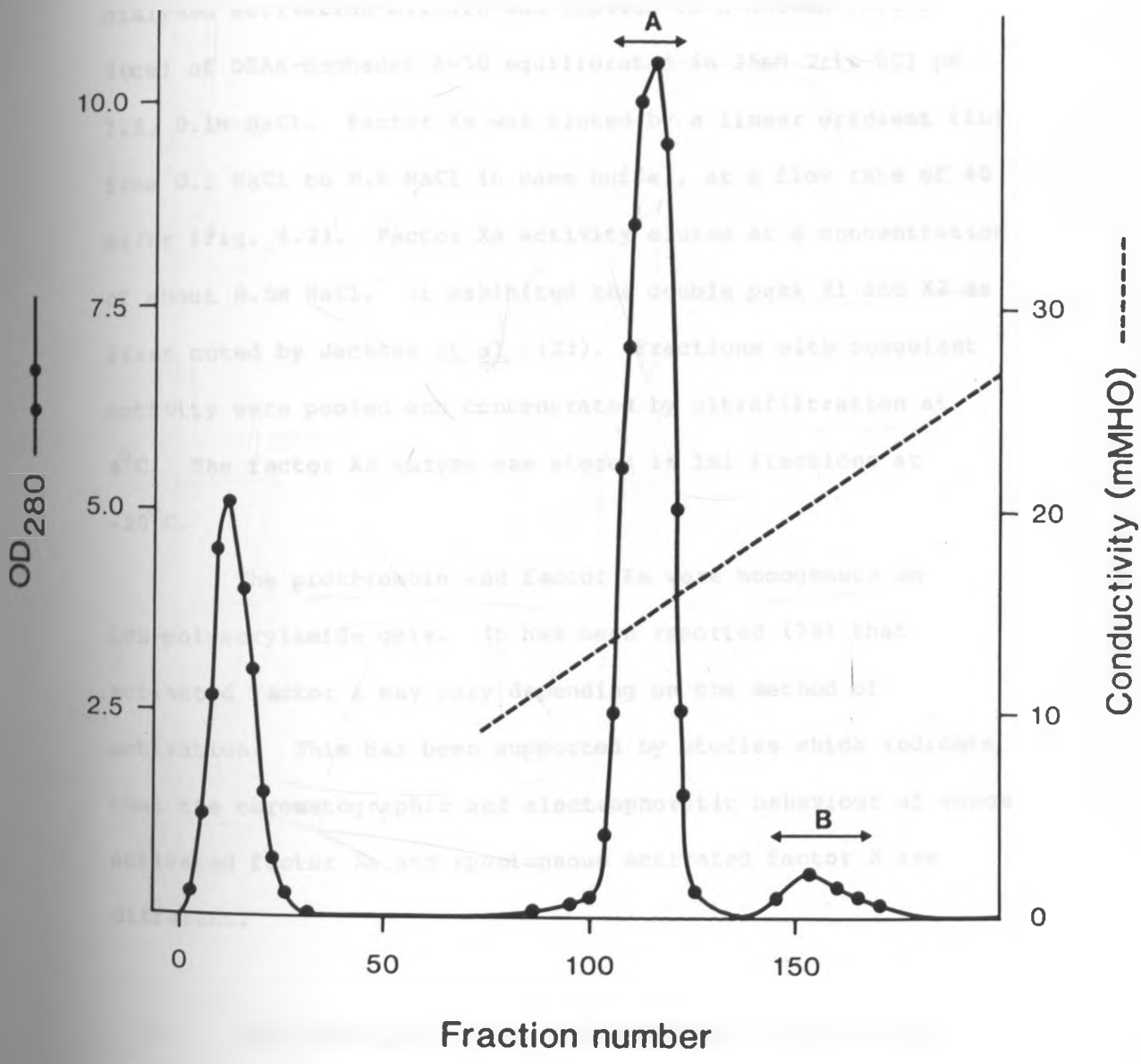

Fig. 4.1: DEAE-Sephadex A-50 column (3.5 x 22cm) chromatography of vitamin K-dependent protein concentrate. Details of the procedure are given in the text. Fraction A contained the major portion of prothrombin and fraction B contained factor X.

Activation time was about 30 min. When no further increase was observed in the amidase activity, the reaction mixture was dialysed against 15 mM Tris-HCl, 0.1M NaCl pH 7.5 (41) for 6 hr at $4^{o}C$. EDTA was added to give a final concentration of 0.1mM and the dialysis was continued for a further 12 hr. The dialysed activation mixture was applied to a column (2.2 x 20cm) of DEAE-Sephadex A-50 equilibrated in 15mM Tris-HCl pH 7.5, 0.1M NaCl. Factor Xa was eluted by a linear gradient (IL) from 0.1 NaCl to 0.6 NaCl in same buffer, at a flow rate of 40 ml/hr (Fig. 4.2). Factor Xa activity eluted at a concentration of about 0.5M NaCl. It exhibited the double peak X1 and X2 as first noted by Jackson et al (133). Fractions with coagulant activity were pooled and concentrated by ultrafiltration at $4^{o}C$. The factor Xa enzyme was stored in 1ml fractions at $-20^{o}C$.

The prothrombin and factor Xa were homogenous on SDS-polyacrylamide gels. It has been reported (78) that activated factor X may vary depending on the method of activation. This has been supported by studies which indicate that the chromatographic and electrophoretic behaviour of venom activated factor Xa and spontaneous activated factor X are different.

4.3.4 Activation of factor IX with Russel's viper venom coupled to Sepharose 4B.

20 mg of a protease in Russel's viper venom in 5ml, 0.1M $NaHCO_3$ pH 8.0, containing 0.5M NaCl was coupled to activated CH-Sepharose 4B according to the precedure previously

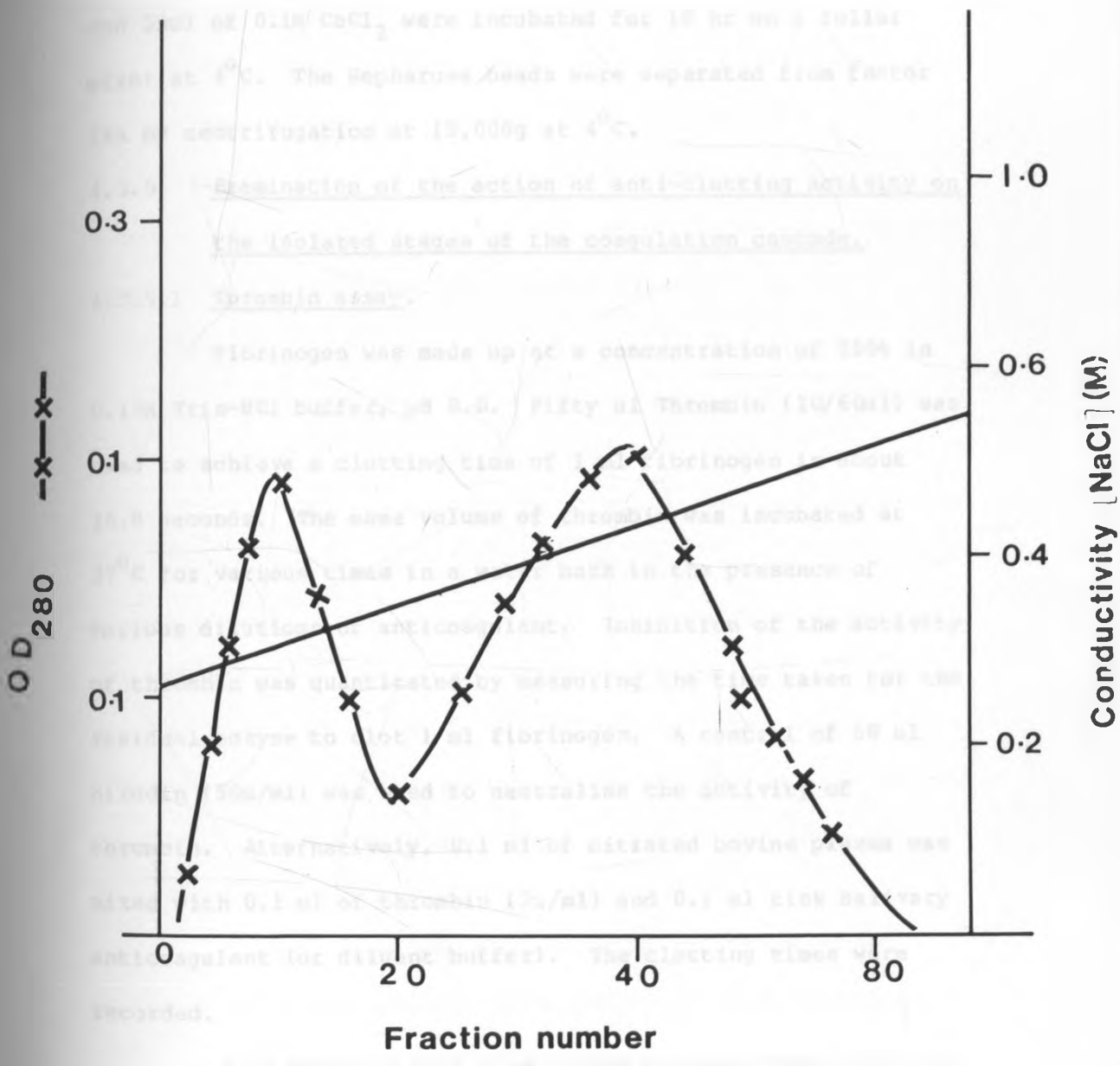

Fig. 4.2 Chromatography of factor Xa on a column of DEAE-Sephadex A-50.

described in section 2.3.7, except that the mixture was stirred for 20hr at 4°C. Bovine blood coagulation factor IX was activated with Russel's viper venom Sepharose beads. Five hundred ul of factor IX (at 0.5mg/ml, 500ul of RVV-Sepharose and 50ul of 0.1M $CaCl_2$ were incubated for 18 hr on a roller mixer at 4°C. The Sepharose beads were separated from factor IXa by centrifugation at 15,000g at 4°C.

4.3.5. Examination of the action of anti-clotting activity on the isolated stages of the coagulation cascade.

4.3.5.1 Thrombin assay.

Fibrinogen was made up at a concentration of 250% in 0.15M Tris-HCl buffer, pH 8.0. Fifty ul Thrombin (1U/60ul) was used to achieve a clotting time of 1 ml fibrinogen in about 15.0 seconds. The same volume of thrombin was incubated at 37°C for various times in a water bath in the presence of various dilutions of anticoagulant. Inhibition of the activity of thrombin was quantitated by measuring the time taken for the residual enzyme to clot 1 ml fibrinogen. A control of 50 ul hirudin (50u/ml) was used to neutralise the activity of thrombin. Alternatively, 0.1 ml of citrated bovine plasma was mixed with 0.1 ml of thrombin (2u/ml) and 0.1 ml tick salivary anticoagulant (or diluent buffer). The clotting times were recorded.

4 ug thrombin (541 u/mg enzyme protein) was incubated with anticoagulant (or buffer control) and its ability to hydrolyze Tos-Arg-Ome or chromozym TH was assayed in a Gilford model 260 spectrophotometer. The reaction was carried out in 50 mM Tris-HCl, 0.2M NaCl, pH 8.0.

4.3.5.2 Fibrinogenolytic, fibrinolytic and Plasminogen activator assays.

For fibrinogenase test, 0.1 ml of 2.5 mg/ml thrombin -clottable fibrinogen in 0.05M Tris-HCl, 0.01M NaCl, pH 7.4 in a series of tubes was incubated with an equal volume of the tick anticoagulant (or buffer when anticoagulant was ommitted) for various periods ranging from 0.5 to 2 hr at 37°C. After the specified time, 0.1 ml of bovine thrombin (100u/ml) was added to the tube and the clotting time determined as a measure of clottable fibrinogen. The control clotting time was approximately 10 sec.

Agar-gel-plate containing fibrin was made as described by Gebrand et al (134). Thrombin (40NIH Units) was added to 20 ml of 0.5% (w/v) agar and 0.1% (w/v) fibrinogen in 0.05M sodium barbital-HCl buffer, pH 8.0, containing 0.1M NaCl. A pattern of wells was cut into each gel made in the plates. Gels with and without the anticoagulant molecule were incubated in humid chamber for 20 hrs at 37°C. A fibrinolysin would produce a colourless zone of lysis against a blue background with a given diameter. The plates were stained with coomassie brilliant blue R-250 and destained with 10% acetic acid.

Amidolytic activity of activated plasminogen was measured (OD 405nm) using a chromogenic substrate, chromozym PL at a final concentration of 0.6 mM in 50 mM Tris-HCl buffer containng 0.01M NaCl, pH 7.4 at 37°C. The reaction mixture contained 4 ug/ml bovine plasminogen, with salivary anticoagulant (7 ug) or streptokinase (1000 U/ml) as a control.

4.3.5.3 Factor Xa and Factor IX clotting assays.

A 0.1 ml of tick salivary anticoagulant appropriately diluted in buffer (50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl in 1mg/ml BSA) was mixed with purified factor Xa. The mixture was incubated for 5 min at 37°C. 0.1 ml of prewarmed factor X deficient plasma was added followed immediately with 0.1 ml of 25 mM $CaCl_2$. The clotting times were determined.

The inhibition of factor IXa coagulant activity was performed by incubating factor IXa (0.017U) that was obtained by activating purified factor IX with Russel's viper venom with 0.1 ml salivary anticoagulant at 37°C for 3 mins. 0.1 ml citrated bovine plasma and 0.1 ml 25 mM $CaCl_2$ was added subsequently before the clotting time was recorded. The purified factor IX and the test system without tick anticoaguant were used as controls.

4.3.5.4 Spectrophotometric assays of anticoagulant against factor Xa in the presence of either heparin and/or antithrombin III.

Principle of assay:

Bz-Ile-Glu-Gly-Arg-pNA $\xrightarrow{\text{factor Xa}}$ Bz-Ile-Glu-Gly-Arg-OH + pNA.

The method for the determination of factor Xa activity was based on the difference in the optical density between pNA of the sensitive chromogenic substrate S-2222 formed and the original substrate. The rate of pNA formation i.e the increase in obsorbance per second at 405 nm is proportional to the factor Xa enzymatic activity. The coagulation inhibitor (3.5

ug/ml) was preincubated with 50 ul factor Xa (0.4 units/ml) for 3 min at 37°C, prior to admixture with 100 ul of S-2222 (100 ug) in a total volume of 2.5 ml of 10mM Tris-HCl pH 8.0, 0.15M NaCl. The time dependent hydrolysis of the substrate was determined in the presence of the tick salivary anticoagulant alone and in the presence of bovine antithrombin III. A control experiment was performed in which 100 ul factor Xa (15ug/ml) was mixed with 100ul antithrombin III (0.2 u/ml) and 100 ul heparin (1.5 u/ml) before assay.

4.3.5.5 Conversion of factor X to factor Xa and prothrombin to thrombin by snake venoms.

Purified factor X activating enzyme from Russel's viper venom was used to convert factor X to factor Xa *in vitro*. To 0.1 ml (0.06U) of Russel's viper venom protease in diluent buffer was incubated with citrated bovine plasma at 37°C for 30 min, in the presence of the purified anticoagulant molecule (10ug/ml). This was followed by the addition of 0.1 ml Ca^{++} (25mM) solution. The plasma clot time was noted and compared to control assay without tick salivary anticoagulant. The clot times in this assay are proportional to the concentration of factor Xa present in the incubation mixutre.

It was also determined if the ability of Taipan snake venom to convert prothrombin to thrombin is affected by tick salivary anticoagulant. The assay mixture contains 0.1 ml of 0.6% fibrinogen, 0.1ml Taipan snake venom solution (diluted

1:1500 with 0.15M Tris-HCl buffer pH 7.4) and 0.1 ml of prothrombin solution containing 1mg/ml in the same buffer. The first two components, tick salivary anticoagulant and Taipan snake venom were mixed at 37°C and prothrombin solution added 3 minutes later.

Factor V activating enzyme from Russel's viper venom was incubated with the salivary anticoagulant (or buffer control) in citrated bovine plasma for 5 min at 37°C, followed by addition of calcium ions (25mM Ca^{++}).

4.3.5.6 Measurement of inhibition of tissue thromboplastin activation of the extrinsic coagulation pathway.

Tissue thromboplastin (extracted from rabbit brain acetone powder) was reconstituted according to the Manufacturers instructions. The contents of the vial were reconstituted by injecting 2.0 ml of 37°C deionized water. One hundred ul (final concentration indicated) of tick salivary anticoagulant) was incubated with 0.2 ml tissue thromboplastin-Ca^{++} mixture for varying periods at 37°C before 0.1ml citrated bovine platelet poor plasma was introduced. The prothrombin time of the plasma was also recorded when 0.1ml platelet poor plasma, 0.1 ml tissue thromboplastin, anticoagulant were added subsequently, incubated at 37°C for 3 min and 0.1 ml Ca^{++} ions (25mM) added last.

4.3.6 Measurement of the effect of tick anticoagulant on factor V-, factor VIII-, and factor-VII deficient plasmas.

In these assays, 0.1 ml of diluted anticoagulant was incubated with 0.1ml of each of the deficient plasma for 3 min at 37^{o}C in plastic tubes. Following incubation, the anticoagulant was assayed for it's ability to correct the clotting time of human factor V-, human factor VIII-, and bovine factor VII-deficient plasma following the addition of 0.1ml calcium ions. Duplicate determinations were performed for each sample.

4.3.7 Determination of phospholipases and protease activities on the purified anticoagulant principle.

Phospholipase activity was determined by the method described by Nashijima et al (135) using substrates radio-labelled in the fatty acid moiety. Phospholipases are distinguished by their position specificity on the substrate, generating free fatty acids during their enzymatic activity. Phospholipases A_2 and B were tested. Radiolabelled substrates (L- -Phosphatidyl choline, B-(1^{-14}C) palmitoyl-palmitoyl for phospholipase A assay and L- -phosphatidyl choline B-(1-^{14}C) palmitoyl for phospholipase B assay each in toluene were evaporated to dryness in a gentle stream of dry nitrogen gas. Fifteen nano moles of substrate used for a single assay, was resuspended in 500 ul of 10mM Tris-HCl buffer pH 8.0, 0.4% Triton X-100 and mixed thoroughly by vortexing. About 10 ug

tick salivary anticoagulant or 100 ug of TSGE in Tris-HCl buffer (500ul) was warmed in a water bath at 37°C for 3 minutes and added to the above substrate mixture prewarmed at 37°C for 3 minutes in 10 ml plastic tubes; a stopwatch was started upon the addition of the substrate mixture to the sample. The contents were thoroughly mixed and allowed to incubate at 37°C for up to 20 minutes. The reaction was stopped by adding 500 ul of 20% trichloroacetic acid and 500 ul of 16% Triton X-100 with thorough mixing. This was followed by additin of 5 ml of n-hexane. The contents were further mixed by inversion for 15 sec. at room temperature. Blank controls using 500 ul of Tris-HCl buffer without salivary anticoagulant or purified phospholipase A_2 were indentically processed. The tubes were centrifuged at 600g for 15 minutes and the 200 ul of the upper n-hexane layer withdrawn, placed in scintillation vials containing 5 ml Aquasol for ß-emission counting using Tri-carb 2660 Scintillation counter.

The purified anticoagulant molecule was tested for its activity towards several synthetic substrate (BAEE, BTEE, chromozym PL, and Chromozym TH) as described in section 2.3.8.2.

4.3.8 Iodination of factor Xa with Bolton - Hunter reagent.

Bovine factor Xa was iodinated with ^{125}I-labelled Bolton-Hunter reagent (N-succinimidyl 3-(4-hydroxy, 5-[^{125}I] iodophenyl propionate)) following the instructions provided by the manufacturer. Iodination occurs predominatly at lysine groups of the protein at pH 8.5. Prior to use for iodination, the benzene solvent in the vial was removed at room temperature

from the preparation by directing a gentle stream of dry nitrogen onto the surface. Two syringe needles were inserted through the seal of the vial, one of which can be used as the inlet for the dry nitrogen. The evaporation was carried out inside a well ventilated fume hood. The protein was labelled by conjugation with the hydroxy-succinimide ester of 3-(4-hydroxy phenyl) propionic acid. Protein (5 ug) in 10 ul of 0.1M borate buffer pH 8.5 was added to the dried iodinated ester and the reaction mixture agitated for 15 min at $4^{o}C$. The labelled ester became hydrolyzed in aqueous media. The I^{125}-labelled factor Xa was separated from the other labelled products of the conjugation reaction, namely,glycine - conjugate and 3-(4-hydroxy phenyl) propionic acid by gel filtration on Sephadex G-50 fine.

4.3.9 Binding Studies of anticoagulant molecule and factor Xa as an approach to provide evidence that factor Xa is the target of inhibitory activity by the tick anticoagulant.

The ^{125}I-labelled or unlabelled factor Xa was preincubated with the coagulation inhibitor at $37^{o}C$ for 15 min before electrophoresis under denaturing and non-denaturing conditions as described in section 4.3.6. One portion of the gel was dried and autoradiographed and the other silver stained to reveal complexes if any. In another experiment, the incubation mixtures of factor Xa and tick anticoagulant factor Xa, and anticoagulant were each run separately on a gel filtration column on HPLC. Both the coagulant activity of factor Xa and the anticoagulant effect of the salivary anticoagulant in the collected fractions were determined.

4.4 Results and discussion.

4.4.1 Effect of anticoagulant activity on thrombin clotting times, fibrin(ogen), synthetic substrates and factor V-, factor VIII-, factor VII- deficient plasmas.

The thrombin clotting time of bovine citrated plasma and bovine fibrinogen was not prolonged by the presence of tick salivary anticoagulant in the clotting assay. When the anticoagulant was incubated with thrombin or fibrinogen and portions assayed periodically, there was no progressive neutralisation of thrombin activity nor any progressive fibrinogen lysis. Thus the effect of tick salivary anticoagulant cannot be explained by a direct action on fibrinogen into unclottable derivatives. The anticoagulant did not induce fibrinolysis in fibrin gels nor activate plasminogen to produce plasmin that lysed fibrin.

Further evidence for the absence of a direct antithrombin from salivary glands of R. appendiculatus was obtained from the esterase and amidolytic activity of bovine thrombin. The action of thrombin on Tos-Arg-Ome and Chromozym TH was not inhibited by the tick anticoagulant. In contrast to the Crude TSGE, the purified tick salivary anticoagulant did not possess Tos-Arg-Ome esterase or amidase activities of the crude TSGE. The anticoagulant was also devoid of phospholiase A_2 and B activities. Phospholipase activity through phospholipid destruction is the main causative agent responsible for the anticoagulant action of some snake venoms (90). Phospholipase A_2 binds phospholipids and has a high affinity for the Ca^{2+}-

binding site of phospholipids. The phospholipids serve to provide a catalytic surface on which the coagulation factors are assembled. Thus the salivary anticoagulant is likely not to be inhibiting coagulation by competing with clotting factors for the lipid surface. Crude TSGE also did not possess phospholipase A_2 and B activities. The reported decrease in blood phospholipids produced by tick infestation in cattle (136) could be caused by other factors other than tick phospholipases.

The tick salivary anticoagulant prolonged the clotting time of human factor V-, human factor VIII-, and bovine factor VII-deficient plasmas as shown below.

Table 4.1 Effect of the tick anticoagulant on factor V-, factor VII- and factor VIII-deficient plasmas.

Coagulation factor Deficient Plasma	clotting time (min)
Saline Buffer	10
Factor VII + TAC	>60
Factor V (aged human plasma)	31
Factor V + TAC	>60
Factor VIII	12
Factor VIII + TAC	>60

Clotting reaction mixture contained: 0.1 ml deficient bovine plasma, 0.1 ml tick anticoagulant in diluent buffer incubated for 30 sec at 37°C and 0.1 ml 25mM $CaCl_2$ solution added. TAC is tick salivary anticoagulant.

The tick anticoagulant is not species specific. It maintains human plasma anticoagulated and this observation indicates that salivary anticoagulant evolved with a broad specificity to facilitate the feeding on the blood of a variety of animals. Factor Va is an important cofactor for prothrombin activation and factor VIIIa is an important cofactor for factor X activation. The above results show that the anticoagulant exerts no specific inactivation of factor Va and factor VIIIa. Thus it does not function as activated bovine plasma protein C. Protein C is a vitamin K dependent plasma glycoprotein with similar physicochemical characteristics found in the four other vitamin K-blood coagulation factors. Protein Ca acts as an inhibitor of normal plasma. The action of protein Ca centres around its inactivation of purified preparations of blood clotting factor Va and factor VIIIa by limited proteolysis (88, 89). The possibility that the anticoagulant site of action is on factor V and VII cannot explain the inhibition of a system without factor V and VIII. The tick salivary anticoagulant maintains factor VII deficient plasma anticoagulated. This indicates that it may not be anti-factor VIIa or antithromboplastin.

4.4.2 Inhibition of tissue thromboplastin activation of the extrinsic coagulation pathway.

It was confirmed in section 4.3.5.2 that the tick anticoagulant had no effect on fibrin polymerization through the action of thrombin on fibrinogen. For this reason, the

remaining studies focused on the first phase of clotting, the activation of prothrombin to thrombin. From the results shown in Fig 4.3 it is concluded that the tick anticoagulant inhibits the tissue thromboplastin-factor VII mediated activation of the coagulation cascade. Inhibition of this reaction is, however, weak to account for the total strong anticoagulant by calcium-induced clotting of plasma. This may be because tissue thromboplastin is rich in potent procoagulant phospholipids. In these experiments high levels of brain thromboplastin were able to decrease the clotting time. There was no difference in prothrombin time occurring relative to the time during which salivary anticoagulant was incubated with thromboplastin at $37^{o}C$. The clotting mechanisms encountered by the tick *in vivo*, is that involving tissue damage at the attachment site. It is, therefore, important that the tick secretes an anticoagulant that inhibits extrinsic pathway to enable it to feed successfully as demonstrated by the extension of prothrombin time.

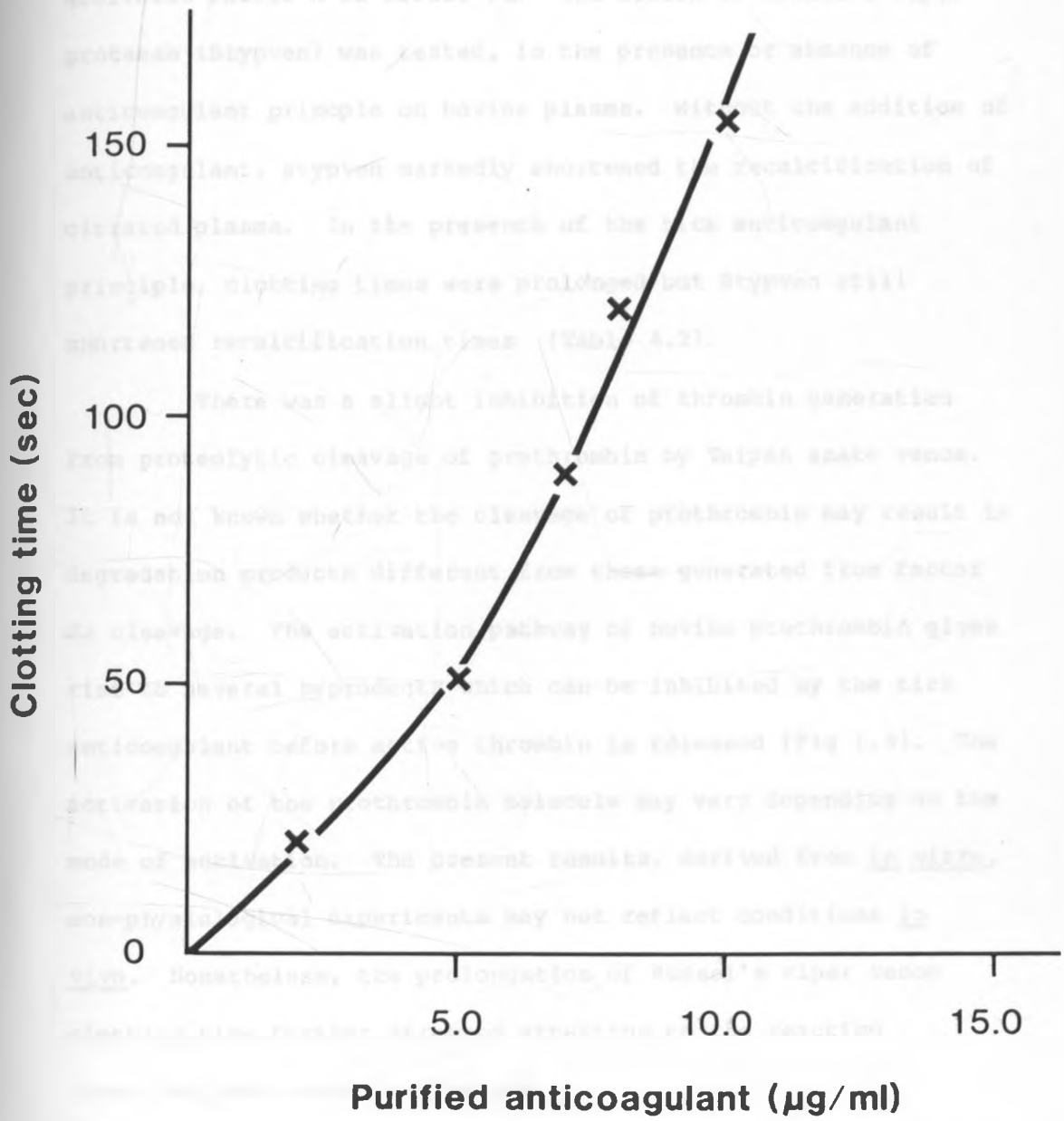

Fig 4.3 Effect of tick anticoagulant on one stage prothrombin time. Clotting system: 0.1 ml platelet poor plasma + 0.1 ml rabbit brain thromboplastin (1/64 dilution) + 0.1 ml anticoagulant principle or saline buffer + 0.1 ml Ca^{2+} (25 mM). The mean of three determinations is presented.

4.4.3. Effect of tick salivary anticoagulant on Stypven time and thrombin generation from proteolytic cleavage by Taipan snake venom.

The protease in Russel's viper venom specifically activates factor X to factor Xa. The action of Russel's viper protease (Stypven) was tested, in the presence or absence of anticoagulant princple on bovine plasma. Without the addition of anticoagulant, stypven markedly shortened the recalcification of citrated plasma. In the presence of the tick anticoagulant principle, clotting times were prolonged but Stypven still shortened recalcification times (Table 4.2).

There was a slight inhibition of thrombin generation from proteolytic cleavage of prothrombin by Taipan snake venom. It is not known whether the cleavage of prothrombin may result in degradation products different from those generated from factor Xa cleavage. The activation pathway of bovine prothrombin gives rise to several byproducts which can be inhibited by the tick anticoagulant before active thrombin is released (Fig 1.4). The activation of the prothrombin molecule may vary depending on the mode of activation. The present results, derived from *in vitro*, non-physiological experiments may not reflect conditions *in vivo*. Nonetheless, the prolongation of Russel's viper venom clotting time further directed attention to the reaction involving prothrombin activation.

When the tick salivary anticoagulant was incubated with increasing concentration of calcium ions upto 250 mM final concentration followed by equilibrium dialysis in 50mM Tris-HCl, pH 7.4, 0.15 NaCl, the anticlotting activity was retained when

Table 4.2 Effect of tick salivary anticoagulant on Russel's viper and Taipan snake venoms clotting times.

Anticoagulant	10ug/ml final concentration.
	RVV+
Saline buffer	20.0 sec.
(TAC)	31.5 sec.
	Prothrombin activation by Taipan snake venom.
Saline buffer	16.0 sec.
(TAC)	42.0 sec.
	Thrombin time*
Saline buffer	15.0 sec.
(TAC)	15.5 sec.

Clotting systems:

+ Factor X activating enzyme (0.06u) from RVV in saline, 0.1 ml anticoagulant or saline, 0.1 ml citrated bovine plasma and 0.1 ml $CaCl_2$ added.

0.1 ml of 0.6% fibrinogen, 10 ul Taipan snake venom (1:500), 0.1 ml anticoagulant, 0.1 ml prothrombin (1mg/ml) in 0.15 M Tris-HCl, pH 7.4, 0.15 M NaCl.

* 0.1 ml citrated bovine plasma, 0.1 ml anticoagulant, 0.1 ml bovine thrombin (0.1 NIH Units).

TAC is tick salivary anticoagulant.

it was subsequently assayed. The activity also appeared in the breakthrough peak when it was passed through a column of hydroxylapatite in a pasteur pipete equilibrated in 0.01M phosphate buffer pH 7.4. This suggested that there is no interaction between tick anticoagulant and calcium ions on the resin. EDTA and other agents function as anticoagulants by chelating calcium. It thus appears that the tick anticoagulant is not a calcium chelator.

4.4.4. Inhibition of the clotting times of factor Xa and factor IXa by the anticoagulant molecule.

Factor IXa and factor Xa (Russel's Viper venom-activated factor X) are plasma glycoprotein constituents of the clotting cascade which are vitamin K-dependent serine proteases containing gamma - carboxyglutamic acid residues. Factors IXa and Xa play crucial roles in blood coagulation.

The tick salivary anticoagulant inhibited activated factor X as indicated by the anticoagulant effect when factor Xa was added to factor X-deficient plasma (Fig. 4.4). The inhibition of factor Xa by the tick anticoagulant was rapid and did not change with time. This suggests a non-enzymatic action. The inhibition of factor IXa by anticoagulant is also shown in Fig. 4.5.

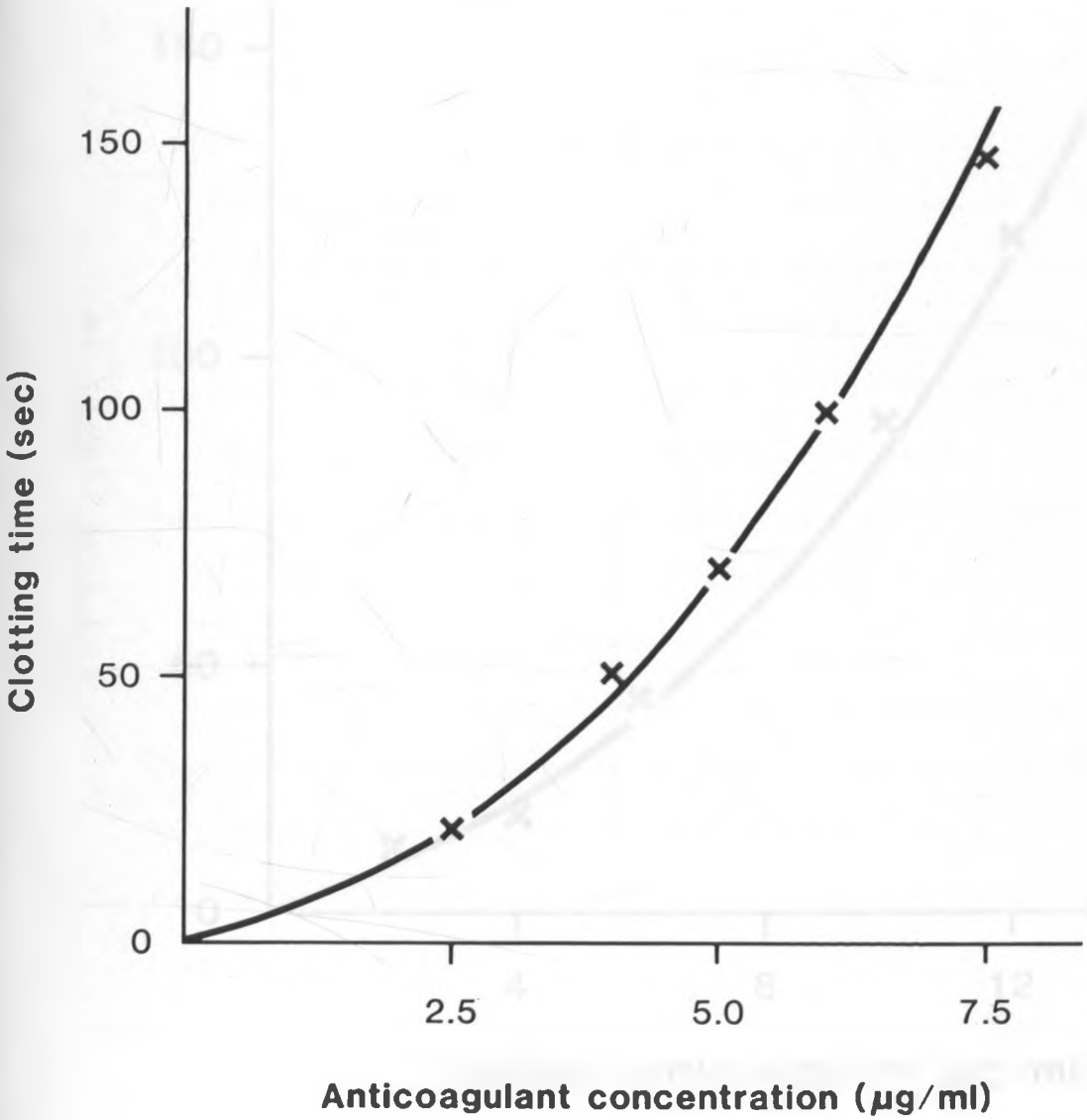

Fig 4.4 Effect of tick salivary anticoagulant on coagulation of factor X-deficient plasma by factor Xa: The incubation mixture contained: 0.1 ml factor X-deficient plasma, 0.1 ml anticoagulant, 10ul (0.04u) factor Xa and 0.1 ml 25 mM $CaCl_2$.

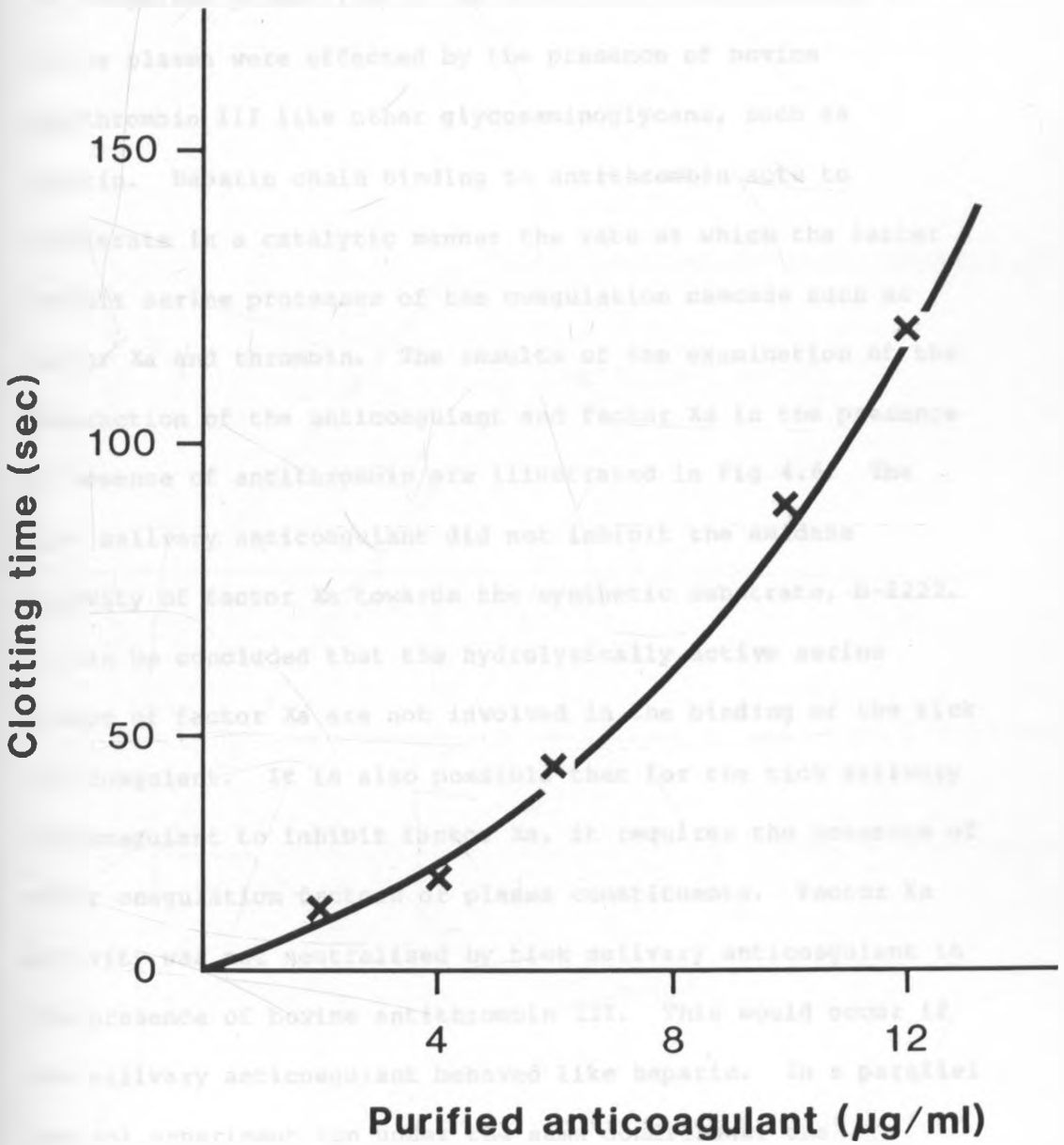

Fig. 4.5 Inhibition of bovine factor IXa by the salivary coagulation inhibitor. Clotting system: 0.1 ml citrated bovine plasma, 0.1 ml anticoagulant and factor IX, 10 ul (0.017U) in saline buffer.

4.4.5 Spectrophotometric assays of salivary anticoagulant against factor Xa in the presence of heparin and/or anti-thrombin III.

Studies were done to determine if the potent anticoagulant properties of the TSGE moiety demonstrated in bovine plasma were effected by the presence of bovine antithrombin III like other glycosaminoglycans, such as heparin. Heparin chain binding to antithrombin acts to accelerate in a catalytic manner the rate at which the latter inhibit serine proteases of the coagulation cascade such as factor Xa and thrombin. The results of the examination of the interaction of the anticoagulant and factor Xa in the presence or absence of antithrombin are illustrated in Fig 4.6. The tick salivary anticoagulant did not inhibit the amidase activity of factor Xa towards the synthetic substrate, S-2222. It can be concluded that the hydrolytically active serine groups of factor Xa are not involved in the binding of the tick anticoagulant. It is also possible that for the tick salivary anticoagulant to inhibit factor Xa, it requires the presence of other coagulation factors or plasma constituents. Factor Xa activity was not neutralised by tick salivary anticoagulant in the presence of bovine antithrombin III. This would occur if the salivary anticoagulant behaved like heparin. In a parallel control experiment run under the same conditions, the inhibition of factor Xa activity by antithrombin III is very rapid and essentially complete within 15 sec.

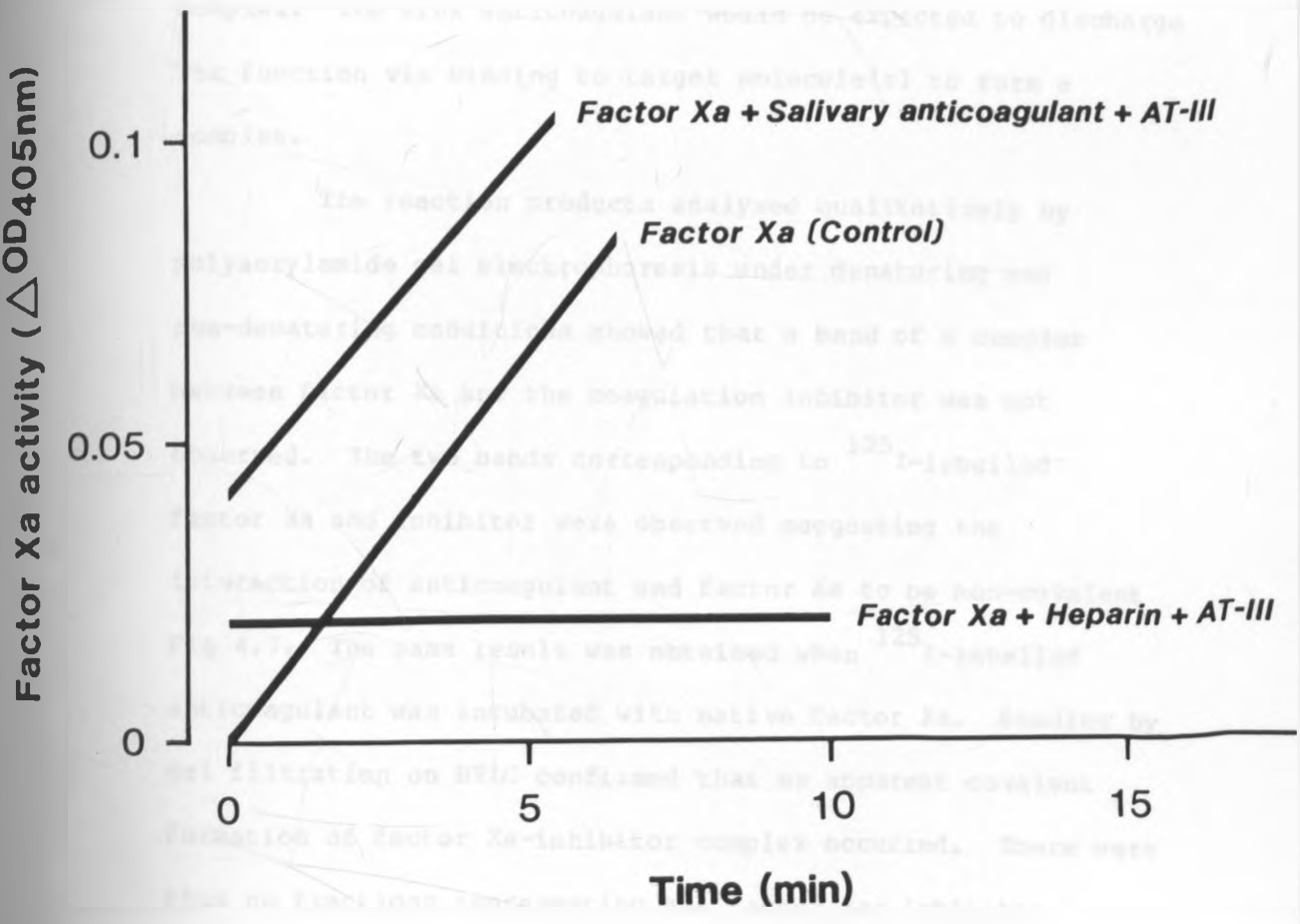

Fig.4.6 Effect of heparin (1.5 u/ml) and tick anticoagulant (3.5ug/ml) on the time course of antithrombin - Factor Xa interaction. 0.02 units of factor Xa was used to hydrolyze 100 ug S-2222 in 2 mls 10mM Tris-HCl pH 8.0, 0.15 M NaCl.

4.4.6 Binding Studies of anticoagulant molecule and factor Xa as an approach to provide evidence that factor Xa is the target of inhibitory activity by the tick anticoagulant.

Having established that salivary anticoagulant possess the power to inhibit both extrinsic and intrinsic pathways, further investigations were confined to the prothrombinase complex. The tick anticoagulant would be expected to discharge its function via binding to target molecule(s) to form a complex.

The reaction products analysed qualitatively by polyacrylamide gel electrophoresis under denaturing and non-denaturing conditions showed that a band of a complex between factor Xa and the coagulation inhibitor was not observed. The two bands corresponding to ^{125}I-labelled factor Xa and inhibitor were observed suggesting the interaction of anticoagulant and factor Xa to be non-covalent Fig 4.7. The same result was obtained when ^{125}I-labelled anticoagulant was incubated with native factor Xa. Studies by gel filtration on HPLC confirmed that no apparent covalent formation of factor Xa-inhibitor complex occurred. There were thus no fractions representing the factor Xa- inhibitor complexes.

These results are indicative of a weak dissociation constant for factor Xa-coagulation inhibitor complex. The complex formed may be transient unlike factor Xa - antithrombin III (in the presence of heparin) which is relatively stable; could possess stability that falls between that of enzyme and

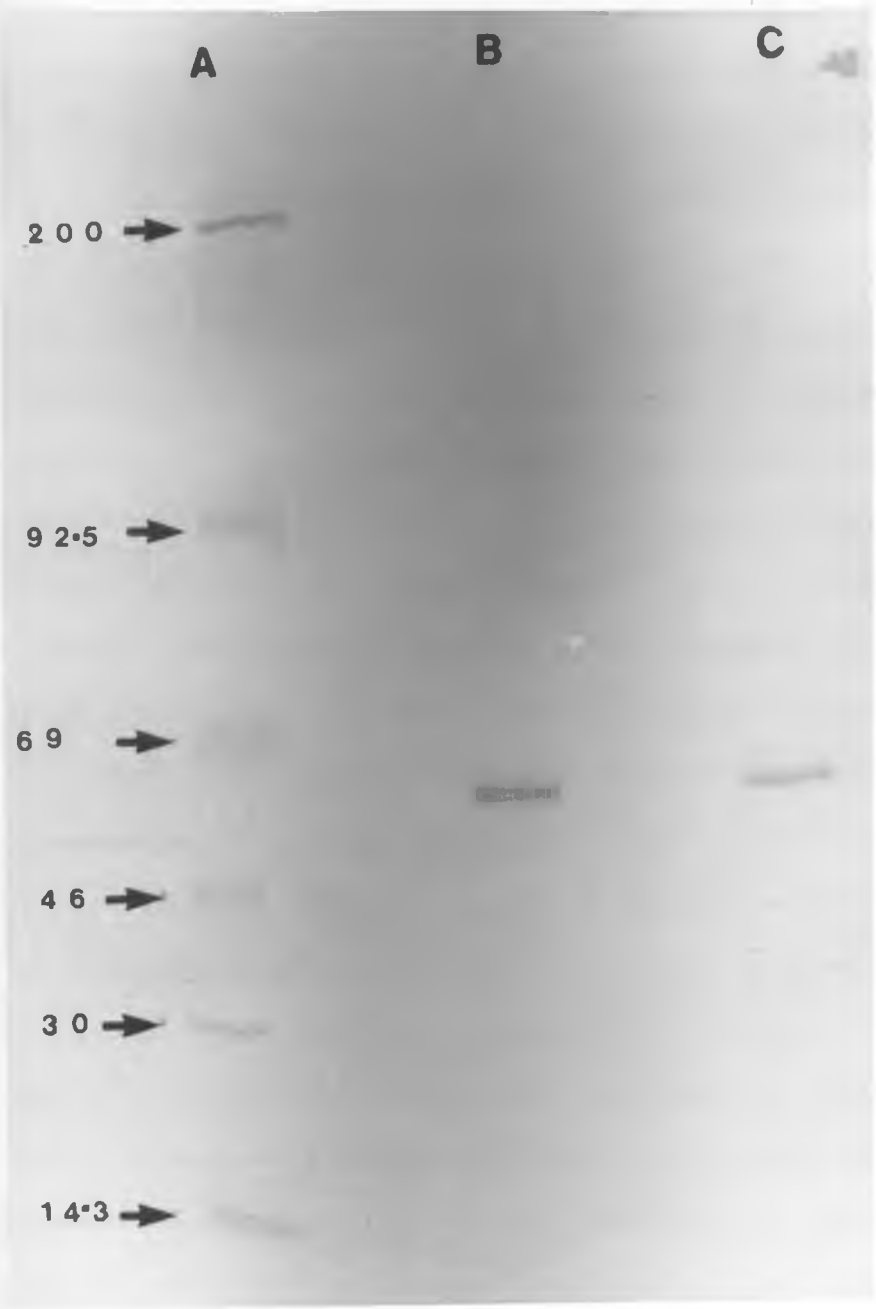

Fig. 4.7 Analysis by SDS-PAGE of tick anticoagulant - ^{125}I-labelled factor Xa reaction for any shift in size due to complex formation.

Lane A: Molecular weight standards in daltons X 10^{-3}

Lane B: ^{125}I-labelled factor Xa

Lane C: Labelled factor Xa incubated with tick anticoagulant.

substrate and factor Xa - antithrombin. It has recently been shown by several investigators that antithrombin - protease complexes dissociate slowly i.e. with half life of several days, to free enzyme and a modified inhibitor that is cleaved at a specific reactive site bond (137). This inhibitory action is restricted to factor Xa as the coagulation inhibitor does not affect the clotting time of thrombin or synthetic substrate hydrolysis by other serine proteases (trypsin, chymotrypsin, plasmin). It is suggested that the anticoagulant action of the tick salivary anticoagulant is due to complex interactions between factor Xa and other constituents of the prothrombinase complex. The formation of the complex (assembled on aggregated platelets) enhance the activation of prothrombin several-fold relative to the rate with factor Xa alone (138). The prothrombinase model is shown in Fig. 4.8. According to current theory, factor Xa, factor Va and calcium form the prothrombinase principle on the phospholipid surface with activated factor Xa as the prime mover. Inhibition of prothrombin activation could thus be a result of inhibition of the prothrombinase complex as a whole, or a result of interference with factor Xa. The active site of factor Xa is not inhibited by the tick anticoagulant <u>in vitro</u>. The tick anticoagulant prolonged the clotting time of the factor V-deficient plasma and was not affected by increasing concentration of phospholipids. Since changing the calcium concentration had no effect on the coagulation inhibitor, the inhibitor does not appear to act by binding calcium.

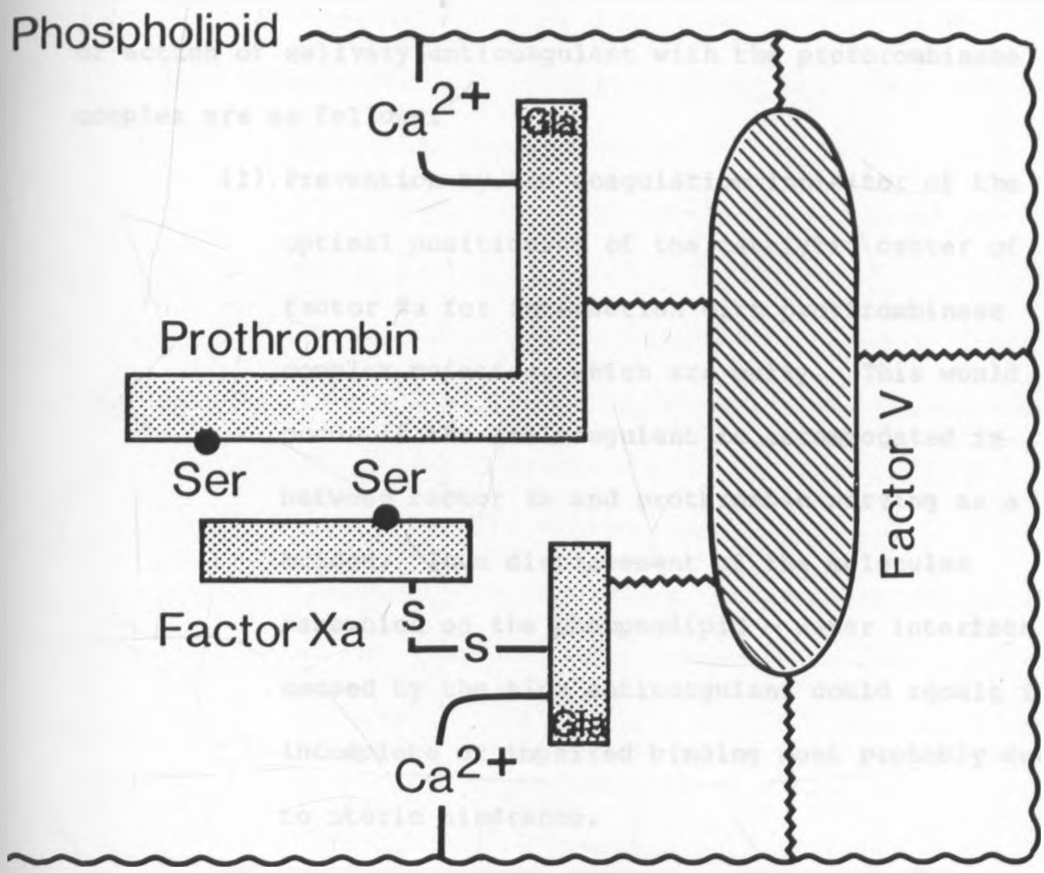

Fig. 4.8 Proposed model of the interaction between factor Xa, Prothrombin, factor V and phospholipid. PL are donated by ~~~ .The mechanisms by which these proteins associate however has remained unclear (90).

In view of the above considerations, the experiments performed which indicate that the coagulation inhibitor interferes with the reactions involving activated factor Xa and present knowledge on the mechanisms by which prothrombinase complex proteins associate, the possible modes of action of salivary anticoagulant with the prothrombinase complex are as follows:

(1) Prevention by the coagulation inhibitor of the optimal positioning of the catalytic center of factor Xa for interaction with prothrombinase complex moieties, which are bulky. This would occur if the anticoagulant is accommodated in between factor Xa and prothrombin serving as a bridge. Thus displacement of the molecules assembled on the phospholipid - water interface caused by the tick anticoagulant could result in incomplete or impaired binding most probably due to steric hindrance.

(2) The anticoagulant molecule could cover GLA residues of the factor Xa that are necessary for phospholipid function. This would happen if the tick anticoagulant binds to a site adjacent or near the phospholipid-Ca^{2+} binding site and sterically hinder the formation of the complex.

(3) It is possible that binding of the anticoagulant to the coagulation factors induces conformational alterations in them thus intefering with the

physical states that favour prothrombinase complex formation. Conformational changes could render coagulation factors more susceptile to the inhibitor and enhanced inhibition. Staphylocoagulase, a extracellular protein produced by Staphylococcus aureus, for example, alters the conformational structure of prothrombin resulting in the formation of an active centre (139).

(4) The anticoagulant could bind to the phopholipid surface thus inhibiting coagulation by competing with clotting proteins for the lipid surface. Substances like phospholipase A, and phospholipids, which have a remarkable affinity for Ca^{2+}, could form a complex with the anticoagulant principle making the surface of the phospholipids inaccessible to blood coagulation factors. This hypothesis is less feasible because of the absence of phospholipase in the salivary anticoagulant and because cephalin had no effect on it's activity.

Chapter V

5. IMMUNOLOGICAL STUDIES.

5.1. Introduction.

Ixodid ticks are very important vectors of pathogens to animals and hence are a constraint to the development of the livestock industry in various parts of the world (Chapter I, section 1.2). The economic losses caused by ticks in animal production call for effective measures of vector control. Because of widespread acaricide resistance, the need for alternative measures is increasingly apparent.

The expression of acquired resistance to ixodid tick infestation by cattle and laboratory animals is known to involve a number of immune effector mechanisms. Cellular responses in the skin of cattle to the attachment of ticks and their relationship to host immune resistance has been described by several authors (92). Other possible mechanism(s) involved in the expression of resistance by animals to ixodid tick infestation have been described in chapter III. Stimulation of the host immune response(s) to induce resistance to tick infestation has considerable potential as an alternative method of control.

Resistance is acquired in the field naturally after multiple tick infestations. Efforts have been directed towards limiting infestation by ixodid ticks by artificial immunization using tick products. The saliva secreted by the tick probably contains the antigen(s) or other physiological components against which the host mounts an immunological response. Resistance to ixodid ticks has been induced by administration

of tick salivary gland immunogens with different results. The previous findings described in Section 1.3.2.2 combined with those of this study indicate that resistance to ixodid tick infestation can be induced with tick antigens. However, despite much work, immunization with crude salivary antigens may not be an optimal approach for the induction of tick resistance because of the poor efficacy in protecting against tick infestation in natural situations. The limited induction of resistance attained with immunisation using such crude salivary preparations may be due to competition of dominant antigens such that the crucial ones involved in resistance elicit low or no antibody response(s). In addition, an immunosuppressive activity has been recently reported in the saliva of the ixodid tick, Ixodes dammini (36).

Few attempts have been made to isolate the important antigens responsible for the induction of resistance to tick infestation. Studies of the biologically active molecules in saliva that may be responsible for the induction of resistance and investigation of their mechanism of immunomodulation might lead to new methods for tick control. In this chapter, non-resistant and tick resistant control animals were included with TSGE immunized animals in order to compare R. appendiculatus larval feeding capabilities. Knowing that crude TSGE induces resistance to subsequent tick challenge, an attempt was made to find the role of the purified anticoagulant contained in TSGE in tick protective responses.

5.2 Materials.

Ticks.

Larval R. appendiculatus ticks were used in the feeding assays. They were grown and hatched in glass tubes as described in section 2.2.1. All ticks used during an experiment were derived originally from one female to help ensure more uniform feeding capabilities.

Host species.

New Zealand white rabbits or rabbits of local breeds obtained from ILRAD were used in this study. The animals were divided into groups of three during experimentation and caged individually. They were fed ad libitum a pelleted commercial diet and provided with water. Outbred female albino Hartley guinea pigs were maintained under the same conditions.

Products and chemicals.

Freund's complete and incomplete adjuvants were obtained from Difco Laboratories, Detroit MI, U.S.A. Agarose was from Serva, London, U.K. and nitrocellulose paper from Schleicher and Schull, West Germany. ^{125}I protein A was purchased from Amersham International PLC, Amersham, England.

5.3. Methods.

5.3.1. Determination of localized salivary anticoagulant at tick attachment sites.

The experiment was performed to determine if the anticoagulant activity is secreted. About 1000 larvae were confined to the left ear of a rabbit in ear bags. When the larvae had fed for five days, the adhesive tape and ear bag was removed leaving the larvae attached to the rabbit in clusters.

The assembly pheromones secreted by ticks induce their clustering. About 500ul of rabbit blood was removed from a commitant left ear vein in the immediate vicinity of the tick bites using a 26 gauge needle and a syringe. A similar amount of blood was obtained from the right ear as control. The time taken for both the samples to form a firm clot was determined.

5.3.2. Preparation of immunogens.

Since the salivary gland represents the site of production of the anticoagulant and other substances required for tick feeding, the extracts of this gland from partially fed adult R. appendiculatus were used as antigen. To optimize the ability to artificially immunize rabbits, female ticks that were fed for four days were selected, since a majority of the biologically active molecules are produced in sufficient amounts at this time and since salivary glands begin to degenerate as feeding terminates. TSGE was processed as described in Section 2.3.3.

Purified functional salivary anticoagulant was used as an immunogen. In addition, the anticoagulant molecule was heat inactivated at 100°C for 10 min, and also treated with cyanogen bromide in 18.7M formic acid, at 4°C, for 24 hrs, and the reaction terminated by dilution with water. The CNBr fragments were used for immunization of rabbits to try to obtain antipeptide antibodies that can bind to the parent molecule.

Equal volumes of immunogen in PBS were thoroughly mixed with complete Freund's adjuvant and this was divided for subcutaneous and intramuscular injection. Incomplete Freund's

adjuvant in the same ratio (1:1 v/v) was used in subsequent booster injections. Because of the possibility that the anti-clotting function in the process of emulsification with the adjuvant oil may undergo denaturation and loose its activity, the anticoagulant was also inoculated in saline without adjuvants.

5.3.3. Determination of the feeding performances of larval ticks on rabbits immunized with TSGE and by successive larval infestation.

Female New Zealand or rabbits of local breeds (weighing 2.5-3.0kg) were pre-bled from the marginal ear vein to obtain pre-immune sera. They were prepared for larval infestation by clipping the hair on the ears short and fastening the hind legs to minimize body movement and grooming by the host; hence any observed changes in tick feeding could be related to the host response and not to grooming activity. The rabbits were randomly separated into four groups. In the first group, 3 rabbits were immunized intramuscularly in both flanks and subcutaneously with 1 ml 100ug of crude TSGE in PBS emulsified with an equal volume of complete Freund's adjuvant. This is about the maximum dose that would allow survival of the rabbits for about 3 months because of tick toxicosis.

The animals were boosted two weeks later with the same dose of crude TSGE preparation in an equal volume of Incomplete Freund's adjuvant. After 10 days blood was collected from the marginal ear vein for preparation of immune sera and the rabbits were ready to be challenged with larval ticks. The blood was allowed to clot at $4^{o}C$ for 18 hrs and serum was

collected and stored at -20°C for antibody screening. The second group of 3 rabbits were infested with 1000 one month old pathogen-free larvae to acquire or express tick resistance. They were restricted to the feeding sites in ear bags fastened with hypo-allergenic adhesive tapes thus confining them to shaved ears. In the initial infestation larvae were fed on the right ear for 5 days followed by 9 tick-free days to allow the ears to recover from bites. A second infestation of similar larval load was applied on the left ear 14 days later. A third group of rabbits were immunized with purified tick salivary anticoagulant. An initial inoculation of 50 ug followed by 25 ug in the second booster was administered as described for the first group. Antibody to the anticoagulant molecule from sera obtained 7 days after the second boost were tested on immunoblotting as described in section 5.3.8.2. A control group of rabbits was injected with adjuvant only as the first group.

To assess the influence of administration of TSGE and immunization by successive larval infestation, all the rabbits including the 3 control rabbits were challenged with about 100 R. appendiculatus larvae. Larval ticks were enumerated into groups of about 100 in the bottom of petriculture dishes chilled on ice and the ticks manipulated only with a fine hair brush to avoid possible damage to the cuticle. At the end of tick feeding period, the ticks were removed from the host after detachment. The expression of resistance was measured by the rabbits ability to prevent the ticks from obtaining a normal blood meal. This was assessed by measuring the engorgement

weights for each batch of ticks obtained from different groups. Because the third group of rabbits, those immunised against purified anticoagulant, failed to elicit antibody response, the rabbits were not challenged with larvae. However, further experiments were attempted to raise antibodies against tick salivary anticoagulant as described in the next section.

5.3.4. Attempts to raise antibodies against the tick salivary anticoagulant.

Rabbits (average Wt. 3.5 kg) were immunized by injection of various amounts of purified salivary gland anticoagulant in complete Freund's adjuvant subcutaneously and intramuscularly in both flanks. After two weeks, booster injections were given several times in same manner in incomplete freunds adjuvant at 14 day intervals (Table 5.1). Rabbits were bled from one week after the third injections and IgG prepared and tested for antibody on the western blotting.

Table 5.1 Preparation of rabbit Sera against the tick anticoagulant.

Rabbit Number	Dose of Inoculum (ug)		
	1st Injection	2nd Injection	3rd Injection
R1	50	30	20
R2	40	25	10
R3	10	20	30

Brown et al (140) showed that salivary gland extracts derived antigens of Amblyomma americanum induced resistance when emulsified with incomplete Freund's adjuvant but had no such

ability when emulsified with complete Freunds adjuvant or administered in saline. In this study also immunization intravenously and subcutaneously with anticoagulant (15 ug) in saline at day 0, 7, 14, 21, and 35 gave no detectable antibody response.

The salivary anticoagulant was boiled for 10 min or treated with cyanogen bromide (as described in Section 5.3.2) with the hope that immunization with smaller fragments of the denatured protein would greatly increase the chances of obtaining region-specific or site specific rabbit sera.

Several experiments were done with reference to the nature, timing and route of anticoagulant presentation that may be required to stimulate the immune response with no success.

5.3.5 Purification of IgG.

Antibodies from immune serum and normal sera were purified by a combination of ion-exchange on DEAE cellulose and Sephadex G-150; The IgG was purified to remove proteases, complement proteins and other components which would interfere with the clotting assay and western blotting analyses.

A column of DE-52 (20 CC DEAE-cellulose per 40 ml serum) was equilibrated with 0.01M potassium phosphate pH7.5, conductivity 1-1.5 mu. Serum was dialyzed at room temperature against three changes of 1 liter of each of 0.01M potassium phosphate until the conductivity of sample was equal to that of the buffer. The dialyzed sample was applied to the column and the antibody activity which is largely associated with IgG molecules washed through the column. This was concentrated by PM30 Amicon membrane and passed through a Sephadex G-150 column

in the same buffer. The final IgG fractions were concentrated to approximately 10-30 mg protein/ml on an Amicon PM30 membrane.

5.3.6. Coupling of rabbit immune IgG to activated CH-Sepharose 4B.

Purified IgG from immune and non-immune antisera, was coupled to activated CH-sepharose 4B according to the Method described in Section 2.3.7. The coupling buffer was 0.1M $NaHCO_3$ containing 0.5M NaCl at pH 9.5.

5.3.7 Immunochemical identification methods used.

5.3.7.1. Ouchterlony double immunodiffusion experiment.

Double immunodiffusion analyses were carried out based on the method of Ouchterlony (141). It was performed in 1% w/v solidified agarose gels containing 0.02% sodium azide in 50mM Tris-HCl, pH7.4. The reaction was carried out on precoated microscope slides or petridishes. The agar was poured when still hot and allowed to cool. Wells of 2mm diameter were cut in the gel at a distance of 10mm from each other and the solidified agarose removed by sunction. The wells were filled with 25ul of antigen source or antisera. Diffusion in gels was allowed to occur at room temperature in a humid chamber on a horizontal surface or at $4^{o}C$ for upto 48 hours. Three (w/v) polyethylene glycol could be included in the buffer to enhance precipitation (142).

After the precipitin lines of antigen-antibody had developed sufficiently, the gels were thoroughly washed in saline (0.9% NaCl) and distilled water to remove unprecipitated proteins at room temperature. The gels were dried and stained with coomassie blue and then destained with 10% acetic acid.

This method was used to assess the reactivity of TSGE directed antibodies in both crude antisera and purified IgG fraction. It was also used to check for the immunological reactivity of purified salivary anticoagulant to the various antisera raised against it.

5.3.7.2. Western immunobloting.

Protein samples were subjected to electrophoresis on a 5 to 20% SDS polyacrylamide slab gels as described in section 4.3.6. The transfer was performed using a Biorad Trans Blot electrophoretic transfer cell apparatus following instructions provided by the manufacturer. Before transfer, sponge/fiber pads, filter paper (Whatmann 3mm chromatographic paper) cut to the same dimensions as the pads was saturated with transfer buffer (25mM Tris, 192mM glycine, pH8.3, 20% (v/v) methanol, 0.02% SDS). SDS was eliminated in transfer buffer when polyacrylamide gels were run. Methanol improves binding of SDS-proteins to nitrocellulose filter. The gel and nitrocellulose membrane (0.45u) were equilibrated in 500ml Tris-glycine buffer for at least 30 min to help prevent shrinking or swelling during transfer. Nitrocellulose paper was allowed to wet by capillary action. This is because abrupt wetting can lead to entrapment of air bubbles in the matrix which can block transfer of molecules by creating points of high resistance.

The pre-equilibrated gel to be transferred was sandwiched between two sheets of pre-wetted Whatmann 3mm filter paper, presoaked in transfer buffer, which are in turn sandwiched between supportive porous foam pads and sponges.

Care was taken to push out all air bubbles between the gel and the nitrocellulose paper. These were removed by rolling a glass pipette on top of the membrane-gel surface to form an adhesive surface.

The sandwich was placed in the gel holder and submerged into slot in transblot tank with transfer buffer pre-chilled prior to transfer. Proteins from SDS-polyacrylamide gels are eluted as anions. Therefore, the sandwich was placed in the apparatus with the gel toward the cathode and the paper towards the anode. A magnetic stirrer bar was placed in the unit to ensure homogenous pH across the gel and to improve heat transfer.

After electrophoresis the proteins in the gels were electrophoretically transferred to nitrocellulose paper which were used as the immobilizing matrix. Transfer was conducted at 200mA for 3 hours or overnight at 6mA. When transfer was complete, the nitrocellulose was rinsed briefly in PBS and then incubated for 1 hour at $37^{o}C$ in a tray containing buffer A (5% - w/v - bovine serum albumin in 10mM Tris, 0.9% NaCl, pH7.4) to saturate free protein binding sites and eliminate background effects. The binding of proteins to nitrocellulose membrane is not clearly understood and probably involves multiple electrostatic and hydrophobic interactions. The transfered proteins were reacted with the appropriate sera or purified antibody diluted in freshly made BSA-Tris-saline with 0.02% azide. The incubations, carried out on a gentle shaker, were done at room temperature for periods varying from one to twelve hours. This was followed by two 10 min washes in

Tris-saline pH7.4 at room temperature. The filters were then washed, with two changes, in 0.05% NP-40 in Tris-saline each wash being 20 min at room temperature) and finally for 10 min wash in Tris-saline to remove unreacted antibody.

The paper was incubated in fresh buffer A containing (^{125}I)-labelled protein A (1 to 5x10^{5}cpm/ml) for 1 hour at 24-25^{o}C. The blot was again washed several times as above to remove excess and non-specific bound probe. The paper was air dried between two sheets of Whatman filter paper and sealed in a plastic bag. It was then exposed against Kodak X-Omat AR film with an intensifying screen, at -80^{o}C. The autoradiogram was developed using Kodak KLX developer and Kodak fixer. Exposure time varied from overnight to several days.

The general procedure for electroblotting to zeta-probe had these slight modifications. The gel was equilibrated in Tris-HCl 5mm Sodium acetate, 0.5mM EDTA, pH7.8, for 30 min while the membrane was soaked in same buffer for 5 mins. The nitrocellulose has higher binding capacity in the absence of alcohol.

5.3.8. <u>An experiment to test if salivary anticoagulant binds to nitrocellulose filter paper</u>.

Tick salivary anticoagulant was subjected to SDS-PAGE under the same conditions described in section 2.3.6. One gel was run under non-denaturing conditions because antibodies may not bind denatured proteins. SDS may also affect antibody binding to antigenic domains of some proteins. One section of the SDS polyacrylamide gel was sliced and silver stained. The other section of the gel was blotted from the gel onto a

nitrocellulose paper to detect antibody reactivity of bound molecules. The blot was incubated with antisera against purified anticoagulant and crude TSGE. The actual processing of the nitrocellulose paper to detect antibody reactivity of bound molecules entails extensive incubations and repeated washes. One variable that was examined was whether the salivary anticoagulant molecule binds to the matrix, and whether, once absorbed to the nitrocellulose, the binding affinity was high enough to be retained on the filters during subsequent incubations. The salivary anticoagulant was radiolabelled with Bolton - Hunter reagent as previously described in section 6.3.8. The labelled anticoagulant molecule was purified using coarse Sephadex G-25 to remove small fragments and unincorporated label. It was subjected to electrophoresis on 5-20% SDS-PAGE and transferred from gels to nitrocellulose filters. The membrane was exposed to X-ray film before and after being subjected to the several incubations and washed as described above for immunoblotting. The utility of Zeta probe, a positively charged nylon membrane, for analysis of this molecule was tested with similar results.

5.4. Results and discussion.

5.4.1 Localisation of salivary anticoagulant in tick attachment sites.

It was desirable to designate this activity as derived from the secretory contents of the glands as opposed to a substance in their supportive structures. The results below indicate that blood remains anticoagulated at the tick bite site during tick feeding. This suggests that the anti-clotting activity is secreted as a component of tick saliva.

1. Control serum - 12 min (normal clotting time)

2. Serum from the tick bite - 55 min

The anticoagulant thus enters the tissues in quantities sufficient to interfere with extravascular coagulation. Most of the plasma fibrinogen extravasated from the blood vessels would be rapidly clotted and cross-linked by the procoagulant effects released in association with the trauma of tissue damage caused by feeding ticks if there was no anticoagulant. The anticoagulant thus seems to leave the gland internally as well as being ejected in the saliva. Recognition that the salivary anticoagulant is detectable in blood and serous fluids from the immediate vicinity of the tick bite site is an aid to investigations of immunity to tick infestation. This may also explain why ticks in nature feed in clusters which enables them to secrete large amounts of the inhibitor necessary for physiological effectiveness at the tick bite site. If one tick were to maintain such concentration of inhibitor in the vicinity of attachment on the host, to achieve beneficial effect, the metabolic cost of the tick would probably be high.

5.4.2 Induction of immune resistance in rabbits infested with larvae and immunized with salivary gland antigen extract from fed adult female R. appendiculatus.

Cattle and laboratory animals are known to acquire resistance to tick infestation (95). This resistance is the ability to interfere with proper tick feeding. As indicated earlier, resistance to infestation has been quantitated as a reduction in the number of ticks attaching and a reduction in

engorgement weights. Immune resistance, when used as immunogen, is transferred with immune serum suggesting that antibodies are involved.

The results in table 5.2 demonstrate that salivary gland extract (TSGE) derived from adult female R. appendiculatus fed on rabbits for four days is capable of inducing minimal protective resistance when used as immunogen, in rabbits never previously exposed to ticks. Induced resistance was expressed by reduced blood meal weights. This resistance is partial in that ticks complete their life cycle successfully with minimal damage to the larvae. The rabbits immunized with TSGE produced low (13.7%) level of tick resistance as compared to animals actively sensitized by larval feeding (two successive infestation) and challenged at the same time. The duration of engorgement was not affected in both cases. This acquired resistance would probably depend on dose, length and route of vaccination. It was observed that about 95% of the applied ticks completed their engorgement successfully. Others were observed dead while attached to the host or did not attach at all, but became engulfed in serous exudate. However, the gross appearance of tick attachment sites was similar for both

Table 5.2 Mean weights of batches of 100 larvae which have obtained blood meals on control, immunized and infested rabbits.

Treatment of rabbits	Mean weight of Larvae (mg)	% Decrease in weight
1. Controls	61.3	-
2. Immunized with TSGE	53.9	13.7
3. Infested with larvae	20.4	66.7

groups of rabbits. Cutaneous reactions were characterized by the presence of profuse serous exudate. These reactions, appearing on the periphery of the bitten sites, were probably caused by release of pharmacologically active substances of the acute inflammatory reactions. This preliminary findings demonstrates the potential for artificial immunization with R. appendiculatus salivary gland extract (TSGE) for an immunologic approach to tick control. However, because they have poor efficacy, it is important, to identify protective antigenic molecules in TSGE and determine how best to present them to the hosts immune system. The above results suggest that a component(s) of the immune resistance response affects the assimilation of the blood meal. Because TSGE from ticks fed for 4 days was less effective, it suggests that the relevant antigens involved in protective responses may be produced during the early development of tick feeding. There is also the possibility that some TSGE molecules reduce immunological

competence of the host with impairment of the function of lymphoid cells. Reduced immunological competence may be manifested as reduced or altered capacity for antibody production and/or reduced cell mediated immune responses. In recent work, immunosuppressive activity has been demonstrated in the saliva of a tick, Ixodes dammini (36). It is obviously of interest to identify immunosuppressive activity in saliva. Other molecules that are worthy of further study in R. appendiculatus are described in section 2.4.1. This study also demonstrates the presence of shared antigen(s) between larval and adult ticks because TSGE from adult ticks is protective against larval infestation. It is thus possible to find core antigens shared by all the life-cycle stages of the tick R. appendiculatus and by several other tick species.

As discussed in chapter III, cell-mediated, antibody and complement-dependent immune effector mechanisms have been implicated in the resistance response. Therefore, each tick host immunological relationship should be examined individually as the responses of the hosts may be varied.

5.4.3. Effect of antisera on anti-clotting activity.

IgG fraction was prepared from normal serum and several immune sera (rabbits that had four successive infestations of larvae or adult ticks, rabbits immunized with crude TSGE or the purified anticoagulant). The immune IgG fractions prepared from sera heated at 56^{o}C for 30 min to inactivate proteolytic enzymes, were used for functional screening for antibodies to the anticoagulant. The

Fig. 5.1 ^{125}I -anticoagulant molecule separated on a 5-20% SDS-polyacrylamide gel, transferred to nitrocellulose paper and the filter exposed before (A) and after (B) washes. Autoradiography of the filters is shown.

anticoagulant activity was same when assayed as described in section 2.3.8.1 with or without anti-tick antibodies.

It is concluded that the anticoagulant activity is not neutralized by the immune sera.

5.4.4. Binding studies of salivary anticoagulant molecule to nitrocellulose.

The electrotransfer studies of ^{125}I-labelled anticoagulant molecule confirm that it binds to nitrocellulose filter paper. Thus the gel composition, transfer time, voltage, buffer type and strength and pH, eluted the molecule from the gel. The band was retained by post transfer washes as shown in Fig.5.1. This ability to visualize labelled salivary anticoagulant on nitrocellulose paper suggests that the transfer efficiency of anticoagulant from gel to nitrocellulose is sensitive enough to detect it at the ug levels.

5.4.5 Ouchterlony immunodiffusion analyses of TSGE and anticoagulant against immune sera.

Ouchterlony double-immunodiffusion analyses were used to characterise the specificity of the antibodies raised to crude TSGE. The immune IgG fraction against crude TSGE gave two strong immunoprecipitation lines on reaction with its homologous antigen (Fig 5.2). There was no reaction between the immune sera and purified salivary anticoagulant.

The double immunodiffusion technique was not sensitive enough to demonstrate all the antigens recognized by these immunized animal sera. The more sensitive western blotting was used in further analyses.

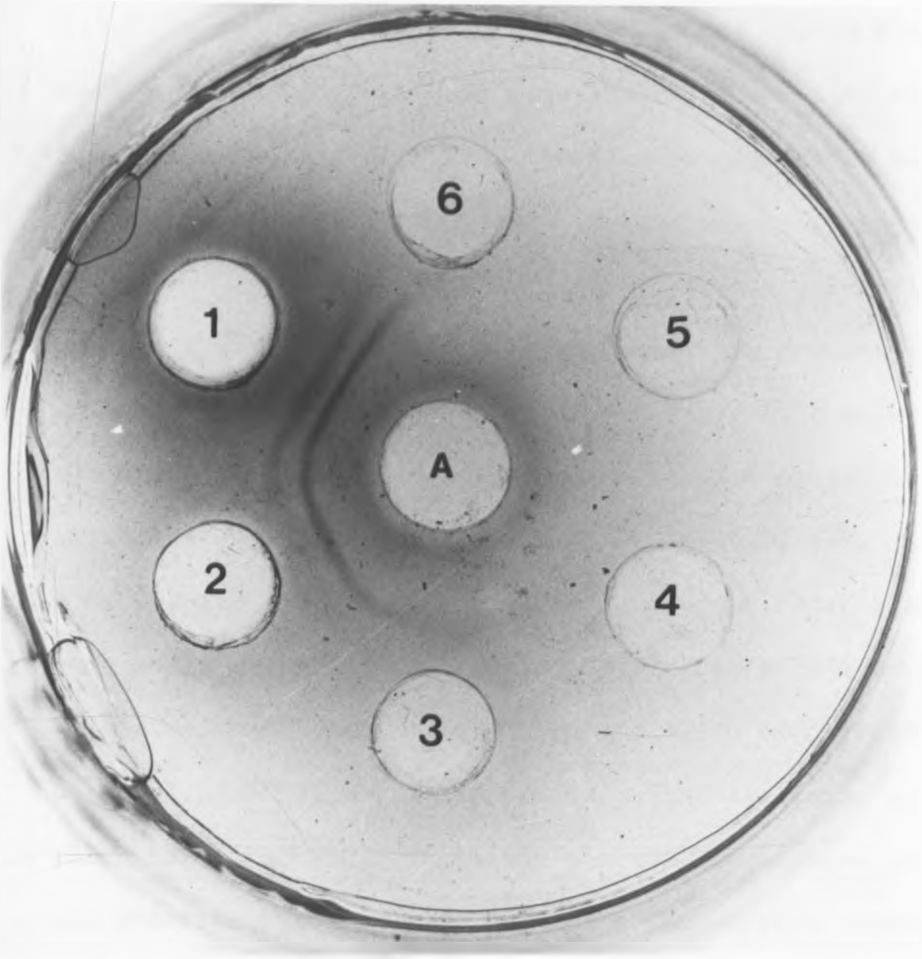

Fig.5.2 A photomicrograph of Ouchterlony antigen-antibody precipitation of crude TSGE against antisera raised against it. The numbers marked 1 to 4 indicate serial dilutions of rabbit immune sera to TSGE (Neat serum (1), 1/2 dilution (2), 1/4 dilution (3) 1/32 dilution (4). Neat serum against salivary anticoagulant (5). The serum dilutions were tested against 50 ug of crude TSGE in central wells (A). No precipitin lines appeared with preimmune sera.

5.4.6 Immunoblot analysis of TSGE and purified anticoagulant reacted with various antisera.

Under the electrophoretic conditions used in section 5.3.7.2., all the TSGE (day 4) proteins are eluted from SDS gels and transferred to the membrane matrices. The results in Fig. 5.3 show that sera from rabbits immunized with total freeze-thawed TSGE, and which conferred low resitance to larval infestation, have a strong reactivity against a restricted number of immunodominant molecules that were present in soluble TSGE as detected by western immunoblotting. The molecular masses of the major tick antigens recognized by IgG of immune serum are 17, 22, 25, 35, 40, 54, 60, 74, 120, 129, 145, 168, 234, 263 and 347kDa. SDS-PAGE was run under denaturing and this means one TSGE component may appear as 2 or more subunits in the gel. An increasingly strong binding of protein A to a polypeptide of 74 kDa was observed. They are fewer molecules which can effectively stimulate the immune response because of the possibilities of competition and cooperation between various antigens. These molecules are, therefore, likely to play a major role in the developement of resistance in this model as immunogens and/or as targets of protective immune responses. Some, of course, may be irrelevant and of no consequence to either the tick or the host.

The antibodies obtained from rabbits and guinea pigs immunized with the purified salivary anticoagulant showed no reactivity on immunoblotting. Failure to detect a signal when the anticoagulant on nitrocellulose was incubated with

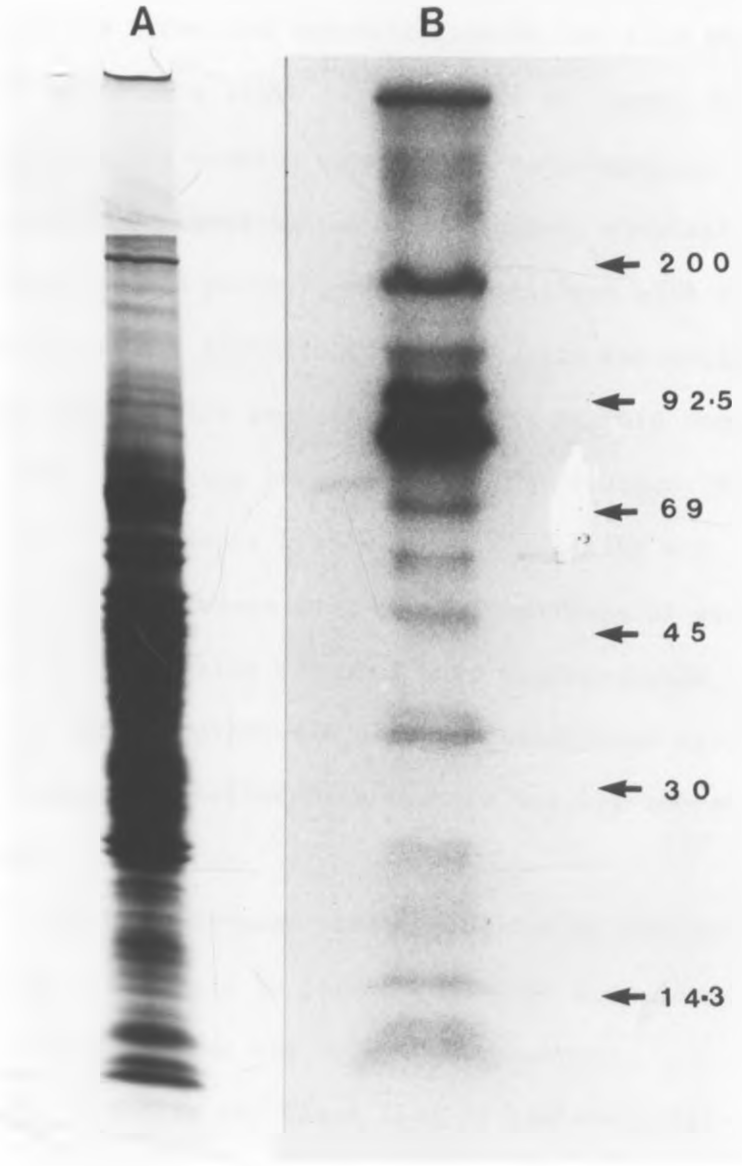

Fig. 5.3 Immunoblot analysis.

A: Coommassie blue stained proteins of TSGE.

B: Antibody-blot method identifies selectively only those proteins recognised by the specific antibodies. Arrows on the margin to the right of the autoradiogram indicate the size of molecular markers (kDa).

^{125}I-protein A is thought to be due to poor antigenic properties of the anticoagulant rather than the insensitivity of the method. An increase in sample load on SDS-pAGE, antibody reaction times and exposure time of the film made no difference. There is a limit to the amount of sample load, because exceeding the binding capacity of anticoagulant to nitrocellulose paper tends to reduce the signal eventually. It is assumed that excess protein, weakly associated with the filter, may be rapidly accessible to react with the antibody in solution, but the ensuing protein A-antibody-protein complex may easily wash off during the washing of the filter. Zeta Bind which has considerably higher binding capacity for proteins than nitrocellulose and good for analysis of greater amounts of protein was also used but gave no detectable reaction. The anticoagulant was also not recognised by antibodies from sera obtained from rabbits heavily infested with larvae or adult ticks.

It is widely believed that the nature of antigenic determinants on a molecule is partly dependent upon the method of antigen preparation and the subsequent analysis. Electrophoretic transfer can cause loss of immunological reactivity. Therefore, a further experiment was done by passing purified anti-clotting function through a column of immune IgG coupled to Sepharose in a Pasteur pipette as described in section 5.3.6. The immune IgG fractions used were from rabbits immunized with crude TSGE and purified salivary anticoagulant. In both cases, the anticoagulant activity was eluted in the fall through protein peak.

The reasons(s) for this apparent lack of immunogenicity are unclear. It is a discouraging finding with regard to vaccine development against tick infestation. It was expected to be immunogenic because intermolecular immune competition is avoided by removing potent immunogens from the salivary extracts. On the other hand, the absence of a positive test may be due to loss of antigenicity during the extensive purification steps such that the sequence(s) and/or three-dimensional features that confer immunogenicity of the protein were lost. The binding and recognition of a protein antigenic site, is highly dependent on the chemical characteristics of the residues constituting the site and the conformational integrity of the site. Thus alterations in the chemical nature of a side chains within a site such as elimination of a hydrogen or disulfide bonds, removal or change of charges, could cause reduction or complete elimination of the immunological reactivity of the site. In view of the fact that the salivary anticoagulant forms aggregates, some of the above phenomena could have occured.

The S. aureus protein A, although useful as a general detection reagent, apparently does not react with IgG of all species and IgG sub-types. These studies do not, therefore, include the entire spectrum of immunoglobulins produced by the rabbit. In addition, no studies have yet been published on specificities of the immunoglobulins involved in protection. However, it is known that mast cell/IgE/basophils or eosinophils may be involved in tick resistance but it has not been reported whether protein A binds to IgE. Recognition of

the antigenic sites is independent of the host species, but it's potency or immunodominance varies with the immunized animals. Anti-tick antibodies induced by active feeding on rabbits, and guinea pigs failed to recognise the salivary anticoagulant molecule. By keeping the above limitations in mind, it can be concluded that the anticoagulant is not involved in antibody mediated mechanisms of protective immunity against tick infestation.

Chapter VI

6. General discussion, conclusions and recommendations.

The ability to control ticks by means of cross-breeding, and acaricide application is very important to the animal production industry. Most ticks in the natural environment feed from previously exposed hosts and are likely to have adapted in some way to host immunity. The strategies adopted by the tick to thwart, subvert or co-exist with the damaging host-protective immune mechanisms are unknown and could be diverse. Understanding of the salivary components that are thought to be essential for tick adaptation to ecto-parasitism is clearly a central question in the quest for molecules that mediate successful tick attachment and feeding and for the ultimate manipulation of these functions.

The ixodid tick R. appendiculatus, has the capacity to remain attached to the host for a period of 5-8 days during which time it appears that it requires a cocktail of biologically active molecules for successful feeding. These bioactive molecules in TSGE and saliva of R. appendiculatus which have hitherto been unreported were identified using different techniques and their significance discussed in section 2.4.1 in terms of the complex host-parasite-vector interaction. Inhibition of the physiological action of these salivary molecules may account partly or entirely for the level of tick resistance induced by immunization of rabbits with TSGE or active sensitization by repeated tick feeding. Antibodies have been implicated in potentiation of protective effects

achieved by immunization. Thus the salivary bioactive molecules identified in this project are considered attractive candidate molecules for immunological intervention.

No reports have been published prior to this study describing the isolation and characterization of R. appendiculatus tick saliva bioactive molecules capable of inducing host immunity to ticks or of eliciting skin responses in animals previously exposed to ticks. It was however, shown in this study that rabbits can be protected against R. appendiculatus by artificial immunization using crude TSGE (section 5.4.2). Because of the complicated biochemistry of the tick saliva, their role in tick feeding and their possible role in induction of host resistance to tick infestation of these molecules, can only be studied individually. Of the activities detected, the tick salivary anticoagulant was studied in detail because of its apparent importance to tick feeding and subsequent disease transmission.

The TSGE anticoagulant, is of major interest because of its ability to prevent coagulation at the site of the tick bite and for maintaining blood anticoagulated in the feeding channels and after ingestion. Balashov (8) showed that blood remains unclotted in the tick gut. In view of the above roles played by the tick salivary anticoagulant, antibodies directed towards inhibition of its physiological action could be useful as vaccine against tick infestation.

The reasons for isolating the anticoagulant from tick salivary gland extract of R. appendiculatus were three - fold: Firstly, purity is necessary for chemical and physical

characterization of the molecule. Secondly, other substances in TSGE may interfere with attempts to define, in detail, the mode of action of the anticoagulant on the clotting system. Finally, the anti-clotting functions of the secreted protein make it a critical component for the feeding processes of the tick and therefore, a potential target for chemotherapeutic or immunological intervention. This assesment of the *in vivo* actions of the anticoagulant would be facilitated if the purified molecule could be inoculated into experimental animals.

The anticoagulant activity levels are present during attachment and the slow feeding first few days. This suggests that the molecule is important in the initial establishment of *R. appendiculatus* on its hosts. The glands reach their peak of anticoagulant concentration on day 4-5 following attachment in parallel with the development of the stage of *Theileria* sporozoites infective for the host in the salivary glands during feeding and with the period of intense feeding. This activity was shown to fall in concentration after detachment and with starvation. Experiments performed demostrated that the anticoagulant is shared by all the life cycle stages of the tick *R. appendiculatus*. The salivary and gut anticoagulant activities of adult female tick, larval and nymphal anticoagulant activities had the same relative location on the elution profiles through Sephadex G-100 and is therefore reasonable to assume to be due to one distinct substance.

The salivary anticoagulant was isolated from the homogenate of salivary glands using Sephadex G-100 gel filtration, ion-exchange on DE-52 cellulose,

aprotinin-Sepharose affinity chromatography and size-exclusion chromatography on HPLC. The purified molecule which appears homogenous has pI in the range of 8.0-8.5 on chromatofocusing and an Mr of 65 kDa on SDS-PAGE. The position of the anticoagulant activity in the sliced gel correspond to that of the protein band in the stained gel. This suggests that the activity is associated with the protein and not due to some trace substance in the preparation. Studies of its properties showed that the anticoagulant is heat labile, stable over a wide pH range, and was inactivated by trypsin and reductive alkylation. These results indicate that the tick anticoagulant is of a protein nature.

Ticks must overcome host haemostasis in order to locate blood and maintain its flow during ingestion. The nature of the mechanism of action of the tick salivary anticoagulant is especially important in the intimate relationship that it has evolved with its host. The effect of anticoagulant on coagulation has been previously described in detail in Chapter IV. The inhibition of bovine plasma was instanteneous and independent of the incubation time. The anticoagulant is therefore unlikely to be a protease or a phospholipase. Phospholipase through phospholid destruction is the main causative agent responsible for the anticoagulant action of some snake venoms (90). Some proteases act as anticoagulants by degrading fibrinogen rendering it incoagulable (129). The anticoagulant did not interfere with the interaction between thrombin and fibrinogen or TOS-Arg-Ome as substrates. However, citrated bovine plasma and factor X deficient plasma

clotting time was prolonged when started with purified factor Xa in the presence of the purified tick anticoagulant . Factor Xa activity towards the synthetic substrate S-2222 was not neutralised by tick anticoagulant with or without bovine antithrombin III. This would occur if the salivary anticoagulant behaved like heparin. It can be concluded that the hydrolytically active serine groups of factor Xa are not involved in the binding of the tick anticoagulant. It is suggested that the anticoagulant action of the tick salivary anticoagulant is due to the inhibition of the complete prothrombinase complex. An interesting and significant finding in these studies is the inhibition of factor Xa by the purified salivary anticoagulant. This is important in tick feeding because the tick anticoagulant has the power to inhibit both the extrinsic and intrinsic pathways of the coagulation system. It has been suggested by Yin (143) that the primary target of physiological control of the coagulation process, rather than being thrombin, is factor Xa. This arises from the unique position of factor X at the convergence point of the coagulation cascade as illustrated in Fig 1.3. Factor Xa can be activated In situ by the extrinsic and intrinsic pathways. The cascade causes the amplification of a small stimuli through linked proteases of increasing concentration. Yin (143) calculated that under optimal conditions inhibition of lug of factor Xa would prevent the generation of 300 ug thrombin. Thus the tick anticoagulant appear crucial for the survival of the tick.

Recognition that the tick anticoagulant is detectable in blood and serous fluids from the immediate vicinity of the tick bite site is an aid to investigations of immunity to tick infestation. In this study, the purified salivary anticoagulant was shown to be poorly immunogenic. By keeping the limitations described in section 5.4.6 in mind, it can be concluded that the tick anticoagulant is not responsible for the induction and elicitation of antibody mediated host immune resistance to R. appendiculatus. If the salivary anticoagulant stimulated the production of immunoglobulins other than IgG or cell mediated responses the techniques used in this study will not be able to identify. In contrast, the immunoglobulins in TSGE immune sera from rabbits recognised a great variety of proteins in TSGE on immunoblotting. The poor immunogenecity of the tick salivary anticoagulant supports the idea that ticks, like other parasites, evade host reactions that would cause rejection by matching its host defences by an appropriate molecular repertoire of its own, and that this adaptation is not only a component of host specificity, but also of other factors such as reduced molecular antigenicity and other modulation of host immune responses. The other antigens detected in TSGE may serve as an alternative to the possibility of immunization against T. parva sporozoites surface coat determinants to protect cattle against East Coast Fever. It is anticipated that the genes encoding the antigenic proteins in tick saliva and TSGE could be located, isolated by recombinant

DNA techniques, introduced into *E. coli* for production of sufficient amounts of these bioactive molecules to examine their utility as defined vaccines against tick infestation.

Thus the limited knowledge of the poor efficacy of TSGE in protecting tick infestation and the evasion of host responses by tick is caused by:

a) a lack of information of the precise biological functions of salivary molecules and molecules with anti-tick reactivity.

b) a lack of information on the nature of mediators with no antigenic specificity which may affect tick feeding.

The work reported, in this project, is one step in the projected goal of purifiying host enzyme inhibitors and other molecules which inactivate host mediators at the tick attachment site thereby circumventing host defense.

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