Characterization of Gaddum's substance R

Douglas, Garry James

Award date:
1991

Awarding institution:
University of Bath

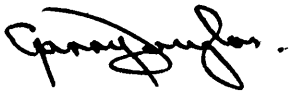
CHARACTERIZATION OF GADDUM'S SUBSTANCE R

submitted by Garry James Douglas

for the degree of PhD

of the University of Bath

1991



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ACKNOWLEDGEMENTS

Firstly I would like to thank Professor Rod Flower for uncovering Gaddum's original discovery of substance R and for his efforts to establish its fate. His enthusiasm, advice and wit were always gratefully received and it was a great pleasure to work with him.

Many thanks to Dr Ivor Williams for his supervision over the past three years. I hope to reciprocate with a fishing trip out on Blagdon Lake one day.

I received a great deal of advice from discussions and during correspondance with Dr Kanti Bhoola at Bristol University, and Professors Julie Chao and Henry Margolius at the Medical University of South Carolina. I am indebted to them for their collaboration using their antisera and antibodies, and to Miss Amber Elderfield for running the electrophoresis gels.

The pharmacology technicians and the animal house staff have always been most helpful and I thank them for that and their constant good humour.

Invaluable support was freely given by Drs Chris Bolton and Sue Peers, and by Mr Gordon Duncan. Many thanks must also go to other members of the department whose friendship I value.

I acknowledge the funding of this project by the SERC.

Lastly, I would like to express my thanks to Dr C. Pouton and Professor M. Schachter for agreeing to be my examiners.

SUMMARY

Perfusion of the rat isolated small intestine via the mesenteric artery liberates a hypotensive oxytocic principle (Gaddum's substance R) which is detectable in perfusate after 30 min and is present in samples collected 8 hours later. The oxytocic activity of substance R is lost after boiling, but is unaffected by treatment with thioglycolate. Furthermore, atropine, methysergide and indomethacin failed to antagonize uterine contractions to substance R. The pharmacological properties of substance R were compared to those of an oxytocic tissue kallikrein. Urinary kallikrein and substance R liberate a kinin from kininogen and share other pharmacological characteristics. The oxytocic and kininogenase properties of both substance R and urinary kallikrein are inhibited by Trasylol. In contrast, SBTI selectively inhibited the oxytocic and kininogenase activities of substance R, but not those of urinary kallikrein. D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, a selective BK_2 bradykinin receptor antagonist inhibited the oxytocic responses of substance R and urinary kallikrein. Gel filtration of substance R resolved a single peak of oxytocic and kininogenase activities with an estimated molecular mass of 40 kDa which was confirmed by SDS-PAGE analysis. A specific radioimmunoassay clearly demonstrated that substance R shares immunological identity with rat tissue kallikrein. Western blot analysis of substance R revealed a single band with a molecular mass of 39 kDa. It is concluded that substance R is a tissue kallikrein which may be distinguished from urinary and other rat tissue kallikreins by its susceptibility to SBTI. The exact nature of this enzyme remains to be elucidated.

CONTENTS

SECTION 1

INTRODUCTION

1.0 PREFACE

Throughout his career Gaddum maintained an interest in naturally-occuring substances and was responsible for a number of important discoveries in the study of neurotransmitters and autacoids and some of his first publications were concerned with their measurement (see Feldberg, 1967). In 1931, together with U.S. von Euler, Gaddum showed that extracts of brain and intestine contained a principle which stimulated smooth muscle (von Euler & Gaddum, 1931). This was identified as a polypeptide and named substance P. Substance P has since been demonstrated to be a neurotransmitter in peptidergic neurones with important central and peripheral actions (see Pernow, 1983). Gaddum and Feldberg (1934) showed that acetylcholine was the neurotransmitter at ganglionic synapses. Throughout the early 1950's the research carried out by Gaddum and his co-workers led to a fundemental understanding of the distribution and function of 5-hydroxytryptamine (5-HT) in the brain (see Feldberg, 1967).

Gaddum also had an enormous impact on the development of modern techniques of bioassay. He applied his interest in mathematics and statistics to the conventional methods of pharmacological research and designed the principles of quantitative bioassay. This work was instrumental in the acceptance of a mathematical approach with the result that pharmacology became a quantitative science in its own right. One of the many techniques which Gaddum refined or introduced was that of superfusion. In 1953 he published a method and an apparatus for its application in what has become a much cited paper entitled *The Technique of Superfusion* (Gaddum, 1953). In addition to his refinements and innovations of experimental bioassay techniques, Gaddum invented a number of pieces of equipment and utilised several unusual tissue preparations in his bioassays. Together with his textbook simply entitled *Pharmacology*, which was first published in 1940 and continues to be updated and reprinted, Gaddum's experimental work and the application of his approach has had a lasting influence on the discipline of pharmacology.

1.1 SUPERFUSION

The Oxford English Dictionary (O.E.D.) (1989) gives several definitions of superfusion, but in medical terms it is defined thus:

The technique of causing a stream of liquid to run over the surface of a piece of suspended tissue, keeping it viable and allowing the interchange of substances between it and the fluid to be observed.

The Dictionary credits Gaddum with its first use, quoting from his 1953 paper *The Technique of Superfusion*,

A piece of tissue may be suspended in air and kept in good condition by a stream of a suitable solution running over its surface... This technique may be called superfusion, since the fluid runs over the tissue, by analogy with perfusion, in which the fluid runs through the tissue.

Although this was the first time that the term superfusion had been used to describe the technique, it was not the first time that the technique itself had been used. Gaddum acknowleged this fact himself, but the O.E.D. omits his reference to Finkleman's experiments (Finkleman, 1930) in the above quotation.

1.2 THE DEVELOPMENT OF THE TECHNIQUE OF SUPERFUSION

Experiments where fluid from an electrically-stimulated nerve or muscle was utilised to prove the transmitter theory of nerve action required a significant reappraisal of modifications to the classical technique of Magnus (1904). The technique of superfusion was first used in 1930 by Finkleman in experiments demonstrating the release of a substance from one tissue to act on a second tissue, so-called "humoral transferance". However, similar experiments had been used before to show the phenomenon of humoral transferance of *vagusstoff* and *acceleranstoff* between frog and later mammalian hearts. Loewi (1921) took the fluid from a frog heart that had been electrically stimulated by the vagus, and stored it. When this fluid was reapplied to the heart in an unstimulated state Loewi found that a decrease in its activity was seen. He concluded that stimulation of the vagus resulted in the generation of a humoral factor which when transfered back to the

unstimulated heart inhibited The inhibitory substance was named *vagusstoff* which was later identified as acetylcholine. Loewi's observations were found by some to be unrepeatable (see Bain, 1932a) and controversy raged for several years (see MacIntosh, 1986) before an elegant modification was made to the method by ten Cate (1924), where perfusion fluid from a stimulated heart passed directly to the second, unstimulated, heart and humoral transferance of Loewi's *vagusstoff* was demonstrated conclusively.

Jendrassik (1924) applied the effluent from stimulated perfused rabbit hearts to isolated intestine. However, he too collected the perfusate before adding it to the conventional isolated intestinal preparation.

In 1930 Finkleman described experiments which showed that inhibition of the rabbit isolated duodenum occured after stimulation of the sympathetic innervation of the mesentery. Use of conventional gut bath techniques did not allow Finkleman to prove that the inhibition was due to the release of a humoral factor, there being too much dilution of the inhibitory substance to demonstrate its activity on a second duodenum preparation. In solving this problem Finkleman developed a technique which used the minimum amount of fluid possible to prevent dilution of the inhibitory factor. Finkleman suspended two lengths of duodenum in a warmed, moist chamber and superfused them in series with Ringer's solution. Thus the fluid passed directly from the first, stimulated length of gut to the second test piece of gut. Hence, Finkleman was able to demonstrate the humoral transferance of the inhibitory substance which he concluded was of an "adrenaline-like character".

A method based on that devised by ten Cate (1924) was soon utilised in conjunction with superfusion to demonstrate the release and action of *vagus substance* on tissues other than the heart. Bain (1932a) showed that vagal stimulation of one frog heart released *vagus substance* which passed directly to and inhibited a second heart. Bain modified his apparatus to show that when the fluid perfusing a stimulated heart flowed over a length of frog stomach muscle, this contracted. This was the first use of superfusion to demonstrate the action of a substance released from one tissue on

a second, different preparation, a technique which has resulted in a number of important discoveries in recent years. Bain (1932b) used the technique of superfusion succesfully a second time to demonstrate the release and humoral transmission of neurotransmitter from the dog tongue.

Gaddum himself used the superfusion technique prior to 1953 to show the action of a substance named *sympathin* on hen's caecum follwing its release from the perfused rabbits ear by stimulation of the "adrenergic" nerves (Gaddum *et al.*, 1939). Gaddum's apparatus differed somewhat to that of Finkleman, requiring as it did only a single piece of gut, but appears to be more practical. Despite being essentially the same as the apparatus described in the 1953 paper, Gaddum used no term to describe the method used.

Kwiatkowski (1941), a co-author on Gaddum's 1939 paper, used the superfused guinea-pig ileum as a "new technique for assaying histamine". The apparatus and method used were referred to as being "based on the principle used for the detection of sympathin", used by Gaddum *et al.* (1939) but gave no further details.

Despite these previous publications, it was not until the appearance of Gaddum's paper *The Technique of Superfusion* in 1953 (figure 1) that superfusion became widely used and the paper has become widely cited. However, less well known is that Gaddum used this publication to report the production by the perfused rat intestine of a factor with potent oxytocic activity which he named *substance R*.

1.3.0 GADDUM'S "TECHNIQUE OF SUPERFUSION" PAPER

1.3.1 The discovery of substance R

Gaddum appears to have returned to the use of the superfusion technique for the same reason that saw its innovation by Finkleman, that is, the problem of dilution of biologically active samples when using conventional methods of bioassay.

Gaddum began his paper by defining superfusion and making reference to the

earlier papers of Finkleman (1930), Gaddum *et al.* (1939) and Kwiatkowski (1941). The apparatus required was then described in detail and the methods and principles of its use, something which Kwiatkowski (1941) omitted to do.

The initial discovery of substance R was made whilst Gaddum was performing an experiment similar in design to that of Finkleman (1930) with modified apparatus, where "two tissues were suspended above one the other and the same fluid was superfused over them both in series". In experiments where a length of rat's small intestine was superfused above a rat's uterus Gaddum observed that

> ...a substance was sometimes liberated from rat's small intestine which caused a contraction of rat's uterus.

This original finding led to experiments where the whole of the small intestine was isolated and perfused with Tyrode's solution through the mesenteric vasculature. Samples of this perfusate were found to be oxytocic, causing a volume-dependent slow, delayed contraction of the superfused rat uterus.

In the results section Gaddum once again gave details of the superfusion technique before briefly describing the assay of histamine. Gaddum claimed that his technique provided a more sensitive assay than that of Kwiatkowski (1941) because Gaddum recommended the application of assay samples directly onto the tissue by pipette whereas Kwiatkowski injected the sample into the stream of superfusate above the tissue. It is interesting to note that the method which has become widely used is the more convenient, the latter rather than the former.

The remainder of the paper is devoted to the results of investigations into the nature of the oxytocic principle present in intestinal perfusate which Gaddum named "substance R". Gaddum demonstrated that the oxytocic activity could not be due to a number of known pharmacologically active substances, including acetylcholine, 5-HT, oxytocin or substance P. Substance R could be precipitated with acetone and crude preparations were made in this way. The oxytocic activity was not lost on dialysis of the perfusate, but it was destroyed by boiling. Gaddum did not consider

that either trypsin or chymotrypsin were present in and responsible for the activity of preparations of substance R, but he could not conclusively prove so. Study of the content of substance R in the gut led Gaddum to suggest that it may be secreted into the intestine by "some gland".

Despite his inability to positively identify substance R with any known substance, Gaddum noted at the end of his discussion that the known properties of substance R were similar to those then recently reviewed by Werle and Berek (1950) for kallikrein. Gaddum finished by stating that this possibility had not been fully explored and that substance R...

> may appear as a complication of experiments on the pharmacological activity of extracts of the intestine, or of experiments designed to detect the release of active substances by the intestine under various experimental conditions.

1.3.2 Subsequent developments to the method

After the publication of Gaddum's paper in 1953, the technique of superfusion became more widely used. Indeed, a modification to the technique, used for the detection of low concentrations of histamine in plasma on the guinea-pig ileum, was published the following year (Adam *et al.*, 1954). Other minor changes in protocol have been made over the years, but the principle remains the same. Some major, if little used, changes have also been made, for example, the sensitivity of the technique can be greatly increased if the tissue is immersed in a bath of oil to minimise the flow rate and volume of superfusate which bathes the tissue in a laminar flow (Ferreira & de Souza Costa, 1976).

Other modifications to the technique have led to important discoveries. Vane (1958, 1964, 1966), using the so-called blood bathed organ technique, developed an on-line method of detection of substances liberated into the circulation. A simple extension of Feldberg's and Gaddum's methods devised by Vane and his colleagues was to contribute to his Nobel laureate (see Vane, 1983). The superfusion of generally up to six different tissues in a cascade system allowed the differentiation of various

endogenous mediators to be made by a characteristic pattern of responses.

Vane's use and various adaptations of superfusion, including the perfusion of an organ such as the lung above the assay tissues, was responsible for the original detections of what were later identified as thromboxane A_2 (Piper & Vane, 1969) and prostacyclin (PGI_2) (Moncada *et al.*, 1976). Such experiments also led to the formation of Vane's hypothesis of the mechanism of action of aspirin (see Vane, 1983). The discovery of lipocortins (Flower & Blackwell, 1979) and the subsequent explanation of steroid action (see Flower, 1984) were also made possible by the use of this technique.

More recently superfusion of a column of cultured endothelial cells above a cascade of vascular tissue has been used for the bioassay of PGI_2 and endothelium-derived relaxing factor (EDRF) (Gryglewski *et al.*, 1986). This method has enabled the half-life of EDRF to be calculated accurately and has contributed to the attempts at identification of the substance responsible for the actions of EDRF.

Today superfusion remains the technique of choice to the pharmacologist where either little of an active substance is available, or a high concentration is required. Whilst the number and complexity of chemical methods of assay has continued to increase, the technique of bioassay of which Gaddum was a great proponent, still provides a valuable and simple tool for the quantification and even discovery of biologically active substances.

1.4.0 THE FATE OF SUBSTANCE R

Gaddum appears to have desisted from further investigation of substance R after 1953 despite maintaining an interest in kallikreins and kinins (Horton & Gaddum, 1958; Gaddum & Guth 1960). In September of that year a symposium was held in Montreal which was attended by many of the leading authorities of what may be called autocoids, including Gaddum himself. The work presented at this symposium dealt with the isolation and characterisation of substances which stimulated smooth

muscle, most of which had been found to be polypeptides. Gaddum edited the volume of papers presented at Montreal under the title of *Polypeptides which stimulate plain muscle* (Gaddum, 1955). It includes an introduction and a chapter on "The pharmacological analysis of tissue extracts" written by Gaddum but contains no reference to substance R, despite the fact that it provided an ideal illustration of a recent identfication of an unkown substance. A chapter by E. Werle on the chemistry and pharmacology of kallikrein makes no mention of either substance R or any kallikrein present in intestine. Perhaps discussions with his contemporaries at this symposium left Gaddum convinced that substance R was a kallikrein and further characterization by him was needless. The content of two similar volumes published soon after (Schachter, 1960; Erdös *et al.*, 1966) reflected the growing interest in substance P and the kallikrein-kinin system but in niether is there any mention of substance R. Throughout the 1950's, Gaddum's interest was also increasingly turning toward 5-HT and other centrally acting neurotransmitters (see Feldberg, 1967). Whatever the reason, Gaddum himself made no further mention of substance R in any of his published scientific writings and its discovery was not even acknowleged by Gaddum's own biographers (von Euler, 1966; Feldberg, 1967; Vogt, 1970; Holmstedt & Liljestrand, 1981).

A search of the literature revealed that substance R has continued to be neglected to the present time. The true identity of substance R was therefore unkown at the begining of the present study. Gaddum's own intuition suggested that substance R and kallikrein were similar, if not identical (Gaddum, 1953). Once the presence of substance R in intestinal perfusate was confirmed, a comparison of substance R and kallikrein was therefore made.

1.5.0 THE KALLIKREIN-KININ SYSTEM

1.5.1 Nomenclature of the components of the kallikrein-kinin system

There have been many names given to the various components of the kallikrein-kinin system since its discovery in Germany in the 1920's and of the bradykinin-bradykininogen system in Brazil in the late 1940's. Kallikrein itself has probably had the most pseudonyms, perhaps the most outrageous being the "Munich kid" (Fritz, 1981). With different groups working simultaneously on the characterization of these substances in different parts of the world at various times, it was perhaps inevitable that some confusion of the nomenclature would occur. In order to clarify and simplify the terminology in use, a meeting was held in Florence in 1965 (Webster, 1966). The background to many of the names currently in use has been described by Webster (1966, 1970b) and Hamburg (1978).

The term kininogenase, e.g. kallikreins, trypsin, pepsin, snake venoms, certain bacterial proteases etc, may be applied in a general sense to any enzyme which forms a kinin from an inactive precursor protein substrate (Webster, 1966).

Kallikreins (E.C. 3.4.21.8) belong to a large generic group of proteins, the serine proteases. Kallikrein was defined as an endogenous enzyme which rapidly and specifically liberates a kinin from kininogen (Webster, 1966, 1970b). The inactive zymogen of kallikrein is known as prekallikrein. In mammals there are two distinct kallikrein systems, those of plasma kallikrein and tissue kallikrein. Although the end products of these two systems are essentially similar and produce the same biological effects, each system fulfils specific physiological roles.

Kininogen is a term first introduced by Schachter and his colleagues (1962) for the stable substrate of kallikrein which was originally crudely prepared by Werle's group in 1937. There are two types of kininogen in human plasma, distinguished by their different molecular masses: low molecular weight (LMW) kininogen and high molecular weight (HMW) kininogen.

Schachter and Thain (1954) introduced the term kinin. Kinins are hypotensive polypeptides which contract most isolated smooth muscle preparations, but relax the rat duodenum (Webster, 1966, 1970b). Kinins also cause bronchconstriction in the guinea-pig, increase microvascular permeability, and produce pain when applied to the base of a skin blister. These low molecular weight polypeptides belong to a closely-related group which are present naturally in wasp and other insect venoms, or are released from a plasma substrate by the actions of kininogenases (Schachter 1980a). Kinins are acted upon by enzymes called kininases ultimately producing inactive peptide fragments.

A simplified scheme demonstrating the interactions of the major components of the kallikrein-kinin system is shown in figure 2.

1.5.2 The discovery of kallikrein

The hypotensive action of urine was first noted during the nineteenth century (see Erdös, 1970). Later, in 1909 Abelous and Bardier confirmed the presence of a hypotensive component of urine which was later partially purified by Pribtan and Herrnheiser in 1920 (see Werle, 1976). However, little attention was immediately paid to this work and it is not until the mid 1920's that the discovery of kallikrein can truly be considered to have taken place.

Whilst investigating post-operative reflex anuria in 1925, the Munich surgeon E.K. Frey found that prolonged hypotension followed intravenous injection of normal dog urine into the anaesthetised dog (Frey, 1926). Frey (1926) also observed that normal human urine produced a similar dose-dependent fall in arterial blood pressure but that this was less potent than dog urine. Frey firmly believed that he had discovered a novel hypotensive agent, despite the assurances of others that this activity was probably due to a number of pharmacologically active substances, amongst which histamine was a prime candidate. Frey followed his conviction however, and demonstrated that since his hypotensive agent remained after dialysis but not after boiling it could not be the low molecular weight, thermostable substance histamine.

Seeking help to isolate the active substance from urine Frey contacted the Nobel laureate R. Willstätter who suggested that Frey collaborate with his fellow worker H. Kraut. Being a chemist with experience in the isolation of high molecular weight, thermolabile substances, Kraut was able to prepare a stable standard preparation of the active hypotensive principle which was then named *Frey-stoff* (or *F-stoff*) and considered to be a circulating hormone (see Werle, 1976). Soon after it was shown that *F-stoff* was inactivated by blood, a process reversible by mild acidification (Frey and Kraut, 1928; Frey *et al.*, 1928). A number of other tissues, in several species, were also found to contain inhibitors of *F-stoff* (Kraut *et al.*, 1930b). If *F-stoff* was a hormone, some organ was producing it and the source of *F-stoff* was therefore sought. It was soon found that pancreatic juice from the fistulae of dogs and men and the content of a human pancreatic cyst produced hypotension in the dog (Kraut *et al.*, 1930a; Frey *et al.*, 1930). Extracts from the normal pancreas of several species, including man, were found to contain considerable amounts of *F-stoff* (Kraut *et al.*, 1930a). Hence, the decision was made to rename *F-stoff* "kallikrein" after the Greek synonym for pancreas, *kallikreas* (Kraut *et al.*, 1930a).

Kallikrein was not unique to the pancreas however, and was soon found in other glands and fluids. Just as the inactivation of *F-stoff* by blood could be reversed by mild acidification, acidification of serum alone was found to activate a substance with the same characteristics as *F-stoff* (Kraut *et al.*, 1928). This kallikrein in plasma was later assumed to be pancreatic kalllikrein, secreted into the blood and circulating in an inactive form, being activated before excretion in the urine. However, it is now known that urinary kallikrein is produced in the kidneys. Salivary glands were also shown to produce a kallikrein. The hypotension which was known to follow intravenous injection of saliva was found to be caused mainly by kallikrein and not acetylcholine as had been assumed previously (Werle & von Roden, 1936). By the late 1930's many other tissues were also found to contain kallikrein activity (see Webster, 1970a; Schachter, 1980a).

1.5.3 The enzymatic nature of kallikrein

That kallikrein was an enzyme rather than a circulating hormone was first recognized in the late 1930's when Werle and his colleagues were investigating its pharmacological actions. In 1928 Frey and Kraut had shown that kallikrein was not responsible for the dilatation of blood vessels which led to its hypotensive activity and another explanation was required (see Werle, 1976). The large size of the kallikrein molecule, coupled with its chemical nature, suggested to Werle that it may be an enzyme (Werle, 1976). Werle (1936c) found that the dog isolated gut was extaordinarily sensitve to kallikrein. However, incubation of the kallikrein with serum for 30 minutes abolished its spasmogenic and hypotensive activities. In contrast, incubation lasting only 2 minutes markedly increased the activity of the mixture (Werle *et al.*, 1936). In addition, this mixture stimulated the guinea-pig isolated ileum whereas neither kallikrein nor serum alone did. Werle concluded that kallikrein was acting as an enzyme, releasing the pharmacologically active *Substanz DK* (*darmkontrahiernde substanz*) from an inactive precursor present in plasma (Werle *et al.*, 1936; see Werle, 1976). Werle (1936b) considered that kallikrein itself circulated in a complex with an inactivator and was freed by another protease which itself was prevented from activating kallikrein by an inhibitor. In 1939 Werle and Grunz showed that *Substanz DK* markedly lowered blood pressure after intravenous injection and its enzymatic breakdown in plasma could be inhibited by cystein (see Werle, 1976; Webster, 1970a). When, in 1948 Werle and Berek suggested that *Substanz DK* be changed to "kallidin", being released from its precursor "kallidinogen" by the action of kallikrein on serum, the basic components of the kallikrein-kinin system were known to exist, and most had been named.

1.5.4 The discovery of bradykinin

In Brazil at this time, research was being carried out into the role of the venom from the snake *Bothrops jararaca* and of trypsin in the release of histamine and circulatory shock in the dog. This led to an important discovery of relevance to the established work in Germany. In 1948 a substance was identified in the blood of

one of the Brazilian group's experimental dogs which produced a slow contraction of the guinea-pig isolated ileum which was not acetylcholine or histamine (Rocha e Silva *et al.*, 1949). This substance was given the name "bradykinin" from the idea of a "slowly acting substance" from the Greek *brady* (slowly) and *kinin* (movement) (Rocha e Silva, 1976a, 1976b). This group also found that the physicochemical properties of bradykinin suggested that it was a polypeptide of low molecular weight, released from the globulin fraction of plasma from an inactive precursor which they named "bradykininogen", and that it could be destroyed by plasma enzymes (Rocha e Silva *et al.*, 1949). However, because of the apparently confusing nature of the literature at this time, bradykinin was not linked immediately with kallidin (Rocha e Silva, 1976a, 1976b).

1.5.5 The structure of kinins

Bradykinin was the first kinin to be purified and synthesized (see Elliott, 1970). After perfecting the isolation of bradykinin from ox blood, in 1959 Elliott and his colleagues deduced its amino acid composition (Elliott *et al.*, 1960). The following year two independent groups synthesized bradykinin and deduced its structure as H-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg-OH (Elliott *et al.*, 1960; Boissonnas, 1962; Boissonnas *et al.*, 1963). By 1962 bradykinin had been characterized using bioassays by comparison with synthetic analogues (see Elliott, 1970).

Soon after the characterization of bradykinin, kallidin was identified as the closely related decapeptide lysyl-bradykinin (Webster & Pierce, 1963). Bradykinin and Lys-bradykinin were shown to be two of the components of the so-called substance Z (see Gaddum & Horton, 1958) found in urine, together with a third, undecapeptide kinin, methionyl-lysyl-bradykinin (Miwa *et al.*, 1969). Met-Lys-bradykinin is thought to be formed by the action of pepsin on kininogen in acidified urine (see Pisano, 1979).

1.5.6 Soruces of other kinins

Several kinins have been identified which are generated as a result of the action of a kininogenase contained in leukocytes (Greenbaum *et al.*, 1978; Greenbaum, 1979). These "leukokinins" range from 21-25 amino acid residues in length but are thought not to contain the bradykinin moiety *per se* since peptide analysis has revealed they contain only one phenylalanine residue whereas bradykinin contains two (Greenbaum, 1979). Okamoto and Greenbaum (1983) discovered an additional kinin, "T-kinin" (Ile-Ser-bradykinin) which is liberated from rat plasma in the presence of high concentrations of trypsin, and more recently Met-Ile-Ser-bradykinin (Met-T-kinin) was discovered (Sakamoto *et al.*, 1989).

Other kinins of mammalian origin have been isolated containing as many as sixteen amino acid residues, but all are very weak agonists compared to bradykinin and there has been some doubt as to their authenticity and physiological relevance (see Pisano, 1970).

Schachter and Thain (1954) were the first to characterize a kinin from non-mammalian sources. The venom of the common European wasp, *Vespa vulgaris*, yielded kinin-like activity which was later shown to be due to the presence of three separate kinins (Mathias & Schachter, 1958). Since these original observations, the structure of the kinins in wasp and several other insect venoms and also kinins from amphibian tissues have been determined. These may have up to eighteen peptide residues in total, built around a bradykinin core, sometimes with substitutions to the bradykinin moiety (see Pisano, 1970, 1979; Bertaccini, 1976).

Kallikrein was found in hen excreta in 1936 (Werle *et al.*, 1936) which was hypotensive only in the hen and not in mammals (Werle & Hürter, 1936). The generation of an "ornithokinin" from bird plasma was later demonstrated by Werle *et al.* (1966). This differed from mammalian kinin in terms of its amino acid composition, molecular weight and pharmacological activity. Ornithokinin was not hypotensive in mammals and it was therefore assumed that ornithokinin was not

closely related structurally to bradykinin (Werle *et al.*, 1966). However, the structure of ornithokinin has recently been reported as [Thr^6,Leu^8]-bradykinin (Kimura *et al.*, 1989). This group confirmed Werle's finding that the pharmacology is markedly different to bradykinin and concluded from these structural and pharmacological differences that residue 8 in the kinin sequence is of critical importance in the specific interaction of kinins with their receptors.

1.5.7 Kinin receptors and antagonists

As early as 1960 Collier and his colleagues (Collier & Shorley, 1960) proposed the existence of more than one bradykinin receptor. Several aspirin-like compounds specifically antagonized the bronchoconstrictor activity of bradykinin *in vivo* in the guinea-pig, but did not specifically antagonize the increased vascular permeability in guinea-pig skin, nor bradykinin's action *in vitro* on the guinea-pig ileum or rat duodenum (Collier & Shorley, 1960). Collier therefore concluded that there were two types of bradykinin receptor, by analogy with the two types of acetylcholine receptor which are antagonized by two different antagonists acting at separate sites (Collier & Shorley, 1960).

Whilst studying the inflammatory action of bradykinin in 1970, Rocha e Silva hypothesised that there were two bradykinin receptors. One bradykinin receptor mediated pain (P receptor) whilst the second (S receptor) which mediated plasma extravasation also appeared to be present in the guinea-pig ileum and some vascular tissues (see Barabé *et al.*, 1977). It had also been suggested that there were structural differences between bradykinin receptors in vascular and visceral smooth muscle (Edery & Grunfeld, 1969). Other evidence gained from electrophysiological and empirical studies supported this idea of two types of bradykinin receptor (see Boschcov *et al.*, 1984).

These distinctions of receptors however, were not based on any sound pharmacological criteria such as those proposed by Schild (Regoli *et al.*, 1978), the most important criterion of which is the demonstration of competitive antagonism.

However, researchers in this field had to await the synthesis of effective competitive antagonists. The analysis of the structure-activity relationship of hundreds of bradykinin analogues over the last 25-30 years has recently led to the development of effective receptor antagonists (see Schröder, 1970; Stewart, 1979; Regoli & Barabé, 1980, 1988; Vavrek & Stewart, 1989; Regoli *et al.*, 1990). Two bradykinin receptor subtypes (BK_1 and BK_2) have been identified in accordance with Schild's recommendations using competitive antagonists and more rigorous criteria (Regoli & Barabé, 1988).

The order of potency of bradykinin, kallidin and their kininase I (see below) metabolites, des-Arg^9-bradykinin and des-Arg^{10}-kallidin was shown to vary between several isolated organs. Thus, it was found that des-Arg^9-bradykinin and des-Arg^{10}-kallidin were more potent in the rabbit aorta than their native precursors (Regoli *et al.*, 1977). This was in contrast to other isolated preparations such as the cat ileum and rat uterus, where the native kinins were more potent than their kininase I metabolites (Barabé *et al.*, 1977). These contradicting results suggested to these authors the presence of two kinin receptors which were named B_1 for those in the rabbit aorta and mesenteric vein, and B_2 for those in the rat uterus, cat ileum and rabbit jugular vein (Regoli *et al.*, 1978).

Bradykinin receptors have since been identified and defined in a number of different tissues (see Regoli *et al.*, 1988). The identification of the two forms of receptor gave further impetus to research into the structure-activity relationship of kinins and soon antagonists were being synthesized and evaluated in bioassays (Regoli & Barabé, 1980), although the development of more specific and potent antagonists was to take several more years.

Study of BK_1 receptor systems revealed that an increase in sensitivity of the rabbit aorta and mesenteric vein preparations occurred gradually during *in vitro* incubation of these tissues (Regoli *et al.*, 1978). This was found to be the result of a *de novo* synthesis of the BK_1 receptors, and could be blocked by the protein synthesis inhibitors actinomycin D and cycloheximide (Regoli *et al.*, 1978). This phenomenon

occurred in all preparations containing BK_1 receptors studied (Regoli & Barabé, 1980). In contrast to these results it has recently been reported that cultured rat aortic vascular smooth muscle cells possess only BK_2 receptors (Hirata *et al.*, 1989). This may be a species difference or may reflect functional changes of cell membranes during culture leading to loss of BK_1 receptors (Hirata *et al.*, 1989). The *de novo* synthesis of BK_1 receptors has also been demonstrated *in vivo*, following intravenous injection of endotoxin into rabbits (Marceau *et al.*, 1983).

The use of competitive antagonists has confirmed the two-receptor hypothesis of Regoli and colleagues (1978). BK_1 bradykinin receptor antagonists, for example, [Leu^8],des-Arg^9-bradykinin (Regoli & Barabé, 1980), are active only in tissues possessing BK_1 receptors and are completely inactive in BK_2 receptor systems (Regoli *et al.*, 1989). On the other hand, BK_2 receptor antagonists are non-selective. These antagonists such as the prototype [Thi^{5,8},D-Phe^7]-bradykinin (Regoli *et al.*, 1986a) are active on both BK_1 and BK_2 receptor systems (Regoli *et al.*, 1989). It is thought that the BK_2 receptor antagonists are converted by kininase I and other carboxypeptidases to metabolites devoid of the C-terminal Arg which are active as BK_1 receptor antagonists (Regoli *et al.*, 1986b; Regoli *et al.*, 1989), an action that is significantly reduced by inhibition of kininase I (Regoli *et al.*, 1986b). Boschcov and colleagues (1984) demonstrated that the relaxant and contractile effects of bradykinin in the rat isolated duodenum are mediated by different sites which are further distinguished by their apparent affinities for agonists and potentiating peptides.

A number of other bradykinin analogues have been synthesized which are specific antagonists of BK_2 bradykinin receptors, one of which, D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin (Regoli *et al.*, 1986a; Schachter *et al.*, 1987), blocks bradykinin-induced vascular permeability in rabbit skin as well as inhibiting responses to bradykinin *in vitro* (Schachter *et al.*, 1987).

More recently three bradykinin analogues have been synthesized which are extremely potent long-acting specific antagonists of bradykinin in a number of

different preparations, *in vitro* and *in vivo* (Lembeck *et al.*, 1991). The most active of these was D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin. All three of these compounds had a long duration of action and it was suggested that they therefore have a high affinity for bradykinin receptors. However, the receptor subtype specificity of these antagonists was not investigated.

Farmer *et al.* (1989) have suggested the presence of a third subtype of bradykinin receptor in lung tissues, the B_3 receptor. BK_2 receptor antagonists were not effective against bradykinin-induced bronchoconstriction in the guinea-pig. It had previously been reported that *in vitro*, BK_2 receptor antagonists were inactive against contraction of airway smooth muscle induced by bradykinin (see Farmer *et al.*, 1989). In addition responses to bradykinin in lung tissues were not inhibited by BK_1 receptor antagonists and thus the effects of bradykinin in this organ are mediated by specific bradykinin receptors which are of neither the BK_1 nor BK_2 subtype (Farmer *et al.*, 1989). Interestingly the new long-acting bradykinin receptor antagonists described by Lembeck *et al.* (1991) were effective inhibitors of bradykinin-induced bronchoconstriction in the guinea-pig.

Recently a study of the vascular actions of bradykinin in the guinea-pig isolated hind brain has also revealed the presence of a third type of bradykinin receptor (Cohen-Laroque *et al.*, 1990). Both selective BK_1 and BK_2 receptor antagonists had similar activities against bradykinin-induced vasoconstriction in this preparation. The receptor mediating this response had other pharmacological characteristics which do not match those previously described for BK_1 or BK_2 receptors and it was concluded it may be of a novel third subtype.

The receptors which mediate the biological responses of bradykinin have as yet not been isolated and characterized as others have, e.g. the nicotinic acetylcholine receptor. However, binding sites for radiolabelled bradykinin and analogues have been identified in a number of tissues, although early studies were complicated by binding to kininase enzymes. Odya *et al.* (1980) demonstrated high and low affinity, specific, saturable binding sites for bradykinin and related peptides in the

myometrium of bovine uterus. The affinities were similar to those reported in a previous publication for rat uterus (see Odya *et al.*, 1980). This group considered that the properties of these binding sites were consistent with those expected of receptors (Odya *et al.*, 1980). Binding sites were also found in guinea-pig ileum, colon, duodenum, heart, and kidney, and rat uterus (Innis *et al.*, 1981). The relative potencies of bradykinin analogues at binding sites in guinea-pig ileum correlated well with their potencies in contracting smooth muscle (Innis *et al.*, 1981). There was not, however, such good correlation in the rat uterus, but it was thought that this may be explained by discrepancies in the contractile potencies of peptides in ileal and uterine tissues which may be due to a difference in recognition sites (Innis *et al.*, 1981). Manning *et al.* (1982) further investigated the binding sites in guinea-pig ileum and found that specific bradykinin receptor binding sites occur in the mucosa as well as the muscle region. Localization of receptors was demonstrated by light microscopic autoradiography, and these results correlated well with the biological sites of action of bradykinin (Manning *et al.*, 1982). The medullary interstitium of the kidney also contains bradykinin binding sites (Manning & Snyder, 1986). Bradykinin binding sites with low and high affinities were demonstrated on cultured rat aortic vascular smooth muscle cells (Hirata *et al.*, 1989) and human fibroblasts (Roscher *et al.*, 1983).

The pharmacological evidence for the existence of a BK_3 receptor subtype in guinea-pig lung was corroborated by binding studies carried out on displacement of radiolabelled bradykinin by BK_1 and BK_2 antagonists. In lung parenchyma 40% of the binding sites were of neither the BK_1 nor BK_2 subtype, whilst in tracheal tissue binding of labelled bradykinin was not displaced by either type of antagonist and thus these sites appeared to be exclusively of the BK_3 subtype (Farmer *et al.*, 1989).

In summary, three different bradykinin receptors, BK_1, BK_2 and BK_3 have been identified and characterized by use of synthetic agonists and antagonists. Membrane binding sites for bradykinin have also been identified in a number of tissues and species and binding studies have corroborated the biological evidence . The location

of the BK_1 and BK_2 receptor subtypes and ther implication in a variety of biological activities has recently been reviewed (Regoli *et al.*, 1989, 1990).

1.5.8 Kininogens

Kininogens are single chain acid glycoproteins synthsised and secreted by the liver, which reside in the alpha-2-globulin fraction of normal plasma. Kininogens are the high molecular weight precursors of kinins and have been purified from a number of species (see Movat, 1979). Crude preparations of kininogen were prepared by early investigators by heating plasma to 60°C for 1-3 hours, but later improved methods were employed and more purified samples were prepared (see Schachter, 1969).

Studies carried out in the 1960's by the groups of Margolis and Vogt (see Movat, 1979) indicated that plasma contains more than one form of substrate for kallikrein. Following on from the work of Margolis, Vogt (1966) postulated that there are two kinin-forming systems in plasma, each containing a separate kininogen and kininogenase. Jacobsen (1966a, 1966b) used gel filtration chromatography to resolve for the first time, two plasma kininogens of different molecular weight which he referred to as substrates 1 and 2. In plasma from a number of species the HMW form of kininogen acted as a substrate for both plasma and glandular kallikreins whereas the LMW form was only a good substrate for glandular kallikreins and not plasma kallikrein (Jacobsen, 1966a, 1966b).

These two forms of kininogen have been extensively studied in a number of species, especially in man. However, there are large species differences in the size of HMW kininogen (see Movat, 1978, 1979; Kato *et al.*, 1981; Müller-Esterl & Fritz, 1984). For a time the presence of HMW kininogen in human plasma was disputed until a condition known as Williams or Fitzgerald trait was described in which there is a deficieny of HMW kininogen (see Movat, 1979). Human HMW kininogen was shown to have a molecular mass of 114 kDa by SDS-electrophoresis, and to consist of a heavy chain of 63 kDa and a light chain of 58 kDa. HMW kininogen circulates

in normal plasma as a complex with prekallikrein, attached to the heavy chain of the kininogen (see Silverberg and Kaplan, 1988). This chain also contains regions which bind to Hageman factor and subendothelial sufaces (Sugo *et al.*, 1980). LMW kininogen was similarly comprised of two chains, of 62 and 5.4 kDa, with the native protein having a molecular weight of 68 kDa (Müller-Esterl & Fritz, 1984). In both kininogens the heavy chain contains the kinin sequence (Müller-Esterl *et al.*, 1986).

A third kininogen has more recently been described in rat plasma. It differs markedly from HMW and LMW kininogen in that it is not susceptible to proteolytic cleavage by kallikreins (Okamoto & Greenbaum, 1984). Trypsin and cathepsin D act on this "T-kininogen" to release Ile-Ser-bradykinin (Okamoto & Greenbaum, 1983) and Met-Ile-Ser-bradykinin is released by an acid protease (Sakamoto *et al.*, 1989). Two forms of T-kininogen have been isolated by gel filtration chromatography and SDS-PAGE with a molecular weight of 69 kDa (Okamoto & Greenbaum, 1984; Greenbaum & Okamoto, 1988).

1.5.9.0 Kinin-forming pathways

Kallikreins can be divided into two classes: plasma kallikreins and tissue (or glandular) kallikreins. The observation by early investigators that *F-stoff* was present in the pancreas led them to believe that it was synthesized there prior to its release into the circulation and subsequent renal excretion (see Werle, 1976). However, it is now known that plasma, pancreatic and urinary kallikreins all differ from one another.

The differences between kallikreins from various sources first became apparent to the original workers in this field, but the significance of their observations was not recognized. It was known, for example, that plasma kallikrein required activation whereas the kallikrein present in urine was already in an active form (Kraut *et al.*, 1928; Werle, 1936b).

In 1955 Werle and his colleagues showed that kallikrein from plasma exists as an inactive precursor, prekallikrein or prokallikrein, which may be activated by enzymatic processes (Werle, 1976). Pancreatic kallikrein was likewise demonstrated to exist in an inactive form, relying on contact with gastric juices for its activation (Fielder & Werle, 1967). Kallikreins from most other glandular sources appear not to require activation (Webster, 1970a), although prokallikrein is also found in the kidney and in urine and some other tissues (Spragg, 1983, 1988; Yakada *et al.*, 1985).

The first known inhibitor of kallikrein was found in human blood by Frey and Kraut (1928). After further investigation it was assumed that the inactive kallikrein in human plasma was formed by a complex of pancreatic kallikrein and the circulating inhibitor. It is now clear, however, that plasma kallikrein circulates as an inactive precursor of kallikrein which is activated by Hageman factor (factor XII) (Werle, 1955; Colman & Bagdasarian, 1976). Thus, plasma prekallikrein is a proenzyme in a cascade system analagous to other zymogens such as prothrombin.

Later, studies of various inhibitors of kallikrein revealed other differences between kallikreins derived from several sources and species which were supported by immunochemical evidence (see Webster & Pierce, 1963; Webster, 1970a). All plasma kallikreins which were studied were found to be inhibited by soy bean trypsin inhibitor (SBTI) whereas those kallikreins derived from other sources were not (see Webster, 1970a).

The two types of kallikrein also liberated different kinins from plasma. Separation of kallidin and bradykinin by chromatography enabled Webster and Pierce (1963) to show that glandular kallikreins generated only kallidin whereas plasma kallikrein liberated only bradykinin from plasma kininogen. These findings were soon confirmed by others (Habermann, 1966; see Webster, 1970a) and further biochemical distinctions were made shortly after.

1.5.9.1 Plasma kallikrein

Using ion-exchange chromatography plasma kallikrein was first separated by Becker and his colleagues (Kagen *et al.*, 1963; Becker & Kagen, 1964). Improvements in biochemical separation procedures have since enabled a greater purification of activated plasma kallikrein and its precursor prekallikrein.

A number of authors, utilizing a variety of techniques, have attributed different molecular weights to human plasma prekallikrein and kallikrein. Gel filtration chromatography of prekallikrein in normal plasma and in a complex with HMW kininogen gave a molecular mass of 285 kDa whereas prekallikrein from HMW kininogen-deficient plasma was isolated with a molecular weight of 115 kDa (Mandle *et al.*, 1976). The following year however, the same group reported two molecular weight variants of prekallikrein, of 85 kDa and 88 kDa using SDS-electrophoresis (Mandle & Kaplan, 1977).

There are several enzymes which may catalyse the activation of prekallikrein. These include trypsin, factor XII, factor XIIa and factor XII fragments (see Heimark & Davie, 1981). Following activation with activated Hageman factor (factor XIIa), and under reducing conditions, SDS-electrophoresis revealed two disulphide-linked, "kallikrein-light" and "kallikrein-heavy" chains of 33 kDa or 36 kDa and 52 kDa respectively (Mandle & Kaplan, 1977). Using radiolabelled inhibitor, the active site of kallikrein was found to reside in the light chain of the molecule (Mandle & Kaplan, 1977), the heavy chain containing a region which binds to the heavy chain of HMW kininogen (see Silverberg and Kaplan, 1988). Other reports have estimated the molecular weight of human prekallikrein to be approximately 100 kDa and both bovine and rabbit prekallikreins are of similar sizes (see Movat, 1979; Schachter, 1980a).

A number of workers have reported that neither bovine, human (see Movat, 1978, 1979) nor hog (see Schachter, 1980a) plasma prekallikrein undergoes a significant change in molecular weight upon activation by Hageman factor. Mandle and Kaplan

(1977) found that there was no difference in either molecular weight or charge between the active kallikrein and proenzyme. It has been assumed therefore that only limited proteolysis occurs to the prekallikrein during activation. On the other hand, activation of rabbit prekallikrein by factor XIIf (see Movat, 1979) and activation of human prekallikrein by kallikrein itself (Mandle & Kaplan, 1977) resulted in cleavage of a fragment of approximately 10 kDa from prekallikrein in both cases.

Circulating kallikrein may be inhibited by a number of plasma-borne inhibitors; C_1 inactivator, alpha-2-macroglobulin, alpha-1-antitrypsin and antithrombin III, but the former two are considered to be the major inhibitors (see Harpel *et al.*, 1975; Schachter, 1980a). To summarise, plasma prekallikrein, the form of kallikrein normally found in plasma, has a molecular weight of approximately 90-100 kDa and consists of two disulphide-linked chains of approximately 35 kDa and 55 kDa. This molecule appears to undergo no significant change in molecular weight or other properties on activation to kallikrein by its normal activator, Hageman factor. The light chain contains the enzymatic active site whilst the heavy chain interacts with HMW kininogen. Plasma kallikrein is inactivated by a number of circulating inhibitors.

The amino acid sequence of human prekallikrein is now known (Chung *et al.*, 1986) and is highly homologous with that of human factor XI (Fujikawa *et al.*, 1986). Chung *et al.* (1986) reported no heterogeneity of the sequence, and the two variants of the light chain reported by Mandle and Kaplan (1977) may be due to differences in carbohydrate content.

1.5.9.2 Tissue kallikrein

Tissue kallikreins have been isolated from a wide variety of species and tissues over recent years. Immunocytochemical studies have revealed that tissue kallikreins are also present in exocrine secretions and in low concentration in plasma (Rabito *et al.*, 1980, 1982; Woodley *et al.*, 1985). Active tissue kallikrein circulating in plasma is

bound to a "kallikrein-binding protein", distinct from the circulating plasma kallikrein inhibitors, which has recently been isolated and characterized (Chao *et al.*, 1986b; Chao *et al.*, 1990).

Tissue kallikreins differ from plasma kallikrein in both physical and chemical properties and are clearly distinctly different enzymes (MacDonald *et al.*, 1988). As one reviewer commented, the relationships between these two groups is not closer than, e.g., those between trypsin and thrombin (Fielder, 1979).

Kallikreins from glandular or tissue sources differ from plasma kallikreins in their substrate specificities and the kinins which they release from kininogens. Bradykinin is liberated from kininogen by plasma kallikrein whereas tissue kallikrein releases kallidin (Webster & Pierce, 1963; Habermann, 1966). Plasma kallikrein acts only on the HMW form of kininogen whilst LMW kininogen is the primary substrate of tissue kallikrein (Jacobsen, 1966a, 1966b).

The two groups of enzymes also differ in their susceptibility to inhibitors. The polyvalent trypsin inhibitor aprotinin (Trasylol), isolated from bovine pancreas and lung, inhibits both plasma and tissue forms of kallikrein (Trautschold *et al.*, 1967), whilst other enzyme inhibitors are more selective (Back & Steger, 1968). It is well documented that plasma kallikreins are inhibited by soy bean trypsin inhibitor (SBTI) whereas all tissue kallikreins tested were not (Werle & Maier, 1952; Werle & Trautschold, 1963; Back & Steger, 1968; Webster, 1970a; Vogel, 1979).

The kallikrein present in most tissues is usually in an active form, whereas that in plasma exists as an inactive proenzyme. However, an inactive precursor of kallikrein has been noted in colon, kidney, urine and other tissues as well as pancreas where the proenzyme was discovered in 1930. Extracts of colon, intestine, lymph nodes brain and nerves of several species have also been found to contain a trypsin-activated tissue kallikrein-like enzyme (Werle & Vogel, 1961).

The inactive form of pancreatic kallikrein is rapidly converted to active kallikrein by

trypsin and this form of enzyme has been extensively studied (see Webster, 1970a; Fielder, 1979). Trypsin also activates the prokallikrein or "latent kallikrein" found in human urine and kidney (see Spragg, 1983 & 1988; Yakada *et al.*, 1985). Whilst Spragg (1988) reported isolation of latent kallikrein with a molecular weight of 55-60 kDa by electrophoresis, Yakada *et al.* (1985) found a molecular mass of 48 kDa by the same method. Other differences appear in these reports. Activation of the proenzyme cleaved a heptapeptide activation fragment and resulted in a drop in molecular weight to 46 kDa (Yakada *et al.*, 1985). Spragg (1983), however, reported the loss of a 10 kDa fragment on activation by trypsin and molecular weight estimation by chromatography, in agreement with a previous report. Yakada *et al.* (1985) found that the most efficient activator of prokallikrein was the metalloprotease thermolysin, with trypsin being more active than plasma kallikrein.

Whilst the above reports differ in some details, both authors concluded that the isolated kallikrein was of a tissue type, the molecular weights, for example, being much lower than expected for a plasma kallikrein.

Recently, human urinary kalikrein was isolated with a molecular weight of 45 kDa by gel filtration which corresponded to that of 45 kDa for the trypsin-activated 48 kDa inactive kallikrein also isolated (Kizuki *et al.*, 1986). Similar results were obtained in the rat where inactive urinary kallikrein was found with a molecular weight of 44 kDa, both urinary kallikrein and trypsin-activated inactive kallikrein being 38 kDa (Takaoka *et al.*, 1986).

Another kallikrein, found in the wall of the colon, however, appears to be a plasma kallikrein. This enzyme was isolated in an inactive form which was activated by trypsin and resembled plasma kallikrein in its molecular weight and inhibition pattern (Seki *et al.*, 1972).

Some tissue kallikreins then, are released in an inactive form, but generally tissue kallikreins appear not to require a proper process of activation (Webster, 1970a). Tissue kallikreins have now been identified in a great many tissues. By the late

1930's Werle and his colleagues had demonstrated the presence of kallikrein not only in blood, urine and pancreas, but also in extracts of salivary glands, intestine, lung and neuronal tissues (see Schachter, 1980a). Urinary, kidney, submandibular salivary gland, salivary and pancreatic kallikreins of many mammalian species have been the most extensively studied (see Schachter, 1966, 1980a; Webster, 1970a; Nustad *et al.*, 1976; Fielder, 1979) whilst the presence of kallikrein in guinea-pig coagulating and prostate glands is well established (Bhoola *et al.*, 1961, 1962). More recently tissue kallikreins have been discovered in a wide variety of tissues (see table 1). However, tissue kallikreins may be even more widely distributed, as recent evidence from the identification of kallikrein genes suggests (MacDonald *et al.*, 1988; Margolius, 1989).

The localization of kallikreins in many tissues has been visualised by immunochemical methods (see Berg-Østravik *et al.*, 1980; Schachter, 1979, 1980b; Schachter *et al.*, 1986; Berg, 1988). Like other secretory proteins, kallikrein is synthesized on the rough endoplasmic reticulum and then glycosylated in the Golgi apparatus. A signal peptide of 17 amino acids is believed to be cleaved from the N-terminal of the newly synthesized molecule, leaving prokallikrein with a short 7 amino acid activation peptide which may then be removed by one of a number of possible proteases (see MacDonald *et al.*, 1988). Granular intracellular stores of tissue kallikrein have been reported (Bhoola *et al.*, 1979). Kallikreins from tissue sources are also of a markedly different molecular weight when compared to plasma kallikreins. Fielder (1979) produced a comprehensive survey of the literature to that date, and despite differences in species and tissue type and techniques, the various estimates of molecular weight were between 25-45 kDa. All the tissue kallikreins isolated were inhibited by aprotinin but were unaffected by SBTI. More recently monoclonal antibodies against a number of tissue kallikreins have been developed. These have enabled highly purified preparations of several tissue kallikreins to be obtained.

The primary structures of some tissue kallikreins are now known, either from amino

acid sequencing of the purified protein, or being deduced from the mRNA sequence where this has been identified.

Porcine pancreatic kallikrein has been purified and sequenced. There are two known forms of this kallikrein, but the amino acid sequence was identical for both (Tschesche *et al.*, 1979). It was reported that there are some slight changes in the amino acid sequences between pancreatic and other tissue kallikreins in the pig, however, the major differences in molecular weights and charge were accounted for by differences in glycosylation (Tschesche *et al.*, 1979).

Some evidence suggested heterogeneity of human tissue kallikreins, including multiple forms of urinary kallikrein. However, Lu *et al.* (1989) showed from sequence data that human urinary kallikrein consists of a single polypeptide chain. The theoretical molecular mass of 26.4 kDa from amino acid composition differed markedly from the two forms isolated by electrophoresis with masses of 38 kDa and 42.5 kDa (Lu *et al.*, 1989). These and other different forms previously reported are due to heterogeneity in glycosylation rather than the primary protein structure.

The sequencing of tissue kallikreins has enabled cDNA libraries to be probed and a kallikrein gene family has now been identified (Gerald *et al.*, 1986; Chao & Chao, 1987). The number of genes encoding the kallikrein gene family is dependant on species, ranging from 4 in man to 27 in mouse (see MacDonald *et al.*, 1988). The kallikrein gene family encodes for serine proteases other than tissue kallikreins *per se* which often differ from kallikrein at key amino acid residues that are responsible for cleavage specificity, but some of these may act as kininogenases. Analysis of the mRNA within species reveals a very high homology (74-89%) between members of the family. Determination of gene structure shows that the tissue kallikrein of different species is very similar, with 59-70% homology, whilst within species all tissue kallikreins share the same gene (see MacDonald *et al.*, 1988). As yet the biological function of all the members of the kallikrein gene family remain to be elucidated, and it is thought that some genes may be redundant.

Tissue kallikreins are also differentiated from plasma kallikrein in that they have little or no proteolytic activity on proteins other than kininogens (Erdös, 1979) whilst plasma kallikrein has a wider spectrum of substrates. This lower substrate specificity of plasma kallikrein is thought to explain its greater susceptibility to inhibitors (Schachter, 1980a).

1.5.9.3 Other kinin-generating systems

An alternative kinin generating system exists in a number of types of leukocytes (Greenbaum, 1979). The acid protease which releases leukokinins is bound within the cell rather than present in plasma and is released to act on the substrate. This is now identified as a cathepsin D-like enzyme (Greenbaum, 1984). The kininogen from which leukokinins are released has a molecular mass of 100 kDa (Roffman & Greenbaum, 1979) and differs from normal HMW kininogen in that it is not acted upon by kallikreins. More recently, the study of leukokininogen levels in human and animal plasmas uncovered a hitherto unknown pathway of kinin generation. The total kininogen content of 1.0 ml plasma was classically measured by kinin liberation by 0.1 mg trypsin (Greenbaum, 1984). However, when a much increased concentration of trypsin was used (up to 1.5 mg ml^{-1}), an unexpected rise in kinin release was seen (Okamoto & Greenbaum, 1983). This additional kinin was found to chromatograph differently from bradykinin, Lys-bradykinin and Met-Lys-bradykinin. The structure of this "T-kinin" ("T" from trypsin) was shown to be Ile-Ser-bradykinin (Okamoto and Greenbaum, 1983). It appears that another enzyme, from granulatomous tissues of rats, may act on T-kininogen to release Met-T-kinin (Sakamoto *et al.*, 1989). The supposed existence of a T-kininogenase has been confirmed by experimental results. The rat and mouse submandibular glands contain a 28 kDa enzyme which is activated by thiol (Barlas *et. al.*, 1987). A very similar protease of 27 kDa was found in rat submandibular gland by another group (Gutman *et al.*, 1988), but they did not comment on its activation by thiol. Other enzymes such as cathepsin D may also release T-kinin (see Barlas *et al.*, 1987).

1.5.10 Inactivation of kinins

Enzymes which inactivate kinins act at both the N-terminal and the C-terminal of the bradykinin chain. The most important of these inactivators are the kininases I and II which cleave the molecule at the C-terminal. Peptides such as bradykinin and kallidin are highly charged and it is unlikely that they can cross the cell membrane. Intracellular proteases therefore probably have little influence over the metabolic fate of kinins, and the most important proteases are located extracellularly. None of the enzymes which act on kinins are substrate specific, but may act on other peptides with susceptible bonds. Thus, there is an interaction between the kinin systems and others such as the complement and renin-angiotensin systems.

The conversion of kallidin to bradykinin was originally observed in human plasma (Webster & Pierce, 1963) and in horse plasma (Erdös *et al.*, 1963). An arginine aminopeptidase present in blood has been isolated which cleaves the N-terminal Lys^1 residue from kallidin and the N-terminal dipeptide Met^1-Lys^2 from Met-Lys-bradykinin, yielding bradykinin (Guimarães et al, 1973). Trypsin is also able to cleave Lys^1 from kallidin, but this is probably unimportant since trypsin is only active in the circulation in certain pathological conditions (see Regoli & Barabé, 1980). There are other enzymes capable of acting at the N-terminal of kinins, iminopeptidase and prolidase, but these are found intracellularly and so are probably of limited importance. It is also generally accepted that enzymes acting at the N-terminal of kinins play a limited role, if any, in their inactivation (Regoli & Barabé, 1980). Thus, cleavage of Lys^1 from kallidin by aminopeptidase merely liberates bradykinin which requires further degradation by other enzymes to render it inactive.

Enzymes which act at the C-terminal of kinins are, however, more important in kinin inactivation. Kininase I (carboxypeptidase N) was first discovered in human plasma (Erdös & Sloane, 1962). This exopeptidase removes the C-terminal Arg from bradykinin and kallidin and is responsible for 90% of the destruction of kinins in plasma, the remainder being destroyed by kininase II (angiotensin converting

enzyme) (see Regoli & Barabé, 1980). Removal of the C-terminal Arg by kininase I reduces the activity of bradykinin proportionally more than that of kallidin.

Pancreatic carboxypeptidase B and chymotrypsin may also inactivate kinins by removing the C-terminal Arg, but the former is far more effective (Erdös, 1979). The plasma concentration of carboxypeptidase B is too low for it to be of any importance in the inactivation of circulating kinins.

There has been another carboxypeptidase-like kininase described which is of unknown origin but which differs in its molecular weight from kininase I and other carboxypeptidases (Erdös, 1979). Erdös (1979) also mentioned a number of other reputed kininases present in a variety of tissues which were presumed to be either kininase I or II.

Both des-Arg^9-bradykinin and des-Arg^{10}-kallidin which are liberated by carboxypeptidase type enzymes are potent stimulants of BK_1 receptor systems. This would suggest that inactivation of kinins by carboxypeptidases (e.g. kininase I) is less efficient than that by kininase II.

Kininase II cleaves the C-terminal dipeptide Phe-Arg from kinins (Yang & Erdös, 1967) before releasing a second dipeptide, Ser-Pro (Dorer *et al.*, 1974a). Bradykinin is the preferred substrate of kininase II, kallidin and other higher homologues of bradykinin being less susceptible, and degraded at a much reduced rate or not at all (Dorer *et al.*, 1974b). This enzyme is located mainly on the cell membrane of endothelial cells, being closely associated with the lungs, kidneys and liver (Ferreira & Vane, 1967; Vane, 1969) and is present in many other vascular beds (Vane, 1969; Erdös, 1979; Regoli & Barabé, 1980). Kininase II provides the complete catabolism of bradykinin; des-Phe^8,des-Arg^9-bradykinin being almost totally inactive both *in vitro* and *in vivo* (as is des-Phe^9,des-Arg^{10}-kallidin) and the shorter peptides having no activity (see Regoli & Barabé, 1980).

1.5.11 Physiological roles of kallikrein-kinin systems

Plasma kallikrein participates in the surface-dependent activation of blood coagulation, fibrinolysis, kinin generation and inflammation. Of these, the involvement of plasma kallikrein in blood clotting following contact activation and subsequent tissue repair is well documented and this is now generally accepted as its primary physiological role (see Pisano, 1975; Movat, 1979; Schachter, 1980a; Müller-Esterl & Fritz, 1984).

HMW kininogen is thought to be an essential component of the intrinsic coagulation cascade. Those patients suffering from Fitzgerald trait, having a deficiency of HMW kininogen, have an extreme prolongation of kaolin-activated clotting time whereas LMW kininogen deficiency has no effect on the clotting time (see Movat, 1979).

The larger plasma kallikreins have less specific substrate requirements than do tissue kallikreins, but the existence of a physiological role for the plasma kallikrein-HMW kininogen system is now considered well established (Müller-Esterl & Fritz, 1984). This function is believed to be an important factor in the intrinsic blood coagulation cascade and fibrinolysis and has been fully discussed elsewhere (Pisano, 1975; Movat, 1979; Müller-Esterl and Fritz, 1984), but will be described brifely. A summary appears in figure 3.

Injury to blood vessels leads to exposure of the negatively charged surfaces of the basement membrane lying beneath endothelial cells. These strongly anionic surfaces rapidly bind to highly cationic sections of both circulating HMW kininogen-prekallikrein complex and Hageman factor. A histidine-rich segment of HMW kininogen (Han *et al.*, 1975) anchors the molecule to the affected site whilst reciprocal activation of prekallikrein and Hageman factor takes place when active kallikrein is the major activator of Hageman factor, and active Hageman factor is the major activator of prekallikrein. Activated Hageman factor (factor XIIa) in turn activates factor XI and so triggers the coagulation cascade. Parallel with this, HMW kininogen undergoes limited proteolysis by activated kallikrein which liberates

bradykinin and a fragment of HMW kininogen. This so-called called "kinin-free HMW kininogen" is more than twice as efficient as a cofactor of the contact phase than is native HMW kininogen and so acts to augment the cascade. The bradykinin liberated by the degradation of kininogen then increases microvascular permeability, and allows blood proteins to pass to the site of injury and so facilitates tissue repair.

Plasma kallikrein has been implicated in the activation of several other regulatory systems: prorenin activation initiates the renin-angiotensin system and thus liberates angiotensin II; activation of plasminogen triggers the fibrinolytic cascade; complement activation (see Movat, 1979; Silverberg & Kaplan, 1987); and is involved in the defence mechanisms against infectious diseases (Colman, 1989).

Both kininase I and II interact intimately with other blood-borne systems. Kininase I has also been referred to as anaphylatoxin inactivator, cleaving the C-terminal Arg from complement fragments C_{3a} and C_{5a} (Erdös, 1979). However, this does not render them totally inactive, e.g., despite being unable to release histamine from mast cells, C_{5a} des-Arg possesses potent inflammatory activity (Jose *et al.*, 1981).

As may be apparent from its pseudonym, kininase II is an integral component of the renin-angiotensin system, but cleaves bradykinin in preference to angiotensin I. This enzyme is involved directly in the regulation of blood pressure by determining the conversion of angiotensin I to the hypertensive peptide angiotensin II. Kininase II also serves to eliminate the hypotensive peptide bradykinin from the circulation. Thus, inhibition of kininase II reduces the plasma level of angiotensin II and prevents bradykinin breakdown.

Tissue kallikreins are known to be present in a large number of tissues and like plasma kallikrein are thought to be implicated in a number of physiological roles. Both tissue and plasma kallikreins are known to be involved in the acute and chronic inflammatory response (see Kaplan & Silverberg, 1988; Kaplan *et al.*, 1989). Kallikrein and bradykinin exert a number of other actions: release of prostaglandins (Terragno & Terragno, 1979) and EDRF (Gryglewski *et al.*, 1986)

from endothelial cells; production of functional vasodilatation in exocrine glands (Gautvik *et al.*, 1976; Bhoola *et al.*, 1979); regulation of systemic arterial blood pressure by prorenin activation (Ward & Margolius, 1979; Sealy, 1980); renal sodium and water balance (Nasjletti & Malik, 1980); and ion transport in the gut (Cuthbert & MacVinish, 1989).

1.6.0 SUMMARY

The kallikrein-kinin systems are thus implicated in many important roles, both physiological and pathological, with many of the components playing an essential part. The plasma kallikrein system interacts closely with other humoral systems, notably that of blood coagulation where its role is considered vital, and others such as the liberation of angiotensin II, activation of plasminogen and initiation of the fibrinolytic cascade, and the activation of the complement system where its physiological relevance has been questioned (Müller-Esterl & Fritz, 1984).

A more complete understanding of the kallikrein-kinin system and its interrelationships with other local mechanisms would therefore be an important step in the understanding of physiological processes and the treatment of pathologiocal states. The recent development of bradykinin receptor antagonists may help in this, but there is still no specific and selective tissue kallikrein inhibitor available.

The identity of an oxytocic principle derived from the isolated small intestine of rat described by Gaddum (1953) remained unkown until the present study. A search of the literature revealed that neither Gaddum nor any of his colleagues pursued the identity of substance R. However, Gaddum (1953) himself suggested that the properties of substance R and kallikrein were similar. Gaddum's original observations have therefore been repeated and extended. His conclusions have been re-evaluated by a comparison of substance R with kallikrein. This has been made possible by the use of biochemical and pharmacological tools and techniques which were unavailabe to Gaddum when he wrote his paper of 1953, *The Technique of Superfusion* and described for the first time the oxytocic principle substance R.

SECTION 2

MATERIALS AND METHODS

2.1 ANIMALS

Male Wistar rats (200-300 g), female Wistar rats (150-200 g), male Dunkin-Hartley guinea-pigs (500-900 g) and New Zealand White rabbits (2.5-3.0 kg) were all supplied by the University of Bath Animal House.

2.2 MATERIALS

Drugs and reagents were obtained from the following sources:

Amersham International, Amersham, Bucks:

^{125}I-human serum albumin, 50 μCi per ml in sterile isotonic saline.

Bayer UK Ltd, Newbury, Berkshire:

Trasylol (aprotinin).

Janssen Pharmaceutical Ltd, Oxford:

Hypnorm (fentanyl citrate, 0.315 mg ml^{-1} and fluanison, 10 mg ml^{-1}).

Kabi Vitrum Ltd, Uxbridge, Middlesex:

H-D-Val-L-Leu-L-Arg-p-nitroanilide hydrochloride (S-2266).

Novabiochem (UK) Ltd, Nottingham:

D-Arg-[Hyp^{3},Thi^{5,8},D-Phe^{7}]-bradykinin.

May and Baker Ltd, Dagenham, Essex:

Pentabarbitone sodium as a 60 mg ml^{-1} solution in ethanol (Sagatal and Expiral).

Pharmacia LKB Biotechnology, Milton Keynes, Bucks:

PD-10 columns

Sephacryl S-200

Sephacryl S-300

DEAE-Sepharose.

Roche Products Ltd, Welwyn Garden City, Hertfordshire:

Midazolam hydrochloride, 5 mg ml^{-1} (Hypnovel).

Sandoz Products Ltd, Hounslow, Middlesex:

Methysergide hydrogen maleate.

Sigma Chemical Company, Poole, Dorset:

atropine sulphate

blue dextran

bovine serum albumin (Cohn fraction V, essentially fatty acid free) (BSA)

bradykinin triacetate

carbamylcholine carbonic anhydrase (EC.4.2.1.1) from bovine erythrocytes

alpha- and delta-chymotrypsin (EC.3.4.21.1) from bovine pancreas

cytochrome-C from horse heart

diethylstilboestrol

Evans blue dye

Folin and Ciocalteu's phenol reagent

heparin sulphate

indomethacin

kallikrein (EC.3.4.21.35) from porcine pancreas

lima bean trypsin inhibitor

molecular weight marker kit MW-SDS-70L: alpha-lactabumin, soy bean trypsin inhibitor PMSF-treated trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin and bovine albumin.

molecular weight marker kit MW-SDS-BLUE: (prestained markers) lactic dehydrogenase, fumarase, pyruvate kinase, fructose-6-phosphate kinase, beta-galactosidase and alpha-2-macroglobulin

ovomucoid (partially purified from chicken egg white)

papain (EC.3.4.22.2) (insoluble, attached to beaded agarose) from *Papaya latex*

soy bean trypsin inhibitor (SBTI)

thioglycolic acid (as the sodium salt)

Trizma base

Trizma hydrochloride

trypsin (EC.3.4.21.4) and insoluble trypsin (attached to cross-linked beaded agarose) from bovine pancreas.

General laboratory grade chemicals were purchased from British Drug Houses (BDH), Poole; Fisons, FSA Laboratory Supplies, Loughborough; and Sigma Chemical Company, Poole.

The following were gifts. A sample of human plasma kininogen was kindly donated by Dr K.D. Bhoola, University of Bristol. Captopril was kindly donated by E.R. Squibb & Sons Ltd, London. Expired human plasma was given by Dr T. Wallington, Regional Blood Transfusion Centre, Southmead Hospital, Bristol.

2.3 BUFFERS, PHYSIOLOGICAL SALT SOLUTIONS AND ANAESTHETICS

Tris buffer. A stock of 500 mM, pH 7.5 tris buffer was prepared at room temperature with 400 mM Trizma hydrochloride and 100 mM Trizma base dissolved in deionized water. The stock was stored at 4°C and working strength buffer (50 mM, pH 7.5) was prepared by dilution in deionized water.

Phosphate buffered saline (50 mM, pH 7.5) (PBS) was made with 12.5 mM $Na_2H_2PO_4.2H_2O$, 37.4 mM $Na_2HPO_4.2H_2O$ and with and 154 mM NaCl in deionized water.

Physiological salt solutuions were prepared with the following compositions:

Tyrode's solution: 137.0 mM NaCl, 5.5 mM D(+)-glucose, 12.0 mM $NaHCO_3$, 2.7 mM KCl, 1.05 mM $MgCl_2.6H_2O$, 0.42 mM $NaH_2PO_4.2H_2O$ and 1.84 mM $CaCl_2.6H_2O$.

De Jalon's solution: 154.0 mM NaCl, 2.8 mM D(+)-glucose, 5.9 mM $NaHCO_3$, 5.6 mM KCl and 0.55 mM $CaCl_2.6H_2O$.

Hypnorm-midazolam cocktail anaesthetic was prepared by mixing 1 part Hypnorm with 2 parts sterile water and this mixture with 1 part Hypnovel.

Sterile PBS and water were prepared with filtered (0.22 μm) polished deionized water and autoclaved.

2.4.0 METHODS

2.4.1 Collection of substance R

The perfused isolated small intestines of male Wistar rats were used for the collection of substance R. Initially in this study rats were anaesthetised with ether. However, subcutaneous administration of the Hypnorm-midazolam cocktail (0.5 ml per 300 g) was found to be more convenient. Anaesthetised rats were then given heparin (1000 u) via a tail vein to prevent blood clotting during the perfusion. Later it was found that the same results could be obtained if animals were killed by cervical dislocation and the use of anaesthetics was abandoned. The bulk of the results presented here were obtained from preparations of substance R prepared without the use of anaesthetics.

Following laparotomy the small intestine was ligated at the duodenal level, distal to the pancreas and also at the ileo-caecal junction (figure 4). The superior mesenteric artery was cannulated and all other vessels, with the exception of the hepatic portal vein, were tied off. The mesenteric vasculature was flushed through slowly with 2 ml of Tyrode's solution containing heparin (200 u). The intestine was sectioned anterior to the duodenal ligature and distal to the caecal ligature, and the mesenteric vasculature was isolated. The intact mesenteric bed was then removed, care being taken to exclude all pancreatic tissue, suspended in a heated jacket (37°C) and perfused through the mesenteric artery with oxygenated (95% O_2, 5% CO_2) Tyrode's solution (0.4 $mlmin^{-1}$) supplied by a peristaltic pump (Watson-Marlow 502S) (figure 5). Effluent from the hepatic portal vein was either collected for consecutive 20 min periods by a fraction collector (LKB Bromma 2212 Helirac or 2112 Redirac) or, after a 30 min equilibration period, perfusate was pooled and used as starting material for the isolation of substance R by the methods described below.

2.4.2 Preparation of substance R

Initially samples of intestinal perfusate were simply dialysed overnight against PBS at 4°C. Small volumes of intestinal perfusate were also desalted using PD-10 columns (Sephadex G-25M). Later, larger volumes were used to isolate the oxytocic principle by chromatographic techniques to obtain purified samples for use in the laboratory. Figure 6 summarises the steps in the purification of substance R.

For preparative purposes pooled perfusates from three intestinal perfusions were used. The pooled perfusates from three such preparations were then dialysed overnight against 10 volumes of ammonium bicarbonate (50 mM, pH 7.8, 4°C). The high molecular weight residue was filtered, freeze-dried and stored at -20°C. Generally the lyophylised perfusate from nine intestinal preparations was redissolved in tris buffer and filtered before application to the ion-exchange column. Substance R eluted from the ion-exchange column was then subjected to gel filtration chromatography.

2.4.3 Anion-exchange chromatography

Anion-exchange chromatography of the soluble lyophyllised intestinal extract (section 2.4.2) was performed using DEAE-sepharose. The sample (15-30 ml) was applied to the column (2.6 x 30 cm) equilibrated in tris buffer at 4°C and washed with 50-150 ml tris. 5.0 ml fractions of eluate were collected (Gilson model 203 fraction collector) at a flow rate of 0.4 ml min^{-1} (Gilson Minipuls 312 peristaltic pump) by a linear salt gradient of 0-1.0 M sodium chloride. Fractions were assayed for oxytocic activity on the superfused rat uterus before measuring absorbance at 280 nm to obtain the elution profile. The fractions containing oxytocic activity were then pooled, dialysed against 50 volumes of ammonium bicarbonate (50 mM, pH 7.8, 4°C) for 12-18 hours and lyophyllised.

2.4.4 Gel-filtration chromatography

Gel-filtration chromatography of the oxytocic principle was carried out routinely on

a calibrated column of Sephacryl S-300 (2.6 x 90 cm) to prepare samples of substance R for use in the laboratory. The column was equilibrated with PBS at 4°C and 5.0 ml of soluble intestinal extract eluted from the ion-exchange column was applied to the column. The sample was eluted at a flow rate of 0.4 ml min^{-1} and 5.0 ml fractions were collected. The oxytocic activty of each fraction was determined by assay on the superfused rat uterus and an elution profile was obtained by measuring each samples absorbance at 280 nm.

Estimation of the molecular weight of the oxytocic principle was made on a Sephacryl S-200 column (2.6 x 90 cm), calibrated with molecular weight markers, using the conditions described above. The fraction containing the peak oxytocic activity (in terms of the per cent maximal contraction per µl of fraction) was determined by assay on the superfused isolated rat uterus.

2.4.5 Calibration of Sephacryl columns

The columns were equilibrated with PBS at 4°C for calibration. Blue dextran or the calibrating proteins, at a concentration of 2.5-5.0 mg ml^{-1}, were loaded onto the column in a volume of 5.0 ml. Fractions of 5.0 ml were eluted from the column at a flow rate of 0.4 ml min^{-1}. The elution profiles of the calibrating proteins and blue dextran were determined spectrophotometrically at 280nm.

The void volume (V_o) of the column was determined with blue dextran. The elution volumes (V_e) of the calibrating proteins (bovine serum albumin (66 kD), carbonic anhydrase (29 kD), cytochrome-C (12.5 kD) and aprotinin (6.5 kD) were then determined. The ratio V_e/V_o was calculated for each of the calibrating proteins and this was plotted against their molecular weights on semi-log paper.

The approximate molecular weight of substance R was determined by comparing the value of V_e/V_o obtained for the fraction containing peak oxytocic activity against the calibration curve.

2.4.6.0 Isolated smooth muscle preparations

Tissues were generally superfused by a modification of the method of Gaddum (1953). Tissues were suspended from isotonic transducers (Ealing) in heated jackets and were superfused with heated oxygenated (95% O_2, 5% CO_2) Tyrode's or de Jalon's solutions supplied by a pump (Watson-Marlow MHRE 22) at a flow rate of 5 ml min^{-1}. Contractions were recorded on a linear pen recorder (JJ Instruments CR650 or CR650S). A cascade system was sometimes employed, where superfusion fluid passed over two tissues in series. Reagents were prepared such that between 10 and 100 µl volumes were injected into the stream of heated superfusion fluid proximal to the tissues. However, sometimes larger volumes of reagent were required and, when this exceeded 250 µl, the pump was stopped and the sample was injected into the injection port at a rate equivalent to that of the superfusion. Antagonists and inhibitors were added to the reservoir of superfusate.

2.4.6.1 The rat isolated uterus

Rat uteri in oestrus were used for the assay of oxytocic activity of samples. Rats were primed 18-24 hours before the experiment by subcutaneous administration of stilboestrol (0.1 mg kg^{-1}) to virgin female rats. The uteri were then prepared according to the method of de Jalon, a thread tied around each end, and mounted in the heated superfusion jacket (30-32°C) under 0.5 g tension. Uteri were superfused with de Jalon's solution (30-32°C, 5 ml min^{-1}) (Gaddum, 1953).

In experiments investigating the effect of inhibitors and antagonists on oxytocic agents, a dose of agonist giving 70-90% of the maximum contraction was used.

2.4.6.2 The rat isolated fundus strip

A strip of rat fundus 4 cm long was prepared from male rats by the method of Vane (1957) and superfused with Tyrode's solution (37°C, 5 ml min^{-1}) under 1.0 g tension.

2.4.6.3 The rat isolated duodenum

The rat isolated duodenum was either prepared by the method of Horton (1959) or the proximal 3 cm of duodenum was removed and was mounted in the superfusion apparatus under 1.0 g tension and superfused with de Jalon's solution (37°C, 5 ml min^{-1}).

2.4.6.4 The guinea-pig isolated ileum

Male guinea-pigs were killed by cervical dislocation and the abdomen opened. A short section of terminal ileum was removed and placed in oxygenated Tyrode's solution. 2 cm lengths of ileum were selected and threads were attached to either end. The isolated ileum was then either superfused with Tyrode's solution (37°C, 5 ml min^{-1}) or was mounted in a standard organ bath in Tyrode's solution (37°C) under 1.0 g tension.

2.4.6.5 Assay of kinin-releasing activity

The kinin-releasing activity of substance R and kallikrein was assayed on the isolated guinea-pig ileum. In these experiments a 2 cm length of ileum was suspended in an 8 ml organ bath and bathed in oxygenated Tyrode's solution containing captopril (4.6×10^{-4} M). The bath was washed out by upward displacement overflow. Bradykinin was used as a standard reference agonist.

2.4.7 Measurement of arterial blood pressure

Guinea-pigs were anaesthetised with phenobarbitone (100 mg kg^{-1}, i.p.) and pentabarbitone (30 mg kg^{-1}, i.p.) and paralysed with gallamine (10 mg kg^{-1}, i.m.). Animals were ventilated (8 ml kg^{-1}, 1 Hz) via a tracheal cannula using a mixture of air and oxygen (50:50, v/v). Blood pressure and heart rate were recorded from the carotid artery using a transducer (type P23Dd, Gould). Agents were injected as a bolus into the jugular vein via an indwelling canula.

2.4.8 Measurement of local plasma protein leakage in rabbit skin

Plasma protein extravasation was measured as the local accumulation of ^{125}I-HSA as described previously (Williams, 1979), with several minor modifications (see figure 7). Rabbits were anaesthetised by an intravenous injection of sodium pentobarbitone (Sagatal), 30 mg kg^{-1} via an indwelling cannula in the marginal ear vein. The hair of the back and flanks was closely clipped and a fixed, balanced site pattern was marked either side of the midline. ^{125}I-HSA (5 µCi) was mixed with 1.0 ml sterile Evans blue dye (2.5% in saline) and was administered 10-15 minutes prior to intradermal injections. Solutions of agents for intradermal injection were prepared beforehand in sterile saline or PBS, and kept on ice until required. Test and control sites were injected in six replicates (0.1 ml), using 1.0 ml syringes fitted with 27g (0.4 x 12 mm) sterile needles. Thirty minutes after the intradermal injections, a blood sample was taken by cardiac puncture into heparin (10 u ml^{-1}) and the animal was killed with an overdose of barbiturate (Expiral).

Skin samples for estimation of plasma leakage were prepared in the following manner. The rabbit was skinned and the skin laid outside-down, blood was expressed from the larger vessels, and excess fat was removed from the skin. Skin sites were excised using a 17 mm steel punch and were placed in polycarbonate tubes ready for counting. Plasma samples were prepared by centrifugation of the heparinised blood at 1,500 g, 10 min. Three 1.0 ml plasma samples in polycarbonate tubes were taken as standards, and together with the skin samples, were counted in a gamma-counter (LKB 1275 Multigamma). Exudate volumes are expressed in terms of plasma volume per skin site, calculated by dividing the count from each skin site by the count for 1 µl of plasma.

Prostaglandin E_2 (PGE_2) was used at a concentration of 3 x 10^{-10} moles per site in all experiments.

2.4.9 Chromogenic assay of kininogenase activity

Hydrolysis of the chromogenic substrate S-2266 was used to assay the tissue kallikrein content of samples (Amundsen *et al.*, 1979). The endpoint method for the determination of kallikrein in urine as described in the Kabi Vitrum Laboratory Instruction booklet was used.

2.4.10 Immunodiffusion assay

In collaboration with Mr Carlos Figueroa at the University of Bristol a double diffusion immunoassay was carried out. Samples of substance R and rat urinary kallikrein prepared in our laboratory were tested against rabbit anti-rat tissue kallikrein antiserum (Rabito *et al.*, 1980).

2.4.11 Radioimmunoassy for tissue kallikrein

Radioimmunoassay for rat tissue kallikrein was performed in Professor Chao's laboratory by a previously published method (Shimamoto *et al.*, 1979: see Chao and Chao, 1988).

2.4.12 Preparation of urinary kallikrein

Initially, crude preparations of rat urinary kallikrein were made by desalting rat urine on a Sephadex-G25M (PD-10) column equilibrated with PBS. Later, for comparative purposes, rat urinary kallikrein was prepared from urine collected overnight from male rats. The urine from individual rats was pooled and filtered before an overnight dialysis against 20 volumes of PBS. The dialysed sample was stored frozen at -20°C. A partially purified preparation of urinary kallikrein was obtained using the same preparative and assay techniques used in the isolation of substance R.

2.4.13 Kininogen preparations

Crude rat kininogen was prepared from blood collected by cardiac puncture from

anaesthetised rats. Plasma was prepared by centrifugation of heparinised blood (10 u ml^{-1}) at 1500 g for 10 min. Serum was prepared by centrifugation of coagulated blood (3,000 g, 15 min). Plasma or serum was heated at 56°C for 3 hours. After centrifugation (3,000g, 15 min) the supernatant was used as a crude preparation of kininogen.

Human plasma kininogen was prepared according to a method modified from that of Bhoola and Lemon (unpublished). Expired anticoagulated human plasma was heated at 56°C for 3 hours. An equal volume of cold (4°C) saturated ammonium sulphate solution was added slowly whilst stirring. The mixture was stored at 4°C for 24 hours and then centrifuged at 1,750 g, 4°C for 20 minutes. The supernatant was decanted and discarded and the pellet was resuspended in a volume of cold 0.9% saline equal to half the original volume of plasma. The material was dialysed against 3 x 10 volumes of deionised water at 4°C over 48 hours. The dialysed sample was centrifuged at 1,750 g, 4°C for 20 minutes. The supernatant was decanted, freeze-dried and stored dessicated at -20°C.

2.4.14 Determination of protein concentration

A modification of the method of Lowry *et al.* (1951) was used to determine the protein concentration of samples. Standard curves were constructed using BSA as the standard.

2.4.15 SDS-polyacrylamide gel electrophoreseis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in our laboratory by Miss Amber Elderfield using an adaptation of the method of Laemmili (1970). Briefly, 20µl aliquots of substance R preparations, containing 10 or 5 µg protein were applied to SDS/polyacrylamide gels and constituent proteins were separated by electrophoresis using a `Mighty Small II' vertical slab gel unit (Hoefer Scientific Instruments). Gels were stained with 0.2% PAGE blue 83 (BDH) to visualise individual bands.

2.4.16 Western blot analysis

Western blot analysis of substance R was carried out in collaboration with Professors Henry Margolius and Julie Chao at their laboratories at the Medical University of South Carolina, USA.

An antigen overlay method was used for Western blot analysis of substance R and rat tissue kallikrein. Electrophoresis was performed on a 7.1-15% linear gradient SDS-PAGE under reducing conditions and the proteins were electrotransferred onto nitrocellulose. The nitrocellulose sheet was incubated with sheep anti-rat tissue kallikrein antiserum (1:500 dilution) followed by ^{125}I-rat tissue kallikrein. The antibody binding sites were visualised by autoradiography.

2.4.17 Statistical analysis

Where necessary data are expressed as mean ± standard error of the mean (mean ± s.e. mean). Statistical significance was computed using the paired *t*-test or analysis of variance (ANOVAR) where appropriate.

SECTION 3

RESULTS

3.1.0 PROPERTIES OF AN OXYTOCIC PRINCPLE FROM THE RAT INTESTINE

3.1.1 Release of an oxytocic princple from rat intestine

Experiments were carried out where lengths of rat isolated small intestine were introduced into the stream of superfusion fluid above the rat uterus in a cascade system. This resulted in the uterus undergoing a series of slow and prolonged contractions (figure 8). In four such experiments the series of contractions began immediately following the introduction of the intestine and lasted between 5 and 20 minutes. In each experiment the uterus did not contract at all spontaneously after the initial series of contractions and each was stable for at least 30 minutes, one uterus being stable for 150 minutes.

Figure 8 also shows that the same phenomenom was observed when a length of guinea-pig ileum was placed above the rat uterus in the cascade system.

Neither a "sham" disturbance of the superfusate flow where a length of cotton thread replaced the intestine, nor the introduction of another uterus in place of the intestine resulted in contraction of the uterus.

The motility of the gut appeared to have no relation to the contractions of the superfused uterus below.

3.1.2 Intestinal perfusion

Perfusion of the rat isolated small intestine resulted in the elution of a light yellow perfusate, tinted red in the first two or three fractions by the presence of erythrocytes. Fractions collected after several hours of perfusion were markedly darker in colour than the earlier ones. A small increase in volume with time was also noted.

3.1.3 Oxytocic activity of intestinal perfusate

The effluent from perfused small intestines was found to contract the rat isolated superfused uterus in a dose-dependent manner (figure 9). In contrast to uterine contractions induced by either carbachol or bradykinin, those to intestinal perfusate were delayed in onset (from 30-60 seconds) (figure 10). Contractions of the uterus to intestinal perfusate resembled those to bradykinin in that they reached a maximum slowly compared with responses to carbachol.

3.1.4 Time-course of release of oxytocic activity from perfused rat small intestine

Figure 11 shows the time-course of release of oxytocic activity from an intestinal perfusion. When consecutive fractions of perfusate from isolated intestines were collected, at least 30 minutes elapsed before oxytocic activity was detectable. However, oxytocic activity was released in increasing amounts thereafter. A maximum concentration of oxytocic activity was reached after 3-4 hours, and this was sustained for at least a further 2-3 hours. In three such preparations the protein concentration of the eluate remained between 1-2 mg ml^{-1} throughout the period of perfusion. Although there was a slight rise observed at the begining of perfusion, there appeared to be no correlation between protein concentration and oxytocic activity. In a number of perfusion experiments carried out at the begining of this study, oxcytocic activity always appeared in the eluate within 20-60 minutes.

Fasting animals overnight prior to isolation and perfusion of their intestines had no influence on the time-course of release of oxytocic activity.

In the first few experiments the isolated gut was supported in the heated jacket by a guaze hammock (Gaddum, 1953). Removal of this accelerated the isolation and perfusion procedure and did not affect the results obtained. Therefore use of the hammock was dispensed with.

3.1.5 Physical properties of the oxytocic principle

The oxytocic principle from the intestinal perfusate was non-dialysable (m.w. cut off 10-15 kDa). Oxytocic samples of perfusate were applied to Sephadex G-25M (PD-10) columns equilibrated with PBS. The eluate from the PD-10 columns contained oxytocic acivity. This provided a reliable method for rapidly desalting oxytocic samples of intestinal perfusate. This material was found to be stable for at least 8 weeks when stored frozen at -20°C.

When oxytocic samples were mixed with 4 volumes of cold (4°C) acetone a white precipitate was formed. After drying off under nitrogen, and redissolving in PBS, the precipitate was found to posess oxytocic acivity.

Heating oxytocic samples of intestinal perfusate in a boiling water bath for 10-15 minutes resulted in a substantial loss of acitvity on the uterus preparation. This was further investigated using purified samples. Boiled samples retained only 5-20% of their preboiled activity.

Thioglycolic acid was used to determine whether oxytocin was responsible for oxytocic acitvty in intestinal perfusate. Samples were incubated at room temperature with a mixture of thioglycolate (0.01M) and sodium bicabonate (0.01M) at pH 7.5 for 30 minutes (Walker, 1967) before assay on the isolated uterus. Treatment of samples containing oxytocic activity with thiogycolate did not result in any loss of activity (figure 12).

Incubation with either insoluble trypsin or insoluble papain (10 enzyme units per ml, pH 7.5, 30 minutes, 37°C) did not result in any change in oxytocic activity of the samples (figure 13).

3.1.6 Pharmacology of the oxytocic principle

Oxytocic activity of samples of intestinal perfusate was not inhibited by addition of atropine (10^{-6}M), methysergide (5.7×10^{-7}M) or indomethacin (10^{-5}M) to the

superfusate.

3.2.0 COMPARISON OF THE PHARMACOLOGY OF SUBSTANCE R AND RAT URINARY KALLIKREIN

The results presented in section 3.1 are very similar to the characteristics of "substance R" reported by Gaddum in 1953. As Gaddum himself observed, these properties resemble those of the then recently described kallikrein. To compare the activities of the two substances further, rat urine was utilized as a readily available source of kallikrein. A commercial preparation of porcine pancreatic kallikrein was also used in some experiments.

Where "substance R" is used in the following sections discussing results, it denotes that results were obtained using a sample of the oxytocic principle from intestinal perfusate which had been purified using chromatographic techniques.

3.2.1 Oxytocic activity

Rat urinary kallikrein induced contraction of the superfused rat isolated utreus. The contractions resembled those produced by substance R, being delayed in onset and slow to reach a maximum. Up to 500 µg of porcine pancreatic kallikrein was found to be inactive on the uterus in an experiment where 40 µl of intestinal perfusate (50-100 µg protein) produced a maximal contraction.

3.2.2 Pharmacological distinction of substance R from trypsin and chymotrypsin

Trypsin, alpha- and delta-chymotrypsin were all found to cause contractions of the rat uterus that were similar to those produced by intestinal perfusate and kallikrein. An attempt was therefore made to distinguish the oxytocic activty in intestinal perfusate.

Figure 14 shows the responses of sections of superfused rat uterus. A supramaximal

dose of substance R had no effect on the sensitivity of the tissue to bradykinin (figure 14a). Trypsin (100 µg) did not induce a contraction of the uterus. A higher dose of trypsin (1 mg) induced a contraction and sensitization of the tissue to bradykinin (figure 14a). The same piece of tissue however, then contracted to and was sensitized by trypsin (1 mg). A supramaximal dose of urinary kallikrein did not induce a sensitization of the uterus to bradykinin (figure 14b).

Like trypsin, both alpha- and delta-chymotrypsin sensitized the rat uterus to bradykinin (figure 15a & b) at similar doses.

3.2.3 Action on other isolated smooth muscle preparations

Neither substance R nor urinary kallikrein contracted the superfused guinea-pig ileum, rat fundic strip or rat ileum at doses up to 10-fold those which were oxytocic. The superfused rat duodenum relaxed to both substance R and urinary kallikrein at doses coparable to those which were oxytocic. However, whilst consistently relaxing to bradykinin, duodenal preparations rapidly became insensitve to both substances.

3.2.4 Cardiovascular actions of substance R

Substance R was hypotensive in the anaesthetised rat and guinea-pig. Figure 16 shows the recording of the arterial blood pressure of an anaesthetised guinea-pig. Intravenous injection of substance R (10, 20 and 50 µl) caused a dose-related reduction in arterial blood pressure. No action on the heart rate or lung function was observed at these doses. At similar doses, substance R did not relax a rat aorta contracted to phenylephrine (endothelium intact) or have any effect on the perfusion pressure or rate of a Langendorf perfused rat heart.

3.2.5 Effect of trypsin inhibitors on oxytocic activity

In each of six experiments, addition of aprotinin (Trasylol) (100 KIU ml^{-1}; 2.2 x 10^{-6}M) to the fluid superfusing the isolated uterus completely inhibited the oxytocic activity of both substance R and urinary kallikrein (figure 17).

Soy bean trypsin inhibitor (SBTI) (100 μg ml^{-1}; 5 x 10^{-7}M) completely inhibited the action of substance R on the isolated rat uterus (99.4 ± 0.3%) whereas the oxytocic acitvity of urinary kallikrein was only reduced by 12.6 ± 2.5% (n=6) (figure 18). Responses to bradykinin and carbachol were reduced by 9.9 ± 1.8% and 3.0 ± 0.8% respectively. Inhibition of uterine responses to substance R by SBTI was significant (P<0.01, ANOVAR). SBTI was found to be effective against substance R at a concentration of 50 μg ml; 2.5 x 10^{-7}M (n=3).

The trypsin inhibitors ovomucoid trypsin inhibitor (100 μg ml^{-1}) and lima bean trypsin inhibitor (100 μg ml^{-1}) were ineffective against the oxytocic activity of substance R and urinary kallikrein.

3.2.6 Effect of D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin on oxytocic activity

Figure 19 shows the inhibition of kinin-dependent oxytocic responses by the BK_2 antagonist D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin. Addition of D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin (10^{-7}M) to the superfusate reduced oxytocic responses to bradykinin, kallikrein and substance R by 63.8 ± 10.7%, 69.5 ± 11.8% and 85.9 ± 8.6% respectively (P<0.01, ANOVAR) (n=8). Responses to carbachol were not reduced in the presence of the kinin antagonist, on the contrary, they were enhanced in all experiments (increasing by 17.9 ± 4.6%, n=6).

3.2.7 Kinin-releasing activity

When desalted intestinal perfusate and desalted rat urine were incubated with rat heat inactivated plasma or serum in the presence of captopril (4.6 x 10^{-4}M) (37°C, 30 min), a substance was released which contracted the guinea-pig isolated ileum.

Figure 20 shows responses of a length of guinea-pig ileum to desalted samples of intestinal perfusate and rat urine. Addition of these alone had little or no effect on the ileum. However, when added to the bath in the presence of heat inactivated plasma, addition of either substance brought about an immediate contraction of the tissue.

Further experiments were carried out with purified preparations of substance R and urinary kallikrein using human plasma kininogen as the substrate. The kinin-releasing activity was assayed on the guinea-pig isolated ileum preparation in the presence of captopril (4.6×10^{-4}M). The ileum was unresponsive to either kallikrein, substance R or kininogen alone. However, when kininogen was first added to the bath, subsequent addition of kallikrein or substance R resulted in contraction of the ileum which was inhibited by the kinin receptor antagonist D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin (10^{-7}M). Complete inhibition of these contractions was obtained when aprotinin (100 KIU ml^{-1}, 2.2×10^{-6}M) was added to the bath (n=3). In contrast, figure 21 shows that SBTI (5×10^{-7}M) produced a small inhibition of the kinin liberation by urinary kallikrein (13.2 ± 6.3%) but significantly (P<0.001, paired t-test) reduced the release of kinin by substance R by 80.4 ± 5.0%, as measured by contraction of the ileum (n=6) (figure 21).

3.2.8 Oedema formation in rabbit skin

Figure 22 shows that intradermal injection of substance R + PGE_2 into rabbit skin resulted in dose dependent microvascular leakage. A 20 µl volume of the preparation of substance R used produced a maximal contraction of the uterus. In a preliminary experiment substance R alone (20 µl) produced a substantial inflammatory response (64 µl extravasation). PGE_2 alone produced 20 µl of leakage. PGE_2 potentiated the response to substance R, giving 201 µl of plasma leakage.

Maximum plasma leakage was obtained to 10 µl of substance R. A 10 µl sample of boiled substance R injected together with PGE_2 gave a response equivalent to a 2 µl volume of unboiled substance R + PGE_2 (figure 22).

3.3.0 BIOCHEMICAL ANALYSIS OF SUBSTANCE R

3.3.1 Anion-exchange chromotography of intestinal perfusate

An elution profile of ion-exchange chromatography (DEAE-Sepharose) of intestinal perfusate is shown in figure 23. Freeze-dried pooled oxytocic fractions of intestinal perfusate were dissolved in tris buffer, loaded onto the column and washed with 100 ml of buffer. A single peak of oxytocic activity was eluted by a linear salt gradient at approximately 0.2M NaCl.

3.3.2 Gel-filtration chromatography of partially purified substance R

During routine preparation of substance R gel filtration of pooled and lyophylised oxytocic fractions eluted from the ion-exchange column was performed on Sephacryl S-300. A single peak of oxytocic activity was eluted.

Determination of the molecular weight of substance R by gel filtration was carried out on Sephacryl S-200. A single peak of oxytocic activity was resolved, with the peak fraction having a molecular weight of 40,000 Daltons (figure 24). There was no detectable oxytocic activity in 200 µl samples of fractions either side of this peak.

3.3.3 Kininogenase activity

The fractions containing the peak of oxytocic activity from a molecular weight determination were assayed for kininogenase activity using the specific tissue kallikrein chromogenic substrate S-2266. A single peak of kininogenase activity coinciding with that of the oxytocic activity was found (figure 25). Very low levels of activity (3-20 nkat l^{-1}) were present in the fractions immediately adjacent to the oxytocic peak, compared with 5.3×10^3 nkat l^{-1} in the peak fraction.

Kininogenase activity of samples of substance R was also studied, in the laboratory of professor Julie Chao, using two other assay techniques. Substance R posessed

kininogenase activity assessed by the method of Shimamoto et al (1979) where kinin production from purified bovine kininogen was measured by radioimmunoassay (Shimamoto et al, 1979). Substance R also hydrolysed the synthetic substrate ^{3}H-Tos-Arg-OMe (Chao, personal communication).

3.3.4 Immunodiffusion study

Figure 26 shows the result of a double immunodiffusion assay using the technique of Ouchterlony. The assay was carried out on chromatographed samples of substance R and urinary kallikrein that were approximately equipotent on the rat uterus. A strong immunoprecipitate band was formed by the reaction of the antiserum with the urinary kallikrein. Another, weaker immunoprcipitate band was formed between the antiserum and substance R. A sample of a non-oxytocic fraction of eluate from the chromatography of substance R did not react with the antiserum.

3.3.5 SDS-PAGE analysis

The result of SDS-PAGE analysis of substance R is shown in figure 27. A number of protein bands were seperated, the major bands having molecular weights of approximately 62,000, 39,000, 26,000 and 21,000 Da. The 39 kDa band was well separated from the others. this band is thought to represent the protein which elutes with a molecular weight of 40 kDa from the gel-Sephacryl S-200 column, i.e. substance R.

3.3.6 Western blot analysis

In Western blot analysis using a highly sensitive and specific antigen overlay method, substance R was recognised by antibody against rat tissue kallikrein as a single polypeptide with a molecular mass of 39,000 Da, under reducing conditions (figure 28). The molecular weight of substance R was slightly larger than that of 38,000 Da tissue kallikrein purified from rat submandibular gland (figure 28).

The molecluar weight of substance R differed markedly from those obtained for

tonin, argenine esterase A or T-kininogenase under identical conditions.

3.3.7 Radioimmunoassay for tissue kallikrein

Serial dilutions of substance R showed complete parallelism to the standard curve of tissue kallikrein in a direct radioimmunoassay (figure 29).

3.3.8 The effect of inhibitors on hydrolysis of S-2266

The results of a study of the effects of various inhibitors on the hydrolysis of the chromogenic substrate S-2266 are summarised in table 2. Aprotinin, SBTI, sheep anti-rat urinary kallikrein antibody, and the monoclonal antibody V4D11 all produced a concentration dependent inhibition of hydrolysis of S-2266. Substance R appeared to be more susceptible to inhibition by SBTI and aprotinin than was kallikrein. The two antibodies were less potent at inhibiting substance R than tissue kallikrein.

SECTION 4
DISCUSSION

4.0 DISCUSSION

In 1953 Gaddum made a little-known discovery of an oxytocic principle from the rat small intestine which he named substance R. The results obtained in the present study confirm and extend those reported by Gaddum nearly 40 years ago. Initially an attempt was made to repeat Gaddum's first observation of the detection of substance R. It was found that when a length of rat ileum was superfused in series above the rat uterus, the latter underwent a series of slow and prolonged contractions. This result is similar to Gaddum's report that a substance was sometimes liberated from the superfused rat intestine which caused a contraction of the rat's uterus, the principle he named substance R.

The slow, delayed contraction of the rat uterus by intestinal perfusate reported in the present study is similar to that described by Gaddum (1953) for substance R. Release of this oxytocic principle from the rat perfused small intestine increases with time from 30 minutes to approximately 3 hours after which the oxytocic activity of the perfusate remains high. The oxytocic activity of intestinal perfusate was unaffected by atropine or methysergide. Thioglycolate inactivates oxytocin (van Dyke *et al.*, 1942; Ames *et al.*, 1950), yet treatment of intestinal perfusate with this substance did not diminish its oxytocic activity. However, boiling samples of substance R resulted in a substantial loss of activity. Substance R was found to be non-dialysable (molecular weight cut off 10-15 kDa) and an acetone precipitate of preparations contained the oxytocic activity. These results are in agreement with those of Gaddum (1953). In addition substance P could not account for the oxytocic activity of substance R (Gaddum, 1953). Thus, the possibility that the oxytocic principle in intestinal perfusate is one of a number of pharmacologically-active substances such as acetylcholine, 5-HT, histamine, substance P and oxytocin was eliminated. In contrast, substance R is a high molecular weight and thermolabile substance.

As previously reported (Douglas *et al.*, 1988, 1989) these results confirm the release of an oxytocic principle from rat intestine which shares the properties described by

Gaddum (1953) for substance R. The nature of substance R has remained unidentified since Gaddum's first report (1953) and was therefore further investigated.

As Gaddum (1953) himself remarked, substance R and kallikrein share a number of properties. In addtion to being of a high molecular weight and thermolabile, neither substance R or kallikrein contract the isolated guinea-pig ileum, rat fundic strip or rat ileum, both relax the rat duodenum and are both hypotensive. To explore this similarity further, desalted rat urine was used as a source of authentic kallikrein. Demonstration of the release of a kinin from its plasma substrate by a substance indicates that it may be a kallikrein (Webster, 1970a). Kinin generation from kininogen preparations was assayed on the guinea-pig ileum. Neither rat serum or plasma, nor the crude preparations of substance R or kallikrein, caused contraction of the guinea-pig ileum when added to the bath alone. However, when kallikrein or substance R were added to the bathing fluid in the presence of plasma or serum, the muscle contracted. Further experiments carried out with purified urinary kallikrein and substance R preparations confirmed the release of a kinin-like substance from human, rat and bovine plasma kininogen. The rate of generation of kinin-like activity by substance R was similar to that by urinary kallikrein. The competitive bradykinin antagonist D-Arg-[Hyp^{3},Thi^{5,8},D-Phe^{7}]-bradykinin (Regoli *et al.*, 1986a; Schachter *et al.*, 1987) inhibited such contractions and it was concluded that a kinin was indeed released by substance R from kininogen preparations. Other evidence of the kininogenase activity of substance R was obtained from the fact that it hydrolysed the synthetic tissue kallikrein substrates S-2266 (Douglas *et al.*, 1991) and ^{3}H-Tos-Arg-OMe (Chao, personal communication).

The proteolytic enzymes trypsin and chymotrypsin contract the rat uterus in a similar way to substance R (Rocha e Silva, 1939; Gaddum, 1953). Gaddum (1953) explored the possibility that substance R was identical to either of these enzymes. He found that crude acetone precipitations of intestinal perfusate were more active

on a weight for weight basis than crystalline preparations of either trypsin or chymotrypsin. Gaddum deduced, therefore, that neither enzyme was likely to be responsible for the oxytocic activity of substance R, but recognized that this possibility could not be discounted. The pharmacological actions of trypsin and chymotrypsin were therefore compared with those of substance R and kallikrein in the present study. Both trypsin and two forms of chymotrypsin, alpha- and delta-, caused a similar delayed, slow contraction of the superfused uterus to that induced by substance R or kallikrein. However, as reported by Gaddum (1953), it was noted that on a weight for weight basis they were considerably less potent than eithet kallikrein or substance R. It has been reported that trypsin, chymotrypsin and other proteases can sensitize certain smooth muscle preparations to the agonist bradykinin (Edery, 1964, 1965; Gilfoil & Kelly, 1966; Edery & Grunfield, 1969). It is not known why this sensitzation occurs, but several explanations have been put forward, such as a proteolytic action on the bonds between smooth muscle cells, allowing greater penetration of bradykinin (Edery, 1965); a direct action on the muscle fibres (Gilfoil & Kelly, 1966); and exposure of a greater number of bradykinin receptors (Edery & Grunfield, 1969).

Trypsin, alpha- and delta-chymotrypsin induced a specific sensitization of the tissue to bradykinin, at or below oxytocic doses. On the other hand, supramaximal doses of either kallikrein or substance R failed to produce any such sensitization. In addition, enzyme inhibitors such as ovomucoid trypsin inhibitor and lima bean trypsin inhibitor which inhibit the actions of trypsin and chymotrypsin were without effect on the oxytocic activity of substance R. These results strongly suggest that substance R is not identical to either trypsin or chymotrypsin.

The actions of various trypsin inhibitors on oxytocic responses to substance R and kallikrein were investigated. Ovomucoid trypsin inhibitor and lima bean trypsin inhibitor were inactive against either urinary kallikrein or substance R. These results are in agreement with previously reported inhibitory activities against tissue kallikreins (see Vogel & Werle, 1970; Vogel, 1979).

The polyvalent trypsin inhibitor aprotinin (Trautschold *et al.*, 1967) has been shown to inhibit most kallikreins (see Vogel, 1979). Aprotinin inhibited oxytocic responses of the rat uterus to both substance R and urinary kallikrein. However, SBTI, which selectively inhibits plasma kallikreins (Werle & Berek, 1952; Back & Steger, 1968; Vogel, 1979) blocked responses of the rat uterus to substance R without causing a significant reduction in those to urinary kallikrein. The release of kinin from kininogen by substance R was likewise inhibited by SBTI and aprotinin. Kinin liberation from kininogen by urinary kallikrein, however, was inhibited only by aprotinin. These results, summarised in figure 30, suggest that substance R posesses kininogenase activity but was unlikely to be a tissue kallikrein. An attempt was therefore made to purify and characterize substance R further.

Initial results from gel filtration chromatography of the soluble extract from intestinal perfusate suggested the molecular weight of substance R was approximately 21 kDa (Douglas *et al.*, 1989). However, a large number of contaminating proteins, as revealed by SDS-PAGE may have affected this estimate. The number of contaminating proteins were reduced by anion-exchange chromatography prior to gel filtration. A single peak of biological activity with a molecular mass of 40 kDa was then resolved. This estimate was confirmed by SDS-PAGE in our laboratory and by Western blot analysis by Professor Chao. This peak of oxytocic activity also corresponded to another of kininogenase activity. This demonstrates that a single substance is responsible for these two biological activities.

This molecular weight estimation suggests that substance R is unlikely to be a plasma kallikrein since these have been reported to have masses of 90 kDa or more (see Movat, 1979; Schachter, 1980a). The possibility that substance R was a tissue kallikrein was investigated. Using an immunodiffusion assay, it was established that substance R preparations share immunological epitopes with rat tissue kallikrein (Douglas *et al.*, 1989), a finding later confirmed by Western blot analysis and radioimmmunoassay.

Further evidence was obtained that substance R is very similar to rat tissue

kallikrein with respect to its enzymatic properties. Substance R releases kinins from rat and bovine kininogens, hydrolyses the specific tissue kallikrein synthetic substrate S-2266, which is inhibited by aprotinin, and also hydrolyses ^{3}H-Tos-Arg-OMe (Chao, personal communication). In addition, hydrolysis of S-2266 by substance R is inhibited by both affinity-purified polyclonal anti-rat tissue kallikrein antibody, and the monoclonal antibody to rat tissue kallikrein, V4D11 (Chao, personal communication). However, substance R was more susceptible to SBTI inhibition of S-2266 hydrolysis than was tissue kallikrein, thus confirming the original findings on the rat uterus (Chao, personal communication).

Substance R also shares other immunological properties with rat tissue kallikrein. Serial dilutions of substance R show complete parallelism to the standard curve of tissue kallikrein in a radioimmunoassay. Other purified members of the kallikrein gene family such as tonin, arginine esterase A and T-kininogenase share 80-85% sequence homology with tissue kallikrein but clearly display non-parallel curves to tissue kallikrein standard in the radioimmunoassay (Chao, personal communication). A number of cDNA clones encoding kallikrein gene family members have been sequenced by the groups of Chao and MacDonald (see MacDonald *et al.* 1989). These kallikrein-related cDNAs share 75-90% sequence homology and are indistinguishable in Northern or Southern blot analysis. The direct radioimmunoassay for tissue kallikrein is therefore regarded as an extremely specific and powerful technique in distinguishing tissue kallikrein *per se* from other kallikrein gene family members (Chao, personal communication). In Western blot analysis of substance R, the antibody to rat tissue kallikrein recognizes only a single band with a molecular mass of 39 kDa. There was a slight difference in molecular weight between substance R (39 kDa) and rat submandibular kallikrein (38 kDa). However, the size of substance R is markedly different from tonin, arginine esterase A or T-kininogenase under identical conditions (Chao & Chao, 1987; Chao, personal communication).

Previous reports have given markedly differing estimates of molecular weight for

tissue kallikrein (between 25-45 kDa). It has long been known that within the same tissue source there may be different forms of the enzyme which are distinguishable by their molecular mass or isoelectric point (see Fielder, 1979). These multiple forms of kallikrein could be the result of differences in the genetic expression of the enzyme. Alternatively, another explanation may be that kallikreins of pancreatic or sublingual origin are absorbed from the intestine into the blood and are excreted by the kidneys (Nustad *et al.*, 1980). This appeared to be confirmed by evidence that the primary structure of human urinary and pancreatic kallikreins are identical (Fukushima *et al.*, 1985). It has been shown, however, that whilst sharing the same primary protein structure such isoforms may be glycosylated differently. This was first demonstrated in pancreatic kallikrein. The two (or more) forms of porcine pancreatic kallikrein share the same amino acid sequence but differ in their carbohydrate content (Tschesche *et al.*, 1979; Moriya *et al.*, 1983). Urinary kallikrein has been intensively studied and multiple forms of this enzyme have also been described. Morichi *et al.* (1984) ascribed the microheterogeneity of human urinary kallikrein to variations in sialic acid and carbohydrate content. These authors suggested this may result in conformational changes within the molecule. Nustad and Pierce (1974), on the other hand, found no variation in the sialic acid residues of the isoforms of rat urinary kallikrein. The many different pI forms of human urinary kallikreins contain just a single polypeptide chain (Lu *et al.*, 1989). The heterogeneity is not the result of multiple forms of kallikrein gene product, but rather a result of variations in the extent of glycosylation (Lu *et al.*, 1989). These authors discussed the possible glycosylation sites within the molecule, deduced from experimental results and amino acid sequence data. It was their conclusion that the two molecular weight isoforms of the enzyme (38 and 42.5 kDa) and minor heterogeneity in isoelectric points, resulted from varying degrees of glycosylation of the Asn-141 residue.

It thus appears that the multiplicity of tissue kallikrein molecules is not derived from expression of various kallikrein gene family products, but is controlled by post-translational modification by events such as glycosylation. Such heterogeneity

of processing would account for the slight difference in molecular mass between substance R and rat submandibular kallikrein as revealed by Western blot analysis. Given that there are a number of potential sites of glycosylation in the tissue kallikrein molecule (Lu *et al.*, 1989), these differences may possibly occur at different residues in kallikreins from various tissue sources which may infer slight conformational changes and tertiary stuctural differences.

It is known that within the same species the same gene controls expression of tissue kallikrein throughout all tissues (see MacDonald *et al.*, 1988). Thus, Chao *et al.* (1986), found that rat kallikreins from brain, spleen, kidney, pancreas, submandibular gland and urine were indistinguishable in their properties of mass, charge and immunoreactivity. Western blot analysis using a monoclonal antibody demonstrated a molecular mass for these kallikreins of 38 kDa (Chao *et al.*, 1986). Key amino acid residues which are conserved through different species are thought to be the primary determinants of the proteolytic cleavage specificity of tissue kallikreins (MacDonald *et al.*, 1988).

The results presented here from Western blot analysis and radioimmunoassay clearly demonstrate that substance R is a tissue kallikrein. The susceptibility to inhibition by SBTI is apparently its only distinguishing feature. There have been many reports in the past of kallikreins in the gastrointestinal tract, but none of those described is identical to substance R.

Werle and Vogel (1961) described a trypsin-activated SBTI-resistant substance in homogenates of colon wall which posessed hypotensive activity. Saline homogenates of duodenal, jejunal, ileal and colonic tissues of rat and rabbit contained an acid-activated kinin-forming activity (Amundsen & Nustad, 1965). Amundsen (1966) demonstrated the release of kinin, kinin precursor, kininase and kinin-forming activity during the early phase of strangulated terminal ileum in rat. The kinin-forming activity was only present in samples which were acidified. However, none of these authors tested enzyme inhibitors on this mucosal tissue extract.

Release of a `kallikrein-like amidase' from rat colon which hydrolysed a synthetic substrate of kallikrein has been demonstrated. Either incubation of tissue segments with bile salts, or perfusion of the colon with bile salts resulted in liberation of this enzyme, the activity of which was inhibited by aprotinin but not SBTI (Al-Dhahir & Zeitlin, 1983). Cat colon kallikrein has been isolated as a 40 kDa protein with several isoforms, inhibited partially by SBTI but not by aprotinin and is similar to other tissue kallikreins from cat in this respect (Fujimori *et al.*, 1986a, 1986b). A trypsin-activated prekallikrein with a high molecular weight and other properties suggesting it is a plasma kallikrein has also been isolated from human, monkey and dog colon tissue (Seki *et al.*, 1972).

It is possible that substance R may be derived from a source other than the ileum. Stomach kallikrein has been purified from rat and human tissues with molecular weights of 29 and 38 kDa respectively. The hydrolysis of a synthetic substrate by both stomach kallikreins was inhibited by aprotinin and DFP but no inhibition was seen with SBTI, LBTI or OMTI (Uchida *et al.*, 1976; Uetsuji *et al.*, 1981). The pancreas is another possible source of substance R, but unlike substance R, rat pancreatic kallikreins described so far have not been inhibited by SBTI (Vogel & Werle, 1970; Hojima *et al.*, 1975; Vogel, 1979).

Of perhaps more relevance to the present study are reports of a tissue kallikrein from rat small intestine. This is a tissue kallikrein distinct from plasma kallikein as defined by its molecular weight of 33 kDa (Zeitlin *et al.*, 1976), its hydrolysis of synthetic substrates (Zeitlin, 1971, 1972), and the action of inhibitors upon it (Zeitlin, 1971, 1972; Moriwaki *et al.*, 1977b; Frankish & Zeitlin, 1980).

Aqueous extracts of rat homogenized intestinal tissue contained a kinin-forming enzyme (KFE) (Zeitlin, 1971). Incubation of these extracts with a stable kininogen preparation liberated kinin. Activation of this enzyme was demonstrated when incubation of the soluble extract at room temperature for 18 hours or at 55°C for 10 minutes increased the KFE activity more than 5-fold. The kinin-releasing activity was inhibited by aprotinin but was not significantly reduced by SBTI (Zeitlin,

1971). Zeitlin (1972) assessed the actvity of the KFE on synthetic substrates of tissue kallikrein and the effect of inhibitors on this hydrolysis. The effect of inhibitors on the KFE was the same as previously reported for their actions on rat salivary and urinary kallikrein. Zeitlin (1972) concluded that rat intestinal kallikrein is a tissue kallikrein and is present as an inactive precursor, located in the intestinal wall.

The kallikrein content of the gut wall along the length of the gastrointestinal tract of the rat was examined by Frankish and Zeitlin (1977a, 1980). There was little variation in the tissue content of kallikrein between different regions except for a peak of activity found in the caecum, which contained more than 5 times that of any other segment. The kallikrein content of the caecum was found to be directly related to the degree of luminal filling. Fasting for 24 hours had little effect on the kallikrein content of most tissues other than the duodenum and the caecum, where it was doubled and halved respectively. In the present study fasting appeared to have no effect on the amount of substance R released into the perfusate, as would be expected from the results of Frankish and Zeitlin (1980) who found little difference in the kallikrein content of the ileum between fasted and fed rats. Frankish and Zeitlin (1980) also noted that 17% of the kinin-forming activity in the caecum was inhibited by SBTI, and suggested this may have been kallikrein derived from plasma, like that described previously by Seki *et al.*, (1972). However, it is interesting to speculate on the possibility that this may have been substance R derived from intestinal tissue.

The site of release of substance R from the perfused intestine is at present unknown. The presence of kininogenase in rat vascular tissues has previously been reported (Nolly & Lama, 1982; Nolly *et al.*, 1985, 1986). However, in the present study, when the arterial vasculature of the mesentery alone was perfused, none of the 20 minute fractions collected over 6 hours contained oxytocic activity. The kininogenase reported by Nolly and his colleagues was not inhibited by SBTI. It thus seems probable that substance R is released from the intestinal tissue during

perfusion, rather than the arterial vasculature. This would be in agreement with the reports of Zeitlin that intestinal kallikrein is present in the mucosal tissue.

As discussed above, kallikreins have been isolated from tissues throughout the gastrointestinal tract in a number of species. Most previous reports on the immunocytochemical localization of tissue kallikreins have concentrated on the cellular localization within exocrine glands such as the submandibular gland, pancreas and guinea-pig coagulating gland. These reports have shown that kallikreins are present in the secretory cells of the coagulating gland and pancreas, and in the duct cells of the salivary gland (see Schachter, 1980b). These kallikreins were found in both secretory granules and free within the cytoplasm, depending on the cell type.

Recently Schachter and his colleagues (1980b, 1983) localized colonic kallikrein in cat, rat and man to the goblet cells alone in this tissue. It was also reported that kallikrein-like enzymes were present in the lamina propria and submucosa of the colon and other regions of the gastrointestinal tract (Schachter *et al.*, 1980b). In a histochemical study of human colonic tissues, Garrett *et al.* (1982) reported that active kallikrein was seen only in the mast cells of the lamina propria. The immumocytochemical techniques utilized by Schachter *et al.* (1980b) may have identified the inactive proenzyme inaccessible to the visualization technique used by Garrett *et al.* (1982). These results are similar to those reported for the localization of a serine protease in the rat small intestine, which was located in the same tissue regions and also in mast cells (Woodbury *et al.*, 1978).

Tissues of the gastrointestinal tract contain all the components of the kallikrein-kinin system, i.e. kininogen (Geiger *et al.*, 1977), kallikrein (Zeitlin, 1971, 1972; Moriwaki *et al.*, 1977b), kinins, and kininases (see Johnson, 1979). The true physiological function of tissue kallikrein within the gut is unknown but has been the subject of some speculation.

The stimulatory action of kinins on isolated intestinal smooth muscle preparations

was one of their first recognized properties, but whether this is an important physiological function of the kallikrein-kinin system is uncertain (see Johnson, 1979; Bertacinni, 1982). However, stimulation of the pelvic nerves of the cat results in contraction of the colon and an associated reduction in the mucosal kallikrein and plasma kininogen contents (Fatsch *et al.*, 1978). The development of kinin receptor antagonists may now make an accurate assessment of the involvement of kinins in gastrointestinal motility possible.

Production and secretion of kallikreins by cells of exocrine glands is well established (see Gautvik *et al.*, 1976; Bhoola *et al.*, 1979). In addition to being secreted into exocrine fluids, it is thought that kallikreins act as mediators of functional vasodilatation within these glands, a role also proposed for the kallikreins of the gastrointestinal tract (Gautvik *et al.*, 1976). Despite the many vasoactive neuropeptides (see Forssmann, 1980) and other vasoactive autocoids present in gut tissues, kallikreins may act in a similar way to that envisaged in exocrine glands. The rapid initial increase in blood flow is probably mediated by vasodilator nerves, whilst the formation of kinins may more closely adjust the blood flow according to the functional and metabolic requirements of the secretory tissues (Gautvik *et al.*, 1976).

Possibly the major physiological function of the kallikrein-kinin system in the gastrointestinal tract is in the regulation of transepithelial transport and intestinal absorption. Kallikrein itself appears to be absorbed from the gut. This was first recognized when oral administration of kallikrein was used clinically in the treatment of several conditions (see Fink *et al.*, 1980). Later intestinal absorption of kallikrein was confirmed experimentally, and active tissue kallikrein absorbed through the gut wall has been detected in the circulation, bound to some plasma components (Moriwaki *et al.*, 1977a; Overlack *et al.*, 1983; Fink *et al.*, 1989). Intestinal absorption of kallikrein may also explain the traces of tissue kallikrein found in plasma by Rabito *et al.* (1980, 1982). Apart from their action of increasing vascular permeability in the gut, kallikreins rapidly increase the rate of amino acid

transportation by intestinal cells both *in vitro* and *in situ* (Moriwaki *et al.*, 1977a, 1977b). Kallidin stimulates sodium ion (Na^+) and glucose transport in the intestine from the mucosal to serosal surfaces (Moriwaki & Fujimori, 1979). Electrophysiological studies in several species have shown that an increase in net ion transport occurs across the gut wall following stimulation with kinins, which are more potent than a number of other agents (see Cuthbert & Margolius, 1982). Hardcastle *et al.* (1976) first established that the increased potential difference across the rat jejenum and colon caused by bradykinin was the result of an increased net chloride ion (Cl^-) secretion. This has since been confirmed in other tissues and species (see Cuthbert & MacVinish, 1989) and was shown to be dependent on calcium (Ca^{++}) (Cuthbert *et al.*, 1984). Cuthbert and Margolius (1982) demonstrated that rat glandular kallikrein and mellitin, a potent activator of membrane-bound kallikrein, stimulated Cl^- secretion. Kinin-stimulated ion secretion from intestinal epithelial cells is stimulated more by applicaton of the agonist to the basolateral surface of the cells than to the mucosal surface (see Cuthbert & MacVinish, 1989). Valine transport was also stimulated more by additon of kallikrein to the serosal surface than to the mucosal surface of the tissues (Moriwaki *et al.*, 1977b). These results are in agreement with the reported location of the bradykinin receptor sites on the serosal surface of intestinal cells (Manning *et al.*, 1982). The influence of kinins on ion transport in epithelial cells is not thought to be a direct action. Indomethacin and phospholipasae A_2 inhibitors block Cl^- secretion (Hardcastle *et al.*, 1976; Cuthbert & Margolius, 1982; Musch *et al.*, 1983). Thus, Cl^- secretion is believed to be mediated by prostaglandin generation by the epithelial cell, stimulated by kinins (see Cuthbert & MacVinish, 1989). However, depending on the species, lipoxygenase products have been implicated in place of prostaglandins (Musch *et al.*, 1982, 1983). Likewise, epithelial Na^+ uptake is thought to be mediated by a secondary factor. In this case, an increased cytosolic Ca^{++} concentration, the major part of which is mediated by phospholipase C stimulation and the inositol phosphate pathway, although stimulation of kinin receptors may directly affect Ca^{++} concentration (see Cuthbert & MacVinish, 1989).

The first activity observed for substance R was the contraction of the isolated uterus (Gaddum, 1953). The oxytocic action of kallikreins was first noted by Frey *et al.* in 1928. Rat tissue kallikreins contract the isolated rat uterus (Beraldo *et al.*, 1966; Erdos *et al.*, 1968), whilst having no effect on the uterus preparation from other species (Beraldo *et al.*, 1966). In contrast, tissue kallikreins from a number of other species are without oxytocic activity on the rat uterus (Beraldo *et al.*, 1966; Whalley, 1979). The mechanism of action of the oxytocic activity of rat tissue kallikrein has been the subject of a long running dispute as to whether kallikrein acts directly itself on the uterine smooth muscle cells or via kinin generation.

In a series of publications Beraldo and his colleagues proposed that this oxytocic action of kallikrein was independent of uterine kininogen content, the enzyme acting on specific kallikrein receptors on the smooth muscle cells (Beraldo *et al.*, 1966, 1971, 1975, 1976; Araujo *et al.*, 1970; Siqueira *et al.*, 1970). On the other hand, Nustad and Pierce (1974) provided evidence that kinin generation from uterine kininogen was responsible for contraction of the smooth muscle. The tachyphylaxis which occurs after repeated additions of kallikrein to uterine preparations (Nustad & Pierce, 1974; Beraldo *et al.*, 1976) was cited as evidence to the contrary by Beraldo. The muscle still contracted, at the end of 8-9 hours of repeated addition of kallikrein, to 30-50 times the original dose (Beraldo *et al.*, 1976). An alteration in the configuration of the kallikrein receptor, rendering it progressively less reactive to kallikrein was suggested to account for the tachyphylaxis rather than a progressive depletion of kininogen (Beraldo *et al.*, 1976). Others have supported Beraldo's hypothesis. The oxytocic action of kallikrein was unaffected by either carboxypeptidase B or a bradykinin antiserum (Chao *et al.*, 1981). This group could detect no kinin in a radioimmunoassay from media in which kallikrein and uterine tissue had been incubated, and so concluded that the oxytocic action of kallikrein is independent of kinin generation. In contrast, it has recently been demonstrated that both caboxypeptidase B and an antibody to bradykinin reduced uterine responses to kallikrein (Orce *et al.*, 1989). Furthermore, Figueiredo *et al.* (1989, 1990) demonstrated the dependence upon uterine kininogen of the contraction of the uterus

by kallikrein. Whalley (1979) reported that a prototype kinin receptor antagonist inhibited the oxytocic activity of trypsin and kallikrein. This has been confirmed recently by Orce *et al.* (1989) and in the present study, using a selective BK_2 bradykinin receptor antagonist. Uterine contractile responses to both urinary kallikrein and substance R were inhibited by D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin. There was no significant difference between the inhibition of responses to bradykinin, urinary kallikrein or substance R by this antagonist, suggesting that the oxytocic activity of each is mediated by the BK_2 bradykinin receptor, and provides further evidence of the co-identity of substance R with tissue kallikrein. Thus, it is proposed that the oxytocic activity of rat tissue kallikrein and substance R is dependent on kinin generation from uterine kininogen.

In conclusion, substance R is a kininogenase enzyme released from the perfused rat small intestine which shares immunological and physical characteristics with rat tissue kallikrein, but differs in its susceptibility to protease inhibitors. The slight differences in molecular weight to other rat tissue kallikreins and perhaps the differences in inhibition by SBTI may be due to variations in carbohydrate composition. The primary protein structure is probably identical to that determined for other rat tissue kallikreins. However, any variation in the carbohydrate content of the enzyme may result in subtle changes in the conformation of the molecule from that seen in enzymes isolated from other sources. Thus it seems probable that any molecular differences are the result of post-translational mechanisms upon the protein and not in its primary structure determined by expression of the tissue kallikrein gene.

In the future it may be possible to determine the origin of substance R in intestinal tissue by immunohistochemical techniques, utilizing polyclonal or monoclonal antibodies to tissue kallikrein. It is also hoped to study differences in the inhibitory profile of substance R further, and in the near future to sequence the N-terminus of the substance R molecule for comparison with other tissue kallikreins. Despite having partially characterized substance R, why Gaddum chose the initial "R"

remains a mystery. Substance P was so-called because the original preparation was labelled "P" (von Euler & Gaddum, 1931). Perhaps "R" stood for rat. If, however, "R" was next in line after "P", it begs the question of what happened to "substance Q"!

SECTION 5

TABLES AND FIGURES

Table 1.

Source of tissue kallikrein	Reference
brain	Chao *et al.*, 1982
pituitary gland	Polivka *et al.*, 1981 Vio *et al.*, 1984
thyroid	Uchida *et al.*, 1981
spleen	Chao *et al.*, 1984
adrenal glands	Scicli *et al.*, 1989
parotid gland	Chao *et al.*, 1987
seminal plasma	Fink & Schill, 1983
uterus	Marin-Grez *et al.*, 1982
mucosal tissues	Baumgarten *et al.*, 1989
gastrointestinal tract tissue	Werle & Vogel, 1961 Amundsen & Pierce, 1965 Zeitlin, 1971, 1972 Uchida *et al.*, 1976 Moriwaki *et al.*, 1977a, 1977b Utsuji *et al.*, 1981 Fujimori *et al.*, 1986a
adipocytes	Mulholland *et al.*, 1986
arterial vascular tissue	Nolly & Lama, 1982 Nolly *et al.*, 1985
skeletal muscle	Shimojo *et al.*, 1987
polymorphonuclear leukocytes	Figueroa *et al.*, 1989

Table 2.

COMPARISON OF RAT TISSUE KALLIKREIN AND SUBSTANCE R: HYDROLYSIS OF THE CHROMOGENIC SUBSTRATE S-2266

		Rat Tissue Kallikrein		Substance R	
Inhibitors		% Inhibition			
Aprotinin	1x10-8M	43.8	49.0	88.1	82.1
	3x10-8M	68.7	82.3	96.4	91.1
	6x10-8M	100	100		
SBTI	50µg/ml	16.7	31.0	82.4	78.3
	100µg	33.3	56.0	86.7	86.2
	200µg	41.6	75.0	91.2	
Sheep anti RUK					
	2.53x10-9M	30.0		4.4	
	6.33x10-9M	57.5		30.2	
	1.27x10-8M	80.0		58.8	
	2.53x10-8M	86.7		92.2	
V4D11	1.47x10-8M	35.7		3.67x10-8M	20.0
	5.14x10-8M	46.5		1.83x10-7M	58.0
	1.25x10-7M	98.9			

Brit. J. Pharmacol. (1953), **8**, 321.

THE TECHNIQUE OF SUPERFUSION

BY

J. H. GADDUM

From the Department of Pharmacology, University of Edinburgh

(RECEIVED JUNE 5, 1953)

The amount of an active substance required for an assay on isolated plain muscle suspended in a bath depends upon the volume of the bath, which cannot conveniently be made much smaller than about 2 ml. A piece of intestine may be suspended in air and kept in good condition by a stream of a suitable solution running over its surface (Finkleman, 1930). This technique may be called superfusion, since the fluid runs over the tissue, by analogy with perfusion, in which the fluid runs through the tissue.

The effect of drugs on such a preparation may be tested by injecting them into the stream of fluid (Gaddum, Jang, and Kwiatkowski, 1939), and such preparations have been used for the assay of histamine (Kwiatkowski, 1941).

Greater sensitivity can be obtained by stopping the flow for a standard interval of time and applying the active solutions undiluted to the surface of the muscle. The object of the present paper is to describe an apparatus which has been developed during the last few years for this purpose and to present some results obtained with it.

METHODS

The apparatus is shown in Fig. 1. The muscle is suspended in air in the middle of a wide glass tube which is immersed in warm water in a small copper bath. This copper bath is attached to a vertical rod and the lever is attached to the same rod. The warm water is mixed by means of a stream of air which is sufficiently violent to cause slight vibration of the whole apparatus and so diminish friction of the writing point on the drum. The vibration may be controlled by finding the appropriate height for the apparatus on the vertical bar. The appropriate salt solution is contained in a reservoir 20–40 cm. above the bath. It runs from this reservoir through a glass tap and a glass-wool filter, and is then warmed by passing through the water in the copper bath. The rate of flow is controlled by a resistance which consists of a capillary tube drawn out to a fine point and held inside a wider glass tube by passing the same rubber tube over both. The flow is adjusted to 1–5 ml./min. by altering the height of the reservoir. The superfusion fluid drips from a glass tube down the cotton which attaches the tissue to the lever. The end of this tube has been filed off at an acute angle in order to facilitate the administration of drugs. The height of the glass tube above the tissue must not be too small, or the solution may run down one side of the tissue and allow the other side to dry. If the height is sufficient (> 10 cm.), each drop is broken up on reaching the tissue, and wetting is even.

Solutions to be tested are contained in small test-tubes (12×65 mm.) which are warmed before use by placing them in a suitable holder attached to the copper bath.

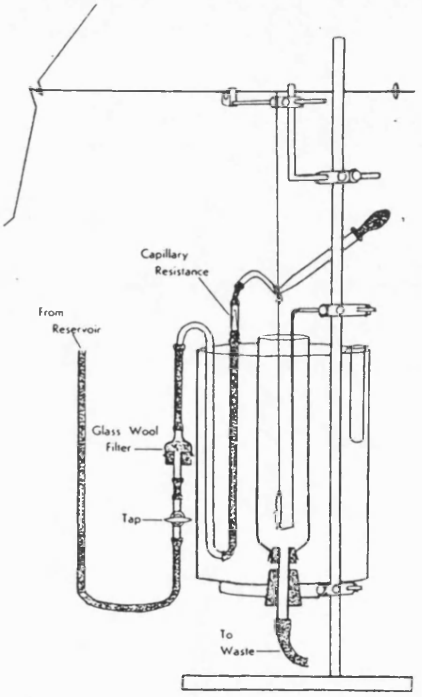


FIG. 1

Figure 1. The title page from Gaddum's 1953 paper on the technique of superfusion in which he described, for the first time, an oxytocic principle which he named substance R.

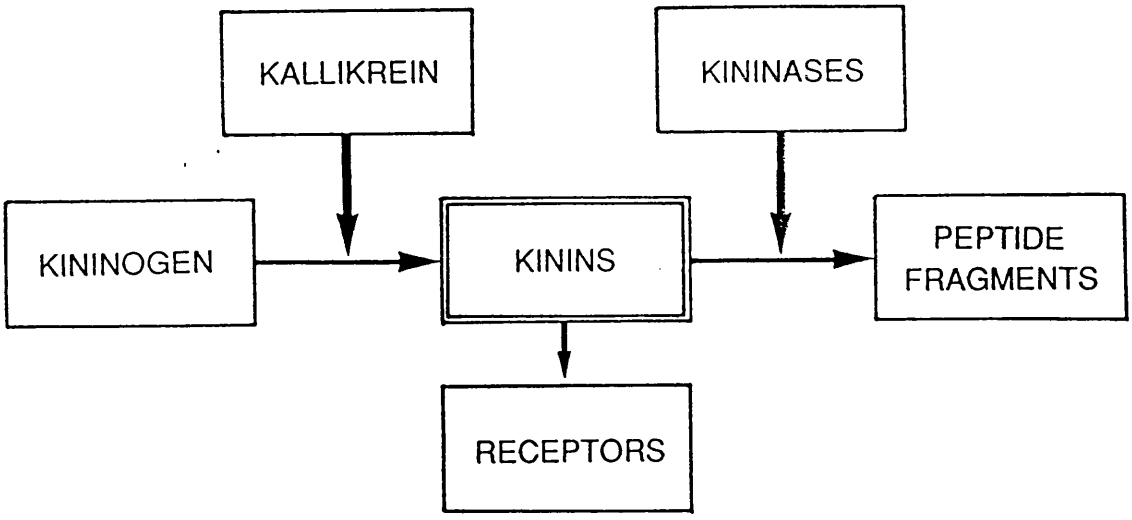


Figure 2. Schematic representation of the interrelationships of the major components of the kallikrein-kinin system. The high molecular weight precursor of the kinins, kininogen, undergoes limited proteolysis by the serine protease kallikrein. This results in liberation of the short chain polypeptide effector kinins which act on receptors located on the surface of the target cells. Kinins are acted upon by kininase enzymes with the formation of inactive peptide fragments.

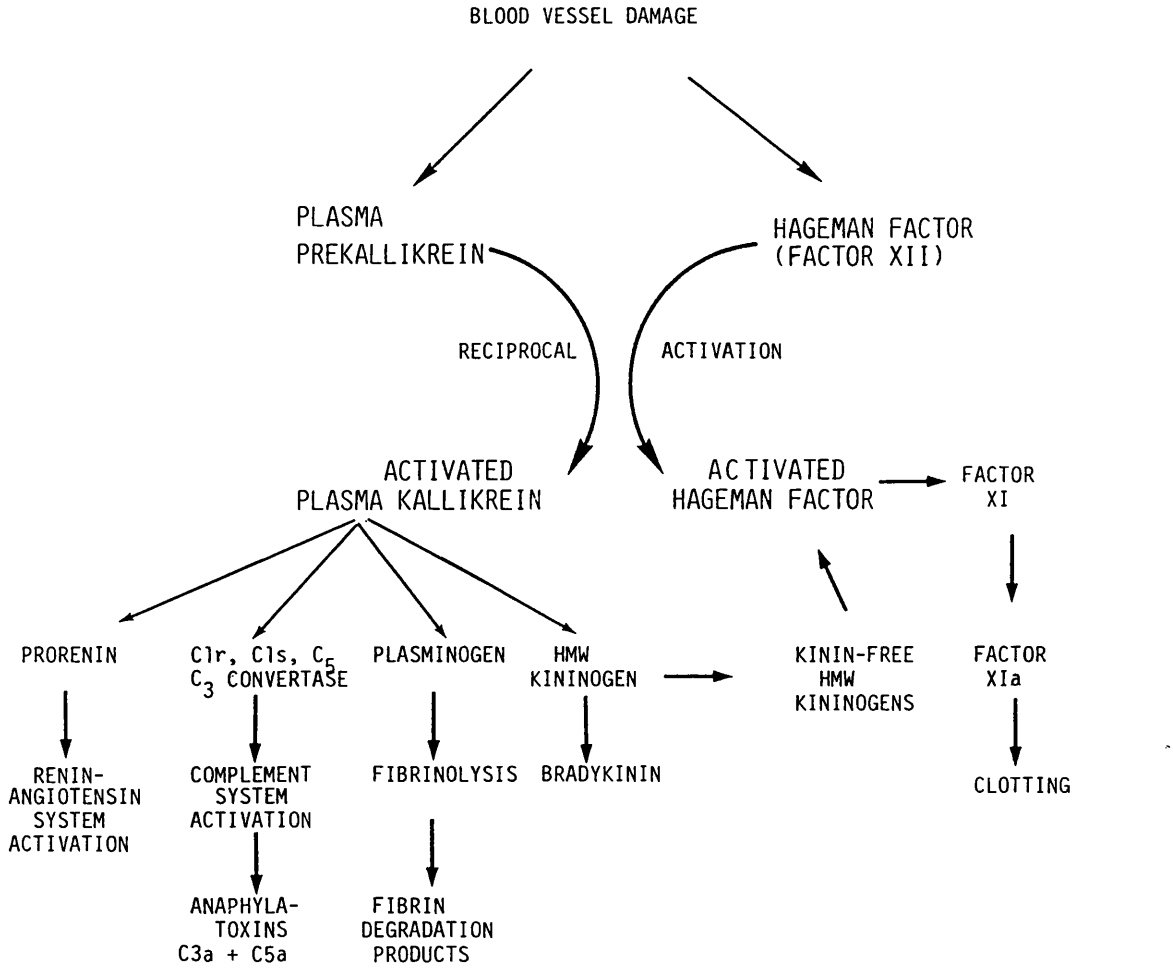


Figure 3. Interrelationship of the plasma kallikrein-kinin system with the blood clotting, fibrinolytic, complement and renin-angiotensin systems.

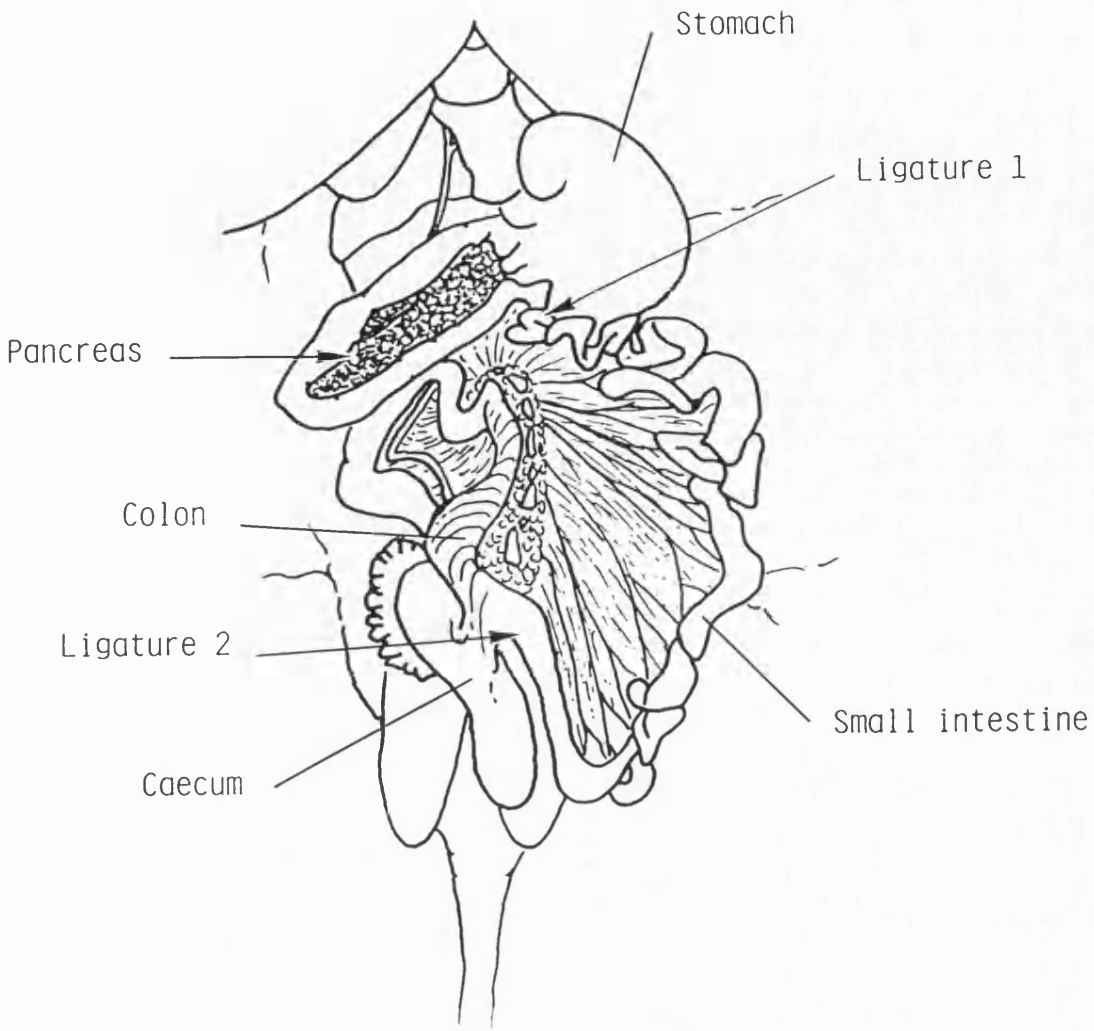


Figure 4. Dissection of the rat small intestine. Following laparotomy, two pairs of ligatures were tied around the intestine, at duodenal level (Ligature 1) and at the ileo-caecal junction (Ligature 2). The superior mesenteric artery was then cannulated and all other vessels, with the exception of the hepatic portal vein, were tied off. The intestine was sectioned between each pair of ligatures and the whole mesenteric bed was removed, care being taken to exclude any pancreatic tissue. The vasculature was flushed gently with heparinised Tyrode's solution before the preparation was mounted in a heated jacket.

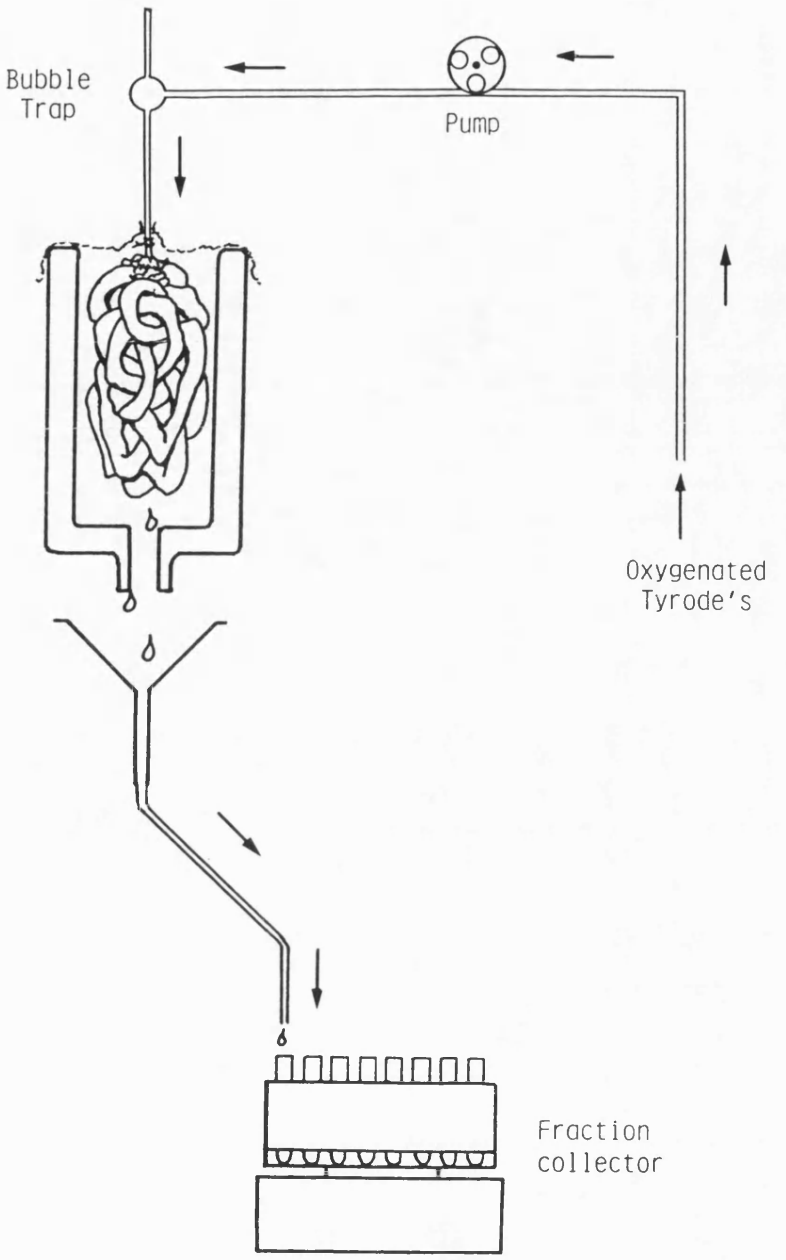


Figure 5. Perfusion of the isolated small intestine of the rat. The dissected intestinal preparation was suspended in a covered heated jacket (37°C) and perfused with aerated (95% O_2; 5% CO_2) Tyrode's solution (0.4 ml min^{-1}, peristaltic pump) via the mesenteric artery. Perfusate from the hepatic portal vein was collected by means of a fraction collector. The oxytocic activity of the fractions of perfusate was determined on the superfused isolated rat uterus.

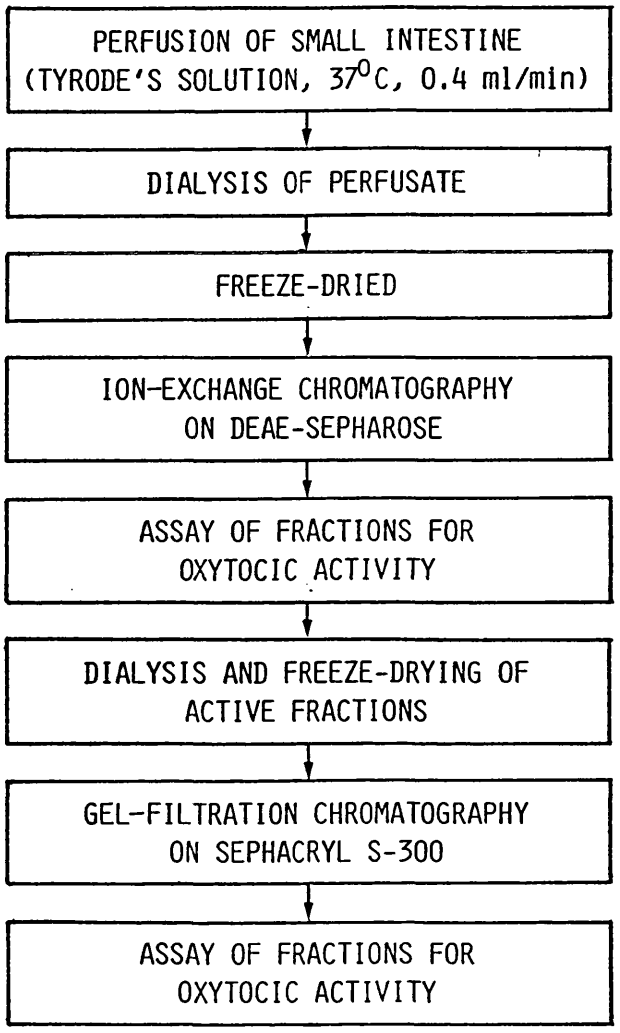


Figure 6. Scheme summarising steps in the purification of substance R from intestinal perfusate.

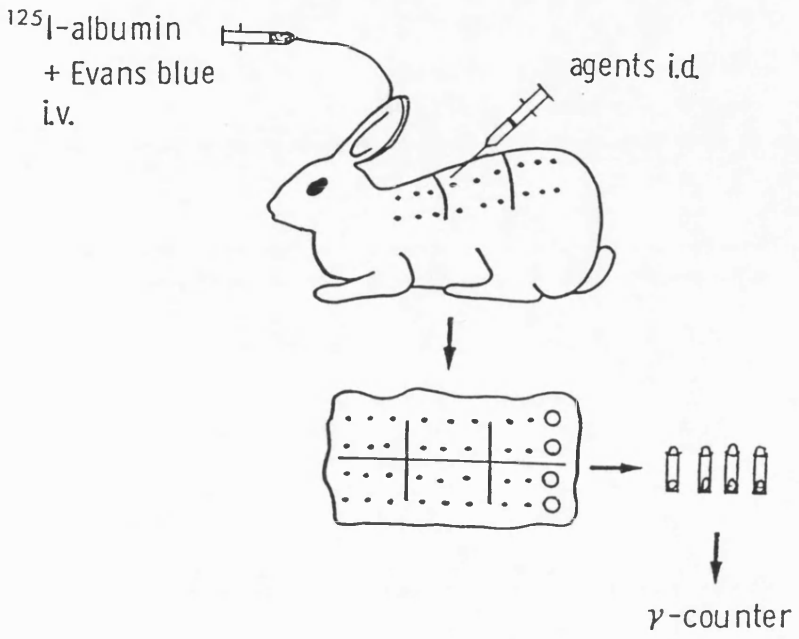


Figure 7. Measurement of oedema in rabbit skin as represented by the local accumulation of ^{125}I-HSA. ^{125}I-HSA is administered via the marginal ear vein, together with Evans blue dye as a visual marker, to an anaesthetised rabbit. Inflammatory agents are injected intradermally into the clipped dorsal skin. The animal is killed after a fixed time by an overdose of barbiturate. A blood sample is taken and from this plasma samples are prepared. The skin sites are excised and their radioactivity determined in a gamma-counter. Exudate volumes are calculated in terms of µl plasma per site.

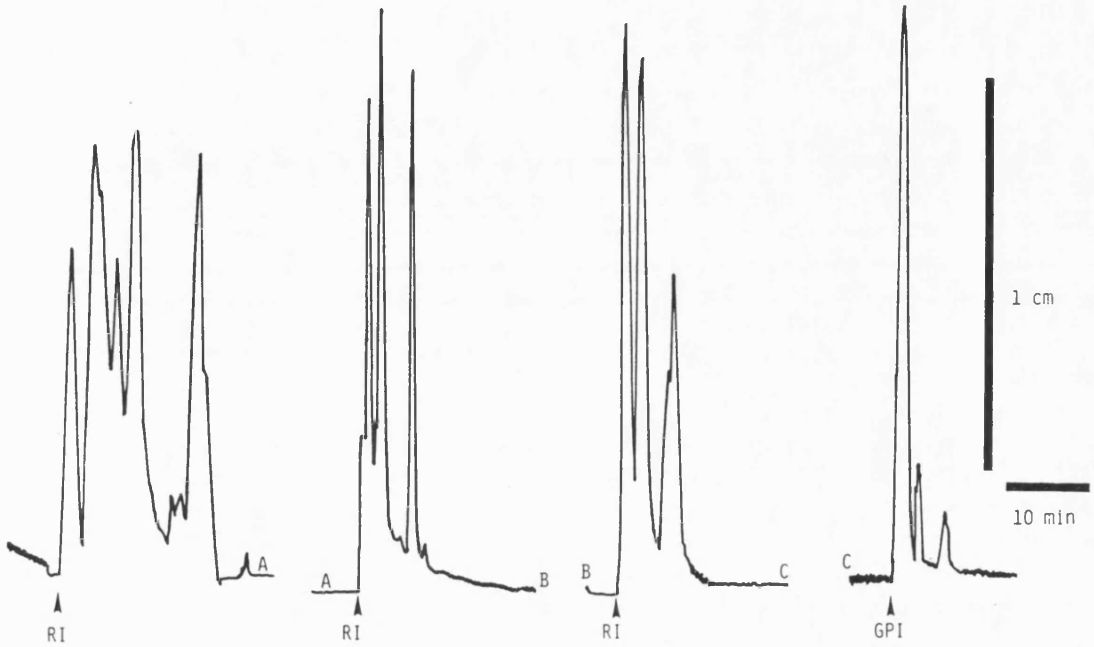


Figure 8. Contractions of the superfused rat isolated uterus. Release of an oxytocic substance from rat intestine. A length of rat intestine was introduced into the stream of superfusion fluid above a rat uterus. A series of slow and prolonged contractions of the uterus were observed. The trace shows the responses of a single uterus over several hours. Lengths of small intestine (RI) were suspended above the uterus as represented by arrows. A-A, B-B and C-C indicate periods (70, 140, 45 min respectively) where the intestine was superfused above the uterus but no activity was released. Perfusion of guinea-pig ileum (GPI) elicited a similar response from the uterus to that elicited by perfusion of rat ileum.

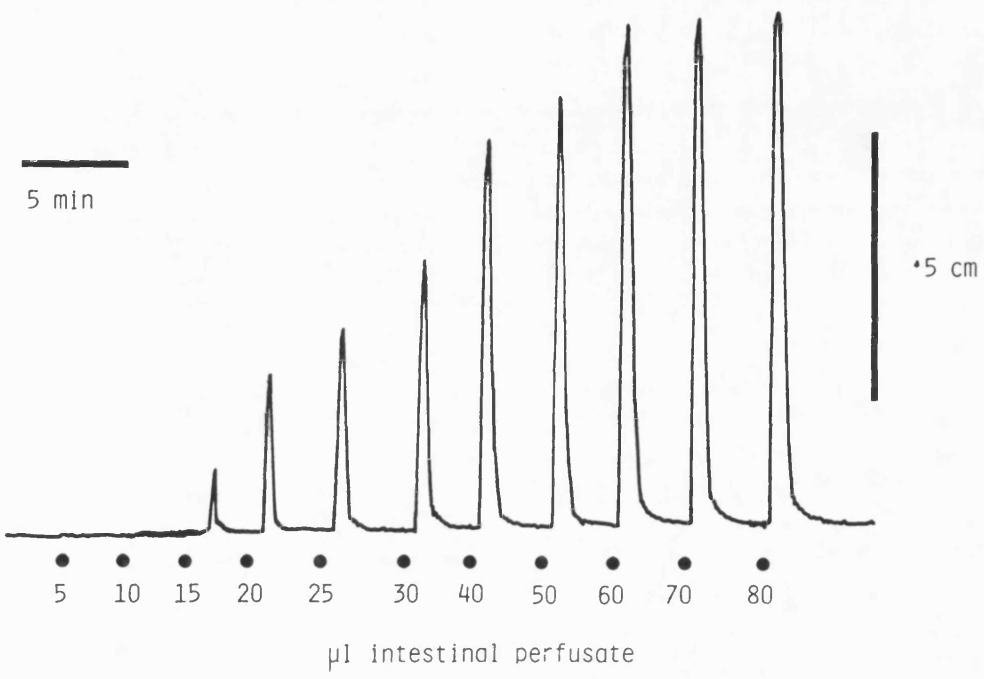


Figure 9. Effect of intestinal perfusate on the isolated superfused rat uterus. Intestinal perfusate causes a volume-dependent contraction of the rat uterus.

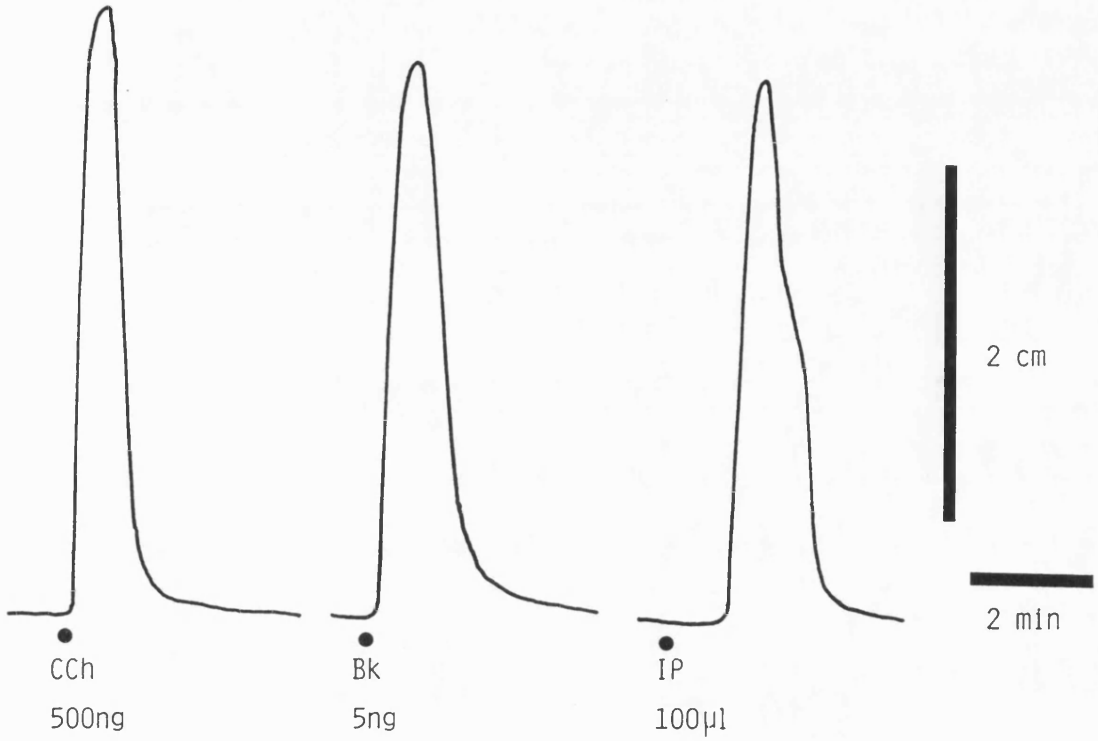


Figure 10. Comparison of the responses of the superfused isolated rat uterus to carbachol (CCh), bradykinin (Bk) and intestinal perfusate (IP). The uterine contractions to bradykinin and intestinal perfusate were similar, being slower to reach a maximum, and of a longer duration than that to carbachol. However, there was a latent period of 30-60 sec after the intestinal perfusate had passed over the tissue before a response was observed.

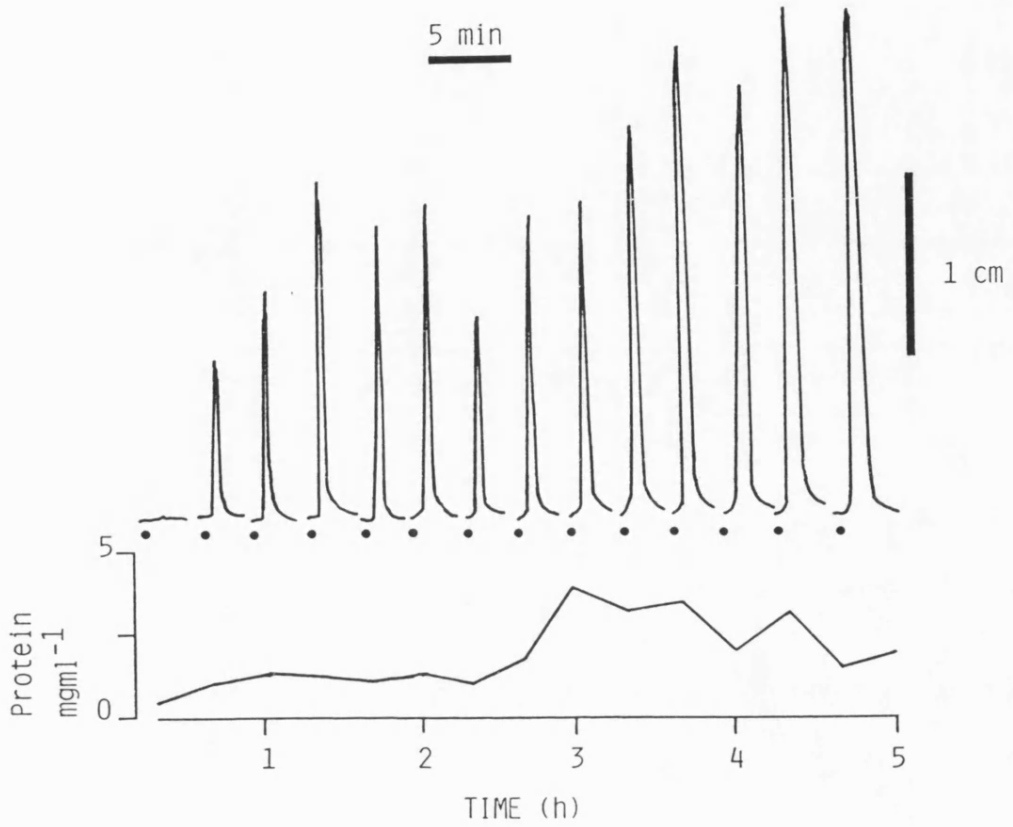


Figure 11. Appearance of oxytocic activity in perfusate from the mesenteric bed. The top panel shows contractions of the superfused rat uterus induced by 100 µl volumes of intestinal perfusate from samples collected over consecutive 20 min periods. No oxytocic activity was detectable during the first 20 min of perfusion, but thereafter the samples contained increasing amounts of activity, reaching a maximum at 3-4 h. The bottom panel shows the protein concentration of the fractions of intestinal perfusate which gradually increased with time. The lower time scale refers to the time of collection of the perfusate.

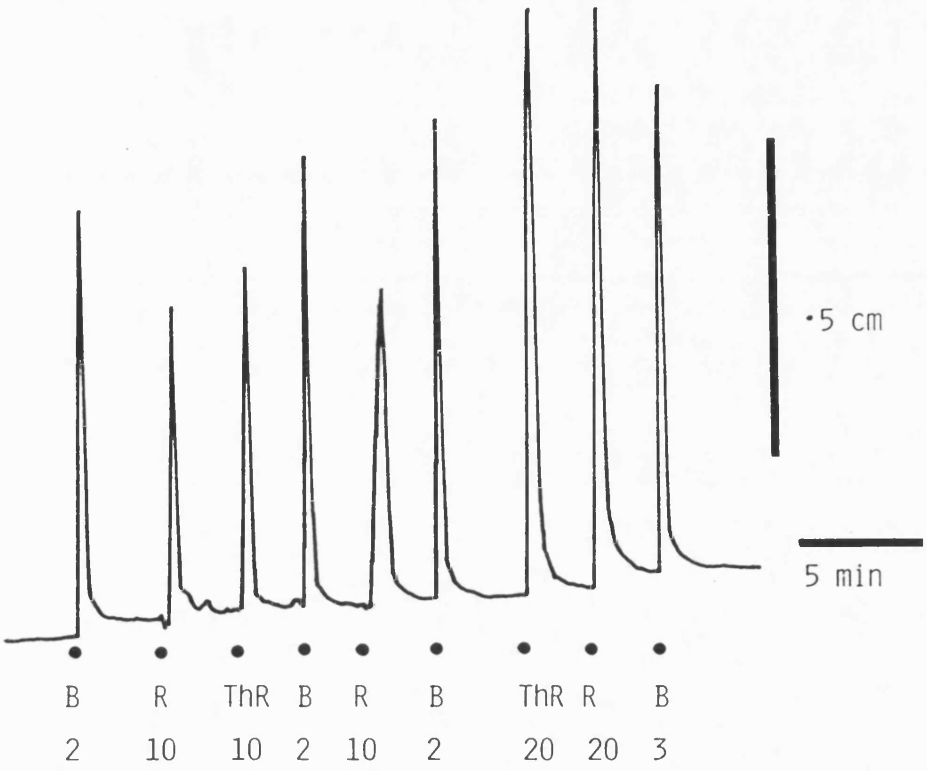


Figure 12. Effect of thioglycolate treatment on the oxytocic activity of substance R. Responses to thioglycolate-treated substance R (ThR, µl) did not differ from those of a control sample of substance R (R, µl). The tissue maintained responsive to bradykinin (B, ng) throughout the experiment.

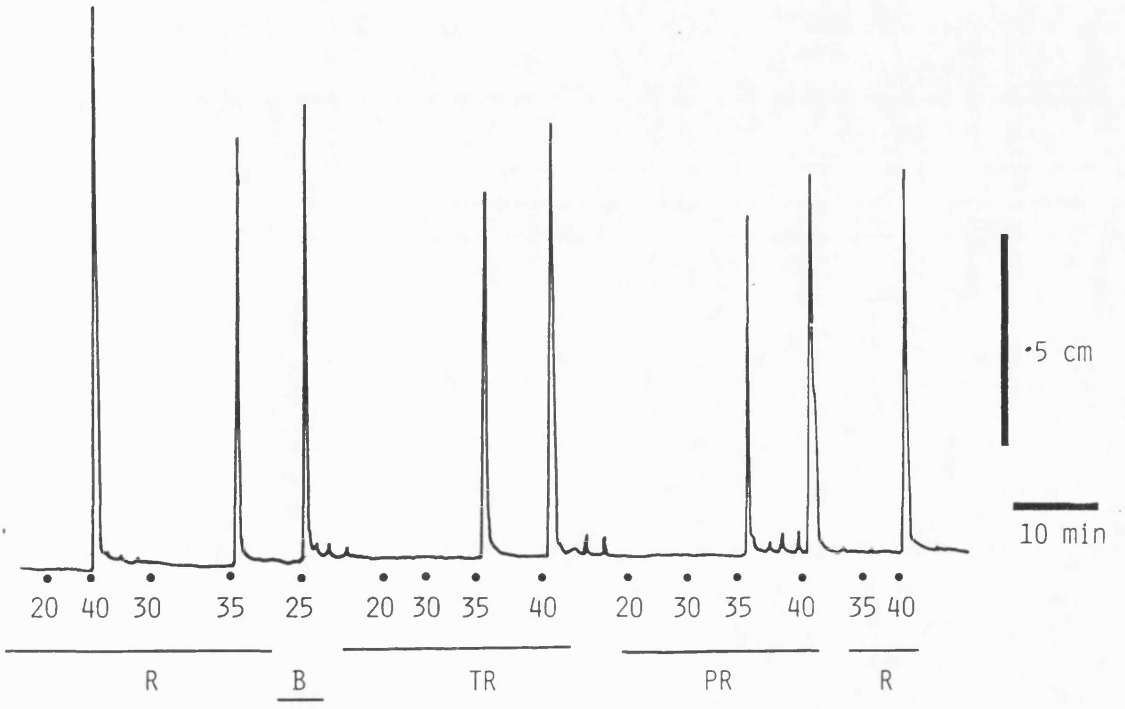


Figure 13. Effect of the proteolytic enzymes trypsin and papain on the oxytocic activity of substance R. Oxytocic responses to substance R (R, µl), bradykinin (B, ng), substance R incubated with insoluble trypsin (TR, µl) and substance R incubated with insoluble papain (PR, µl). The oxytocic activity of substance R was not affected by incubation with either of these enzymes.

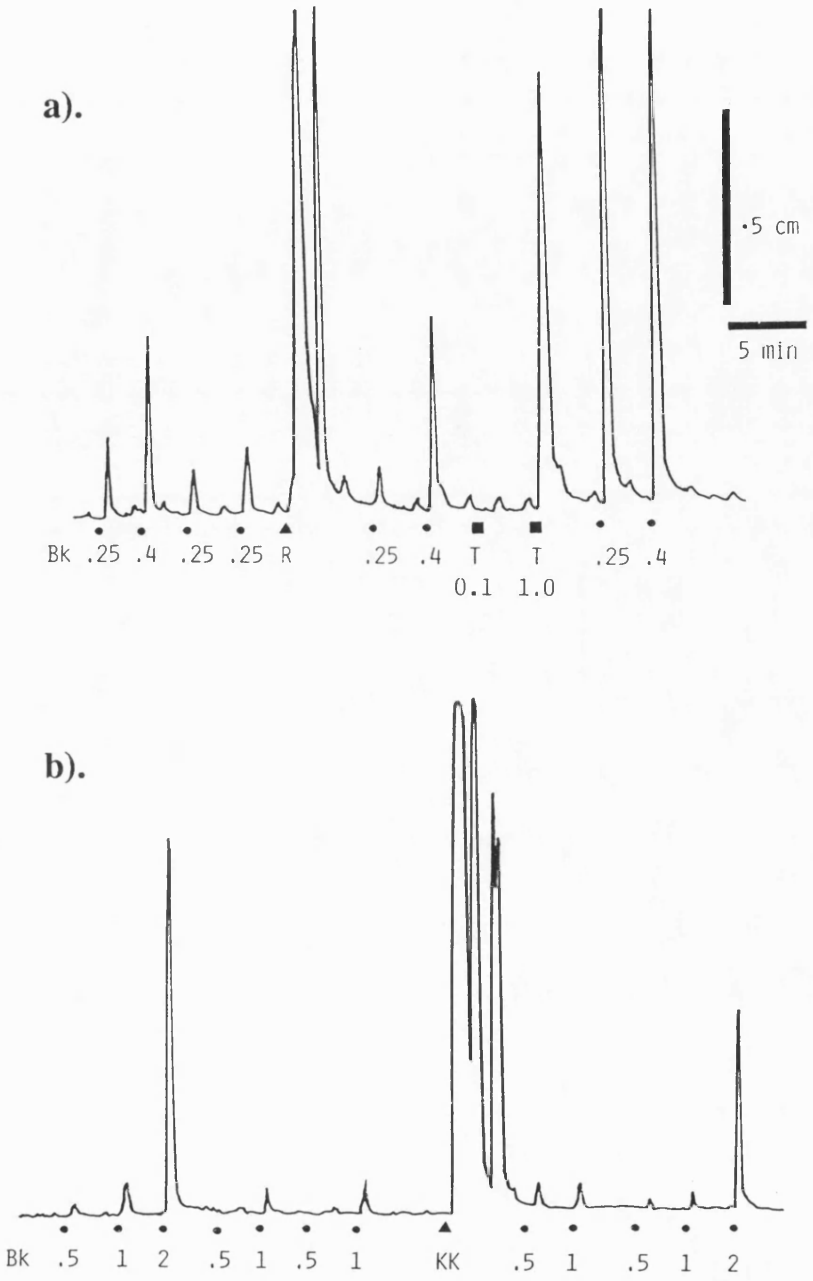


Figure 14. Effect of intestinal perfusate, trypsin and urinary kalllikrein on the superfused rat isolated uterus. **a).** A supramaximal dose of intestinal perfusate (R) has no effect on the responses to bradykinin (Bk, ng). In contrast, trypsin (T, mg) also contracts the uterus, but in addition induces a sensitization of the tissue to bradykinin. **b).** Similar to intestinal perfusate, a supramaximal dose of urinary kallikrein (KK) does not induce a sensitization of the uterus to bradykinin.

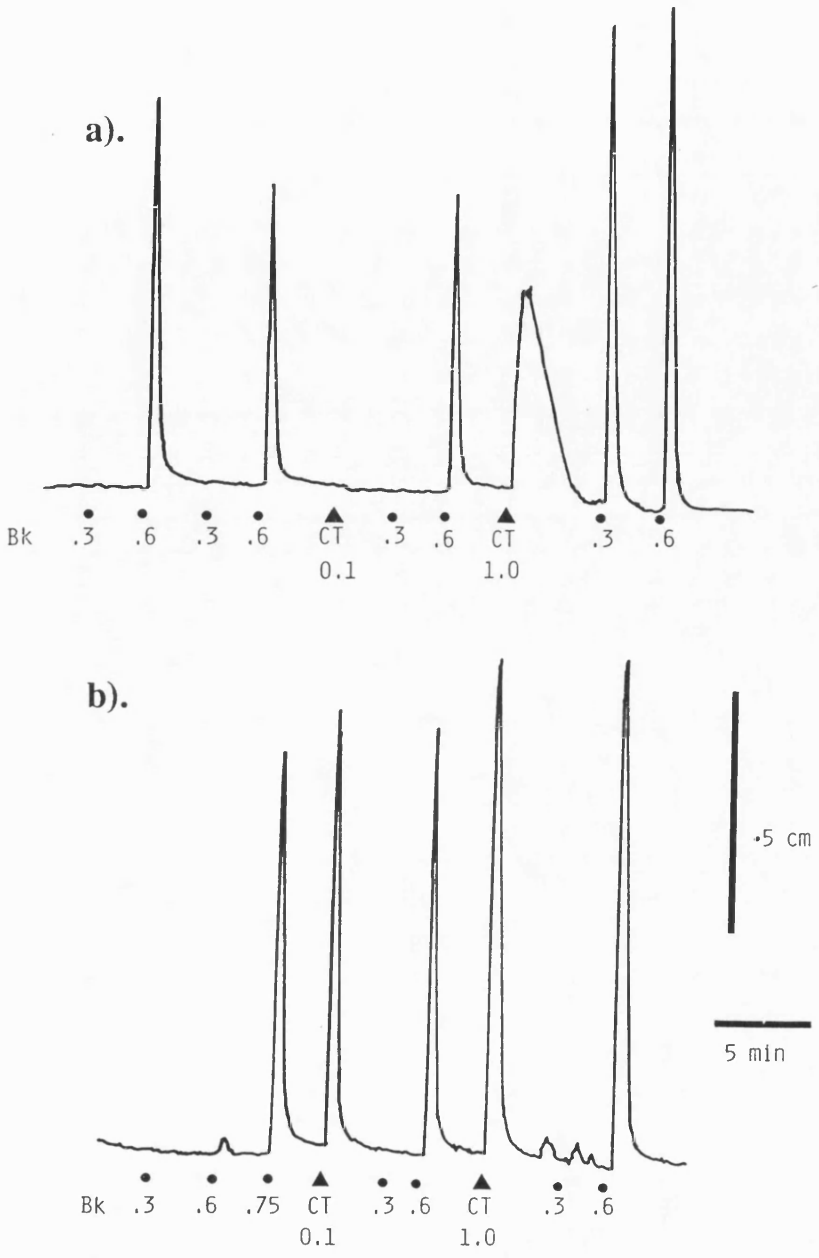


Figure 15. Effect of chymotrypsin on the superfused isolated rat uterus. **a).** Alpha-chymotrypsin (CT, mg) contracts the uterus and induces sensitization to bradykinin (Bk, ng). **b).** Delta-chymotrypsin (CT, mg) is more potent than alpha-chymotrypsin in its oxytocic activity and also induces sensitization of the tissue to bradykinin.

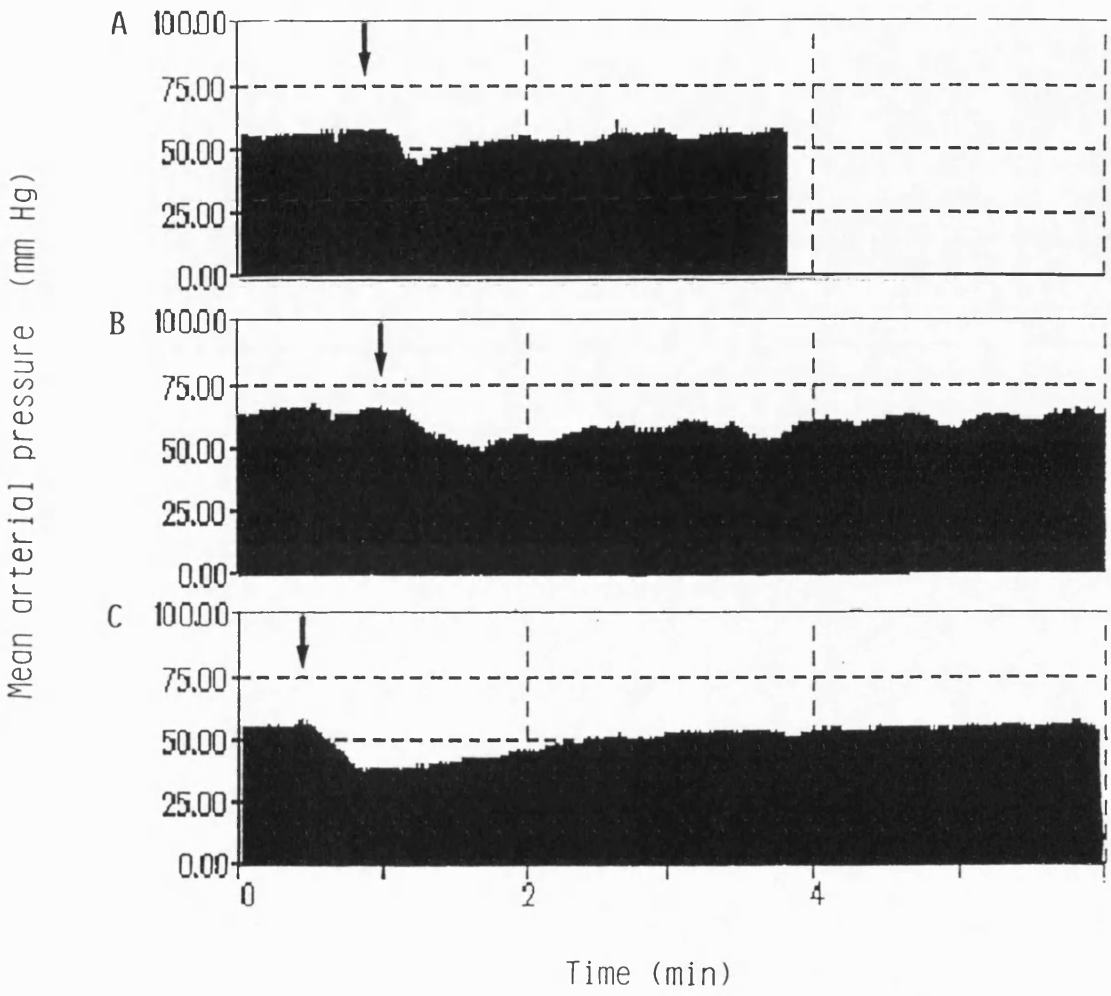


Figure 16. Effect of substance R on blood pressure. Substance R had a dose-related hypotensive action in the anaesthetised guinea-pig. A progessive fall in blood pressure was obtained with **a).** 10 µl, **b).** 20 µl and **c).** 50 µl of a preparation of substance R, injected intravenously as a bolus (200 µl) at the points indicated (↓).

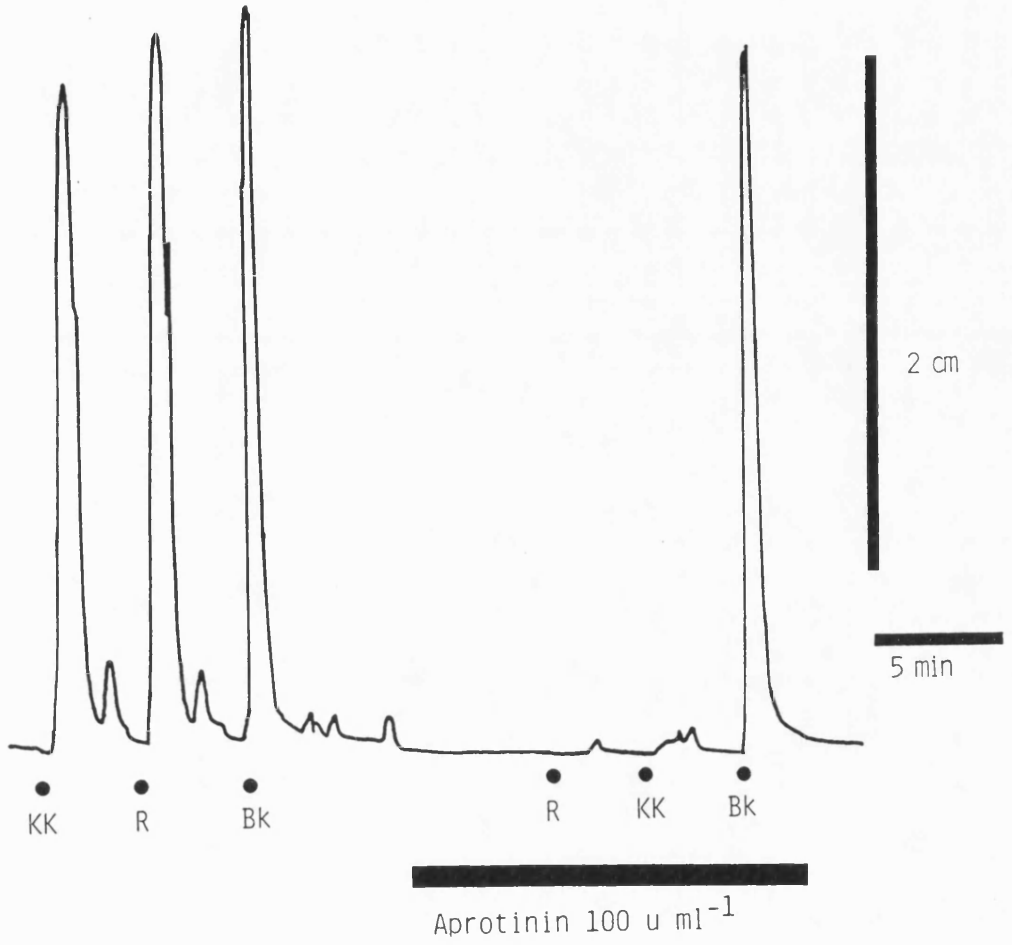


Figure 17. The effect of aprotinin on the oxytocic activities of urinary kallikrein (KK), substance R (R) and bradykinin (Bk). Aprotinin completely inhibited uterine contraction to both kallikrein and substance R, but did not affect responses to bradykinin.

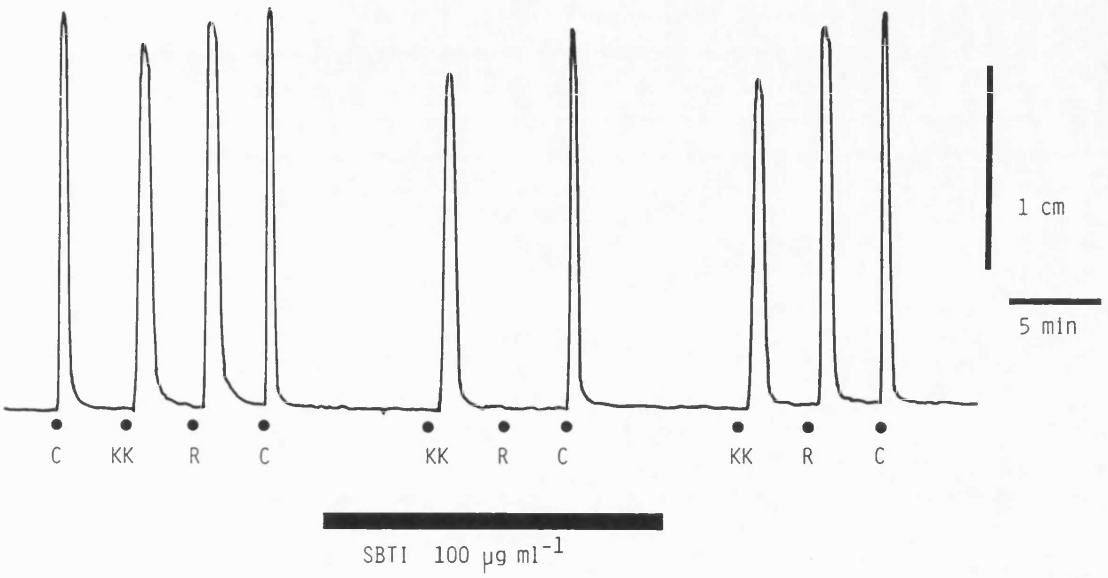


Figure 18. The effect of soy bean trypsin inhibitor (SBTI) on the oxytocic responses of urinary kallikrein (KK), substance R (R) and carbachol (CCh). SBTI reversibly inhibited the response to substance R without a significant reduction in the response to kallikrein or carbachol.

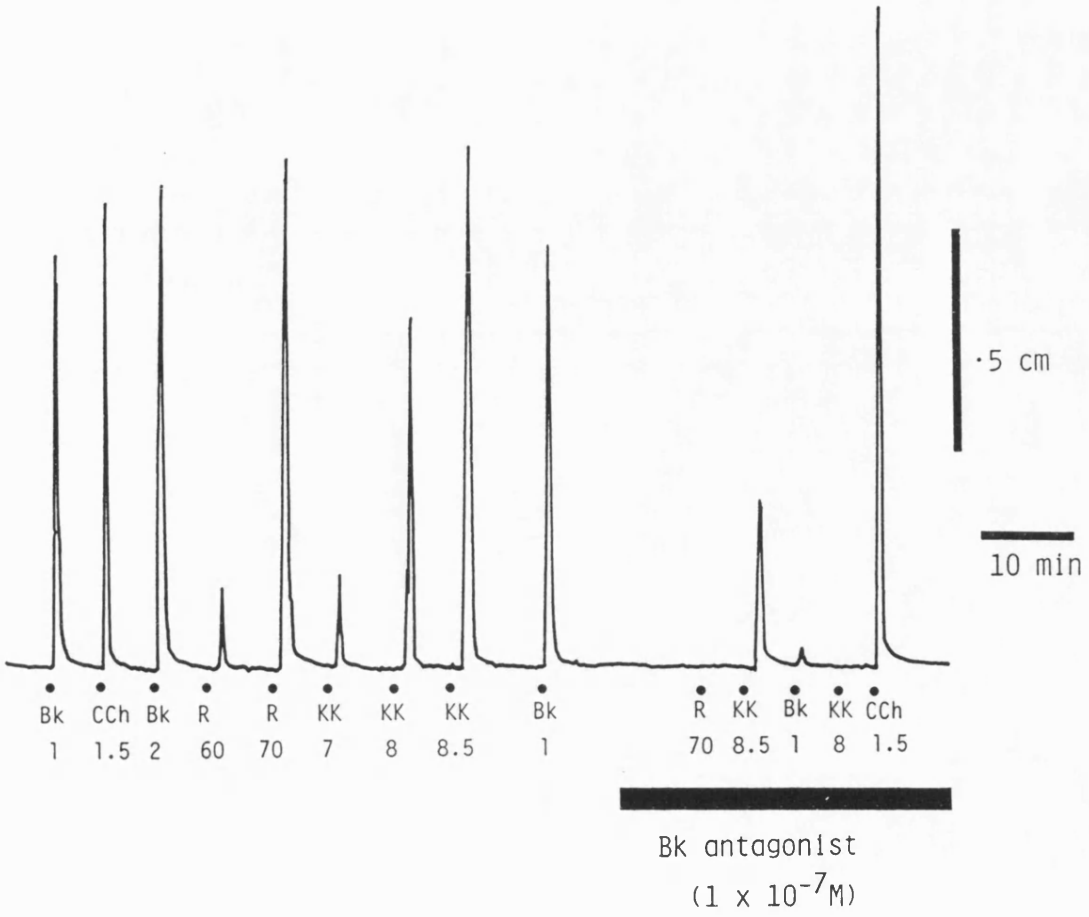


Figure 19. The effect of the selective BK_2 bradykinin receptor antagonist D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin on the oxytocic activities of bradykinin (Bk, ng), carbachol (CCh, ng), substance R (R, µl) and urinary kallikrein (KK, µl). D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin reduces responses to substance R, urinary kallikrein and bradykinin but not those to carbachol.

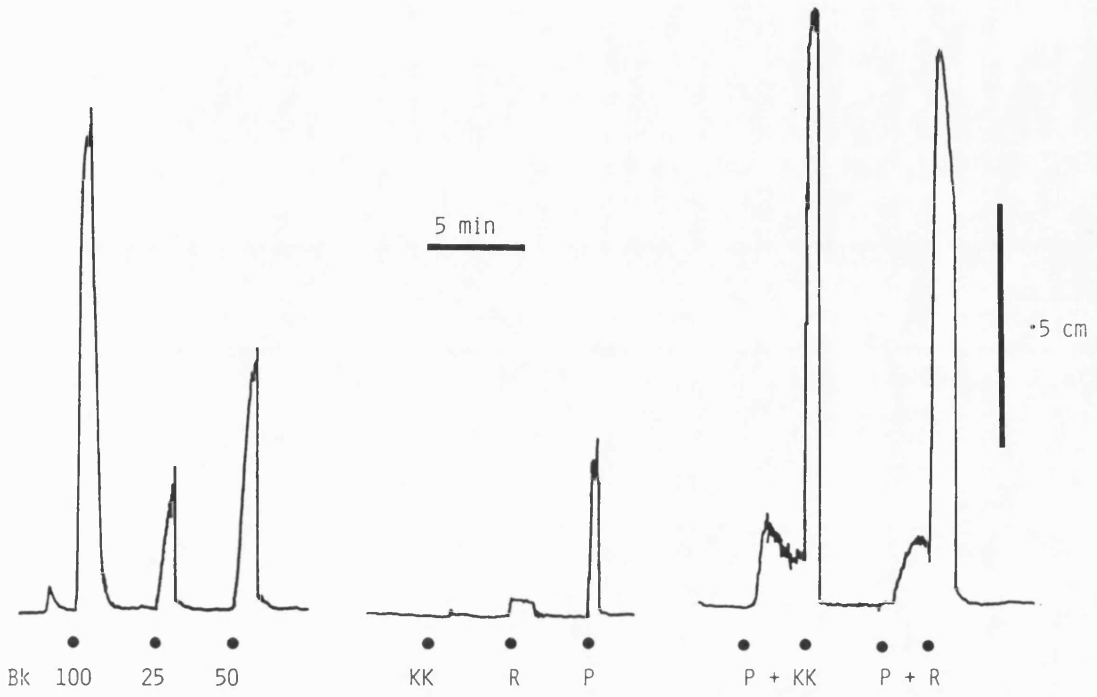


Figure 20. Effect of incubating plasma with kallikrein or substance R on the guinea-pig ileum in the presence of captopril (4.6×10^{-4}M). The guinea-pig ileum contracted to bradykinin (Bk, ng ml^{-1}) but not to urinary kallikrein (KK) or substance R (R) alone and sometimes gave a small contraction to rat plasma (P) alone. However, when kallikrein or substance R were added to the organ bath in the presence of rat plasma, a slow contraction of the ileum was observed.

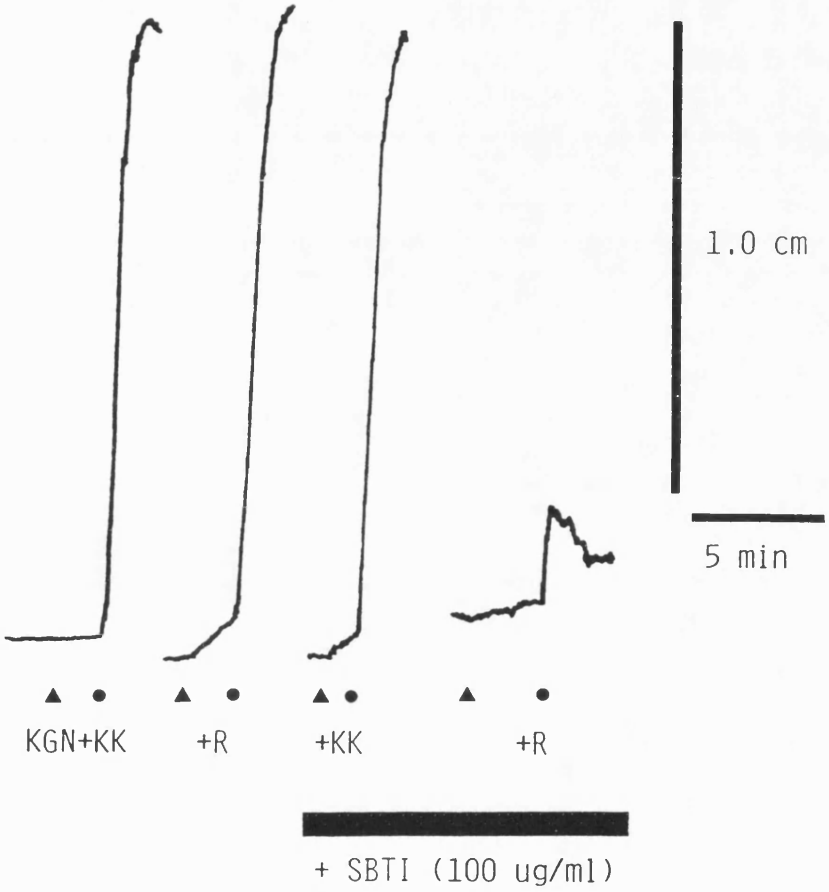


Figure 21. The effect of SBTI on the kininogenase activity of substance R. The experiment was carried out in the presence of captopril (4.6 x 10^{-4}M). Addition of human plasma kininogen (KGN) alone to guinea-pig isolated ileum elicited no contraction. However, when kininogen was present in the organ bath, addition of urinary kallikrein (KK), resulted in a slow contraction of the ileum. Substance R (R) also induced a slow contraction of the ileum in the presence of kininogen (KGN + R). Addition of SBTI to the bath did not markedly affect the response to kininogen + kallikrein. In contrast, the contraction to kininogen + substance R was blocked in the presence of SBTI.

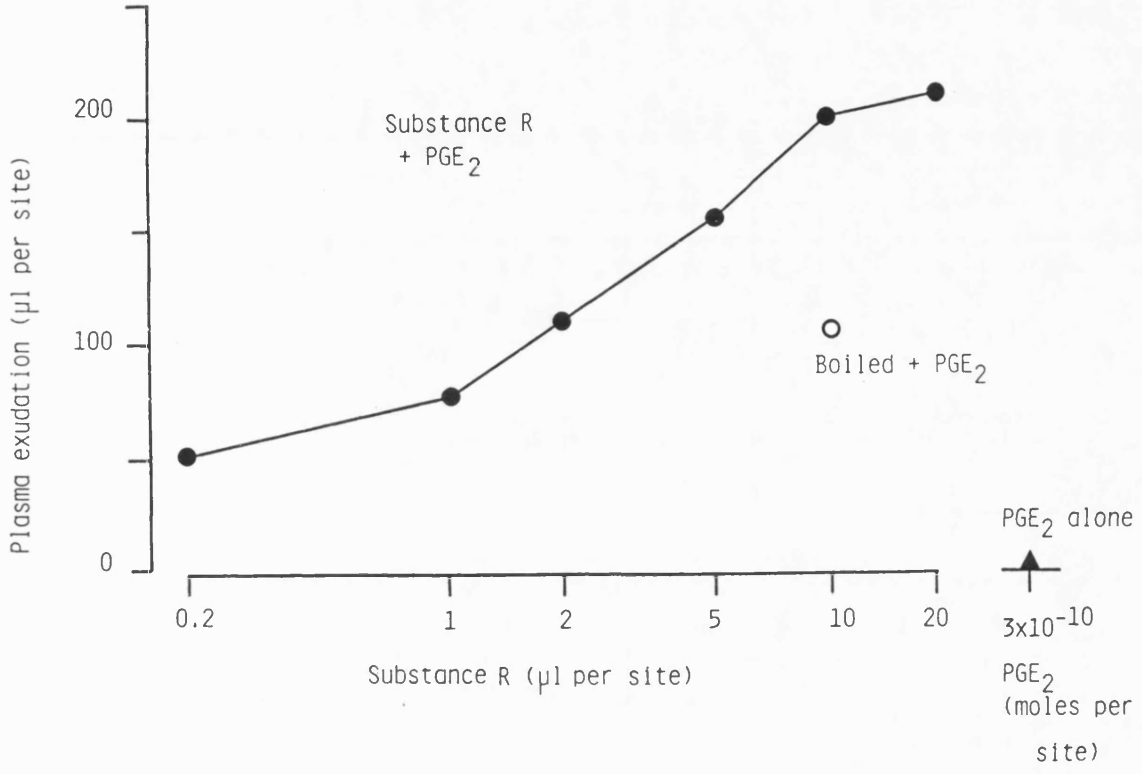


Figure 22 . Oedema formation in rabbit skin induced by mixtures of substance R+PGE_2 (●). Substance R caused a dose-dependent increase in vascular permeability. The response to a boiled sample of substance R+PGE_2 (○) was reduced compared to an unboiled sample. Results are the mean of 6 replicates in a single animal.

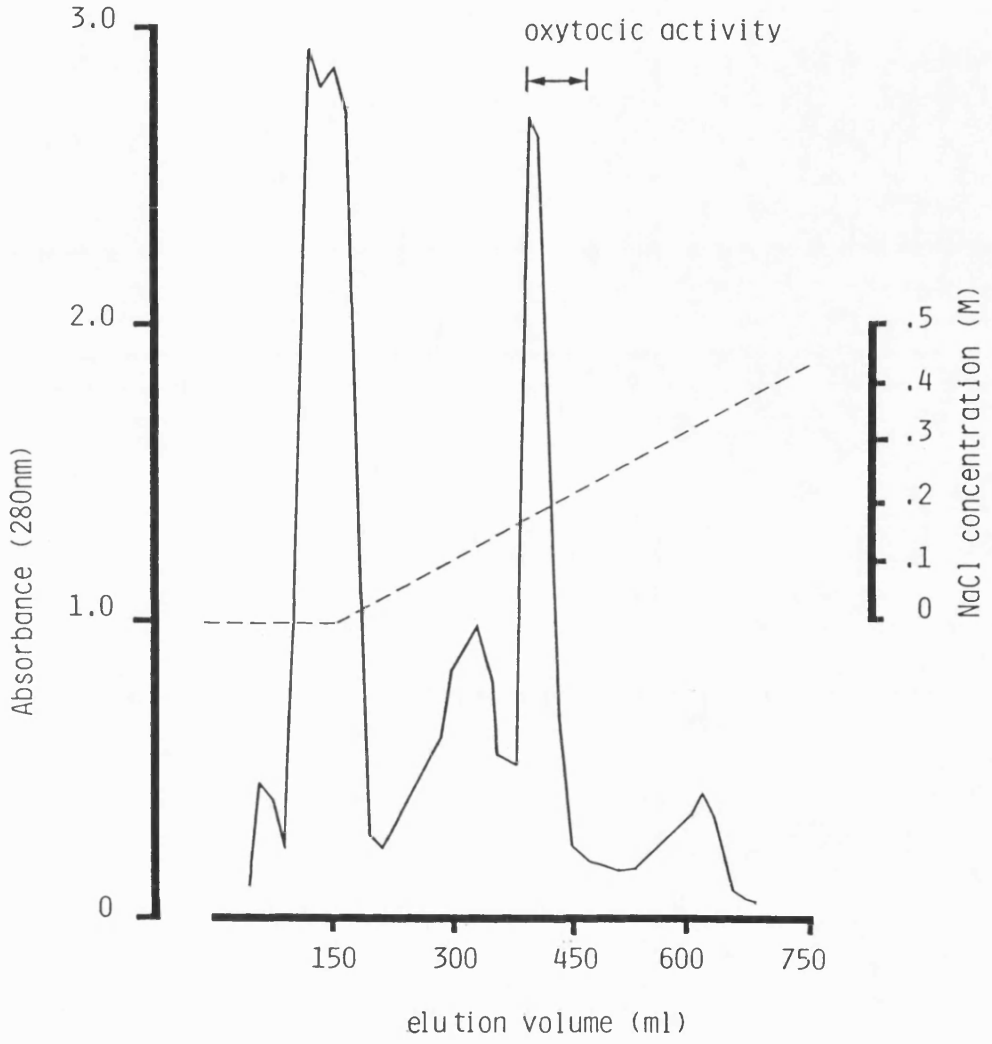


Figure 23. Anion-exchange chromatography of crude intestinal perfusate on DEAE-Sepharose. The sample was applied at V = 0 and was washed with 100 ml Tris buffer (50mM, pH 7.5). Substance R was eluted with a linear salt gradient at approximately 0.2M NaCl indicated by the horizontal arrows above the elution profile. The absobance of fractions was measured at 280 nm and oxytocic activity determined on the superfused rat uterus.

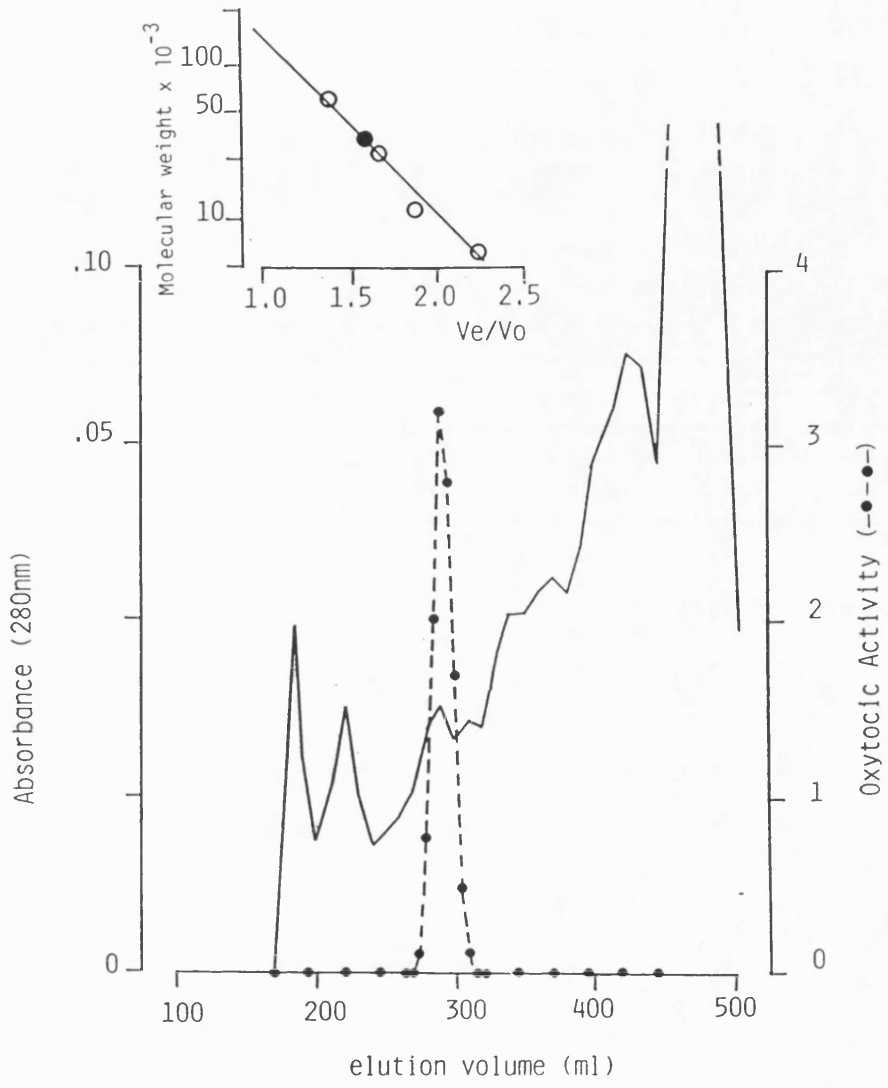


Figure 24. Gel-filtration chromatography on Sephacryl S-200 of pooled oxytocic fractions previously subjected to ion-exchange chromatography. The sample was applied at V = 0 and was eluted with 50mM PBS, pH 7.5. The absorbance was measured at 280 nm and the elution profile is shown by the solid line. Oxytocic activity of the eluted fractions was determined on the isolated superfused uterus of the rat, indicated by the dashed line and is expressed as % maximum contraction per µl eluate. **Inset:** The molecular weight of the substance R peak (●) corresponded to 40 kDa when compared to calibrating proteins (o).

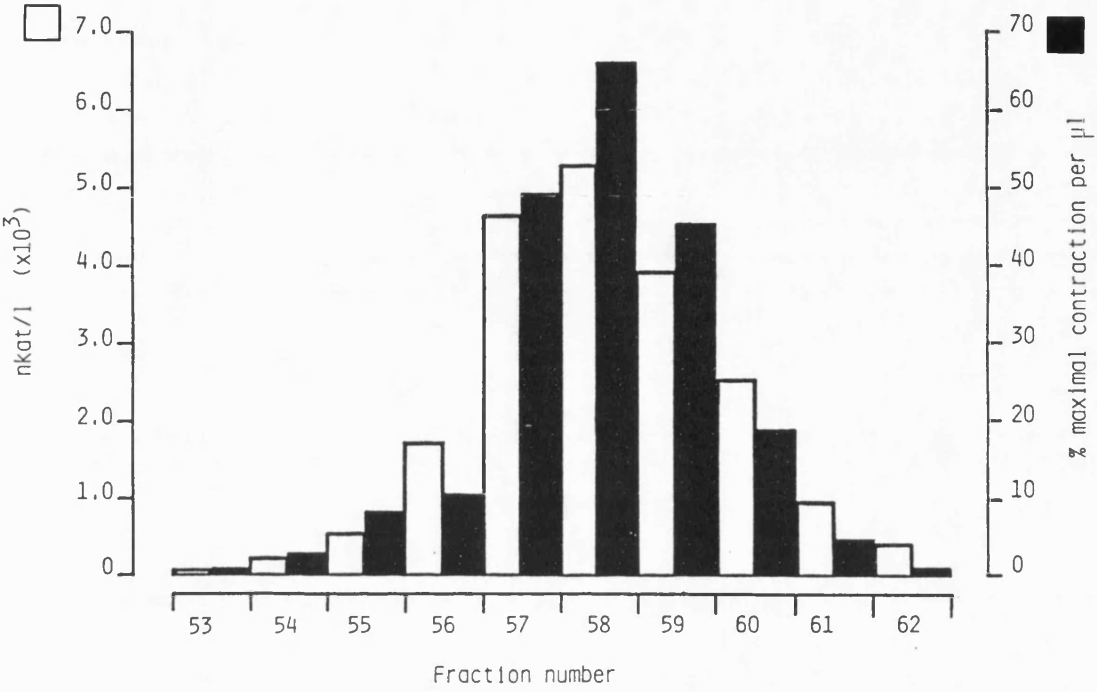


Figure 25. Comparison of the oxytocic activity (■) and kininogenase activity (□) of fractions eluted from the Sephacryl S-200 cloumn. Kininogenase activity was measured by S-2266 hydrolysis (nkat l^{-1}). Oxytocic activity was determined on the isolated rat uterus and is expressed as % maximal contraction per µl eluate. The peak of kininogenase activity corresponds closely to that of the oxytocic activity of substance R.

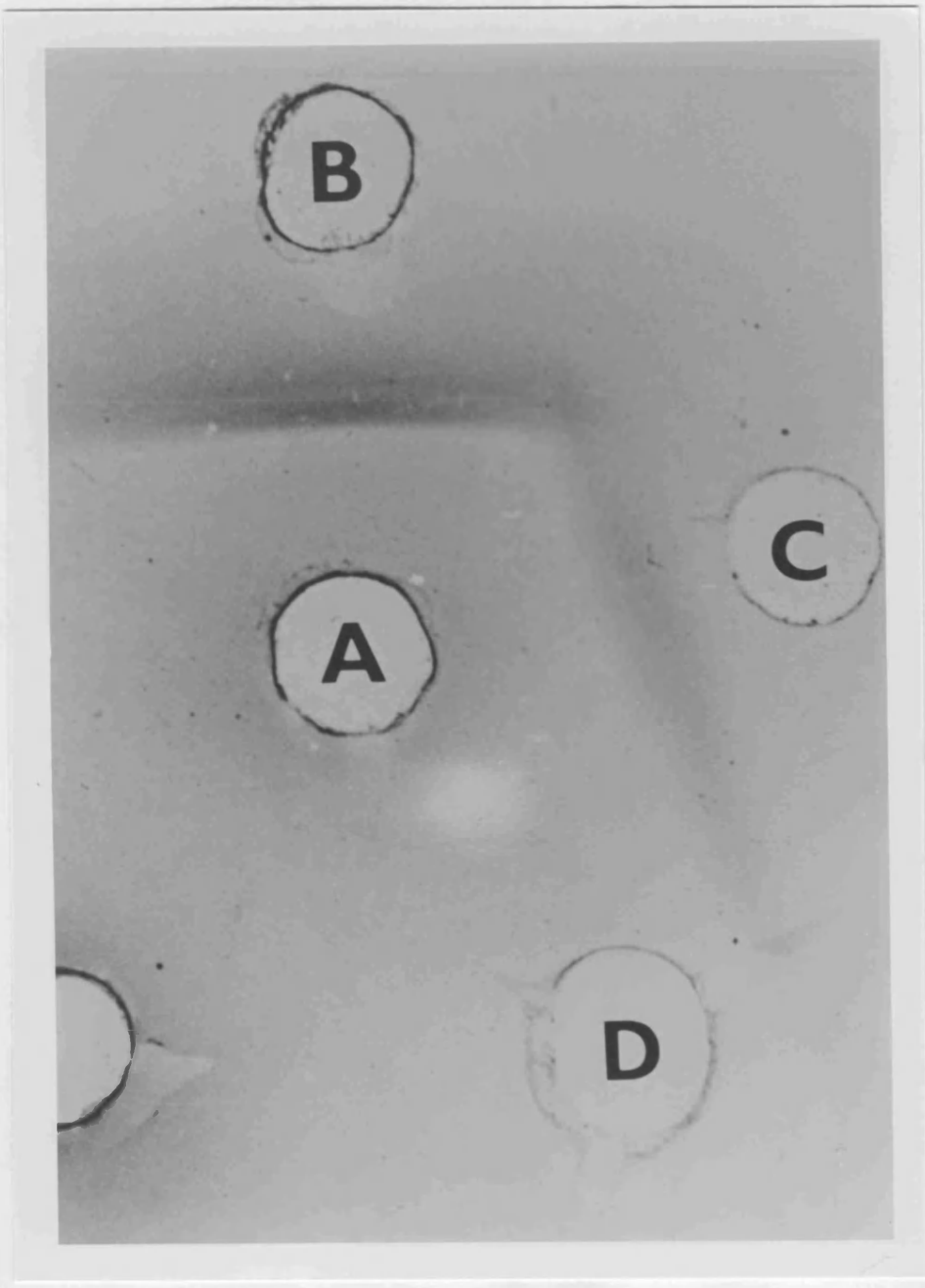


Figure 26. Immunodiffusion study of substance R and rat urinary kallikrein. The central well (A) contained rabbit anti-rat tissue kallikrein antiserum. The other wells contained urinary Kallikrein (B), substance R (C) and a sample from a fraction containing no oxytocic activity (D). A strong precipitation band formed between urinary kallikrein and the antiserum. A precipitation band also formed between substance R and the antiserum but the non-oxytocic sample did not react with antiserum.

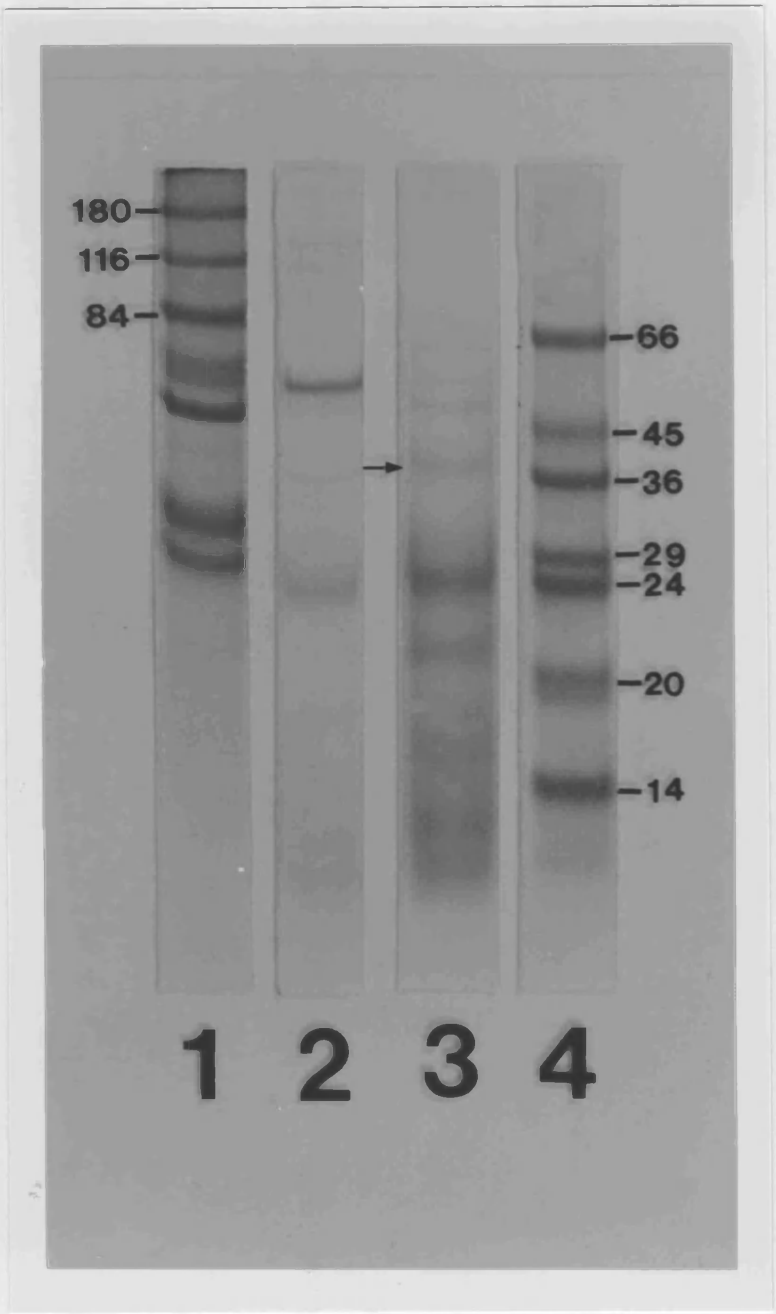


Figure 27. SDS-polyacrylamide gel electrophoresis of substance R. Lanes 1 and 4: molecular weight markers. Lanes 2 and 3: preparations of substance R. The major protein bands in the substance R preparations have approximate molecular weights of 62,000, 39,000, 26,000 and 21,000 Daltons. The band indicated (→) which migrated between glyceraldehyde-6-phosphate dehydrogenase (36 kDa) and egg albumin (45 KDa) confirms the presence of a protein of approximately 40,000 Da, corresponding to the estimated molecular weight of substance R from the peak of oxytocic activity eluted on gel filtration.

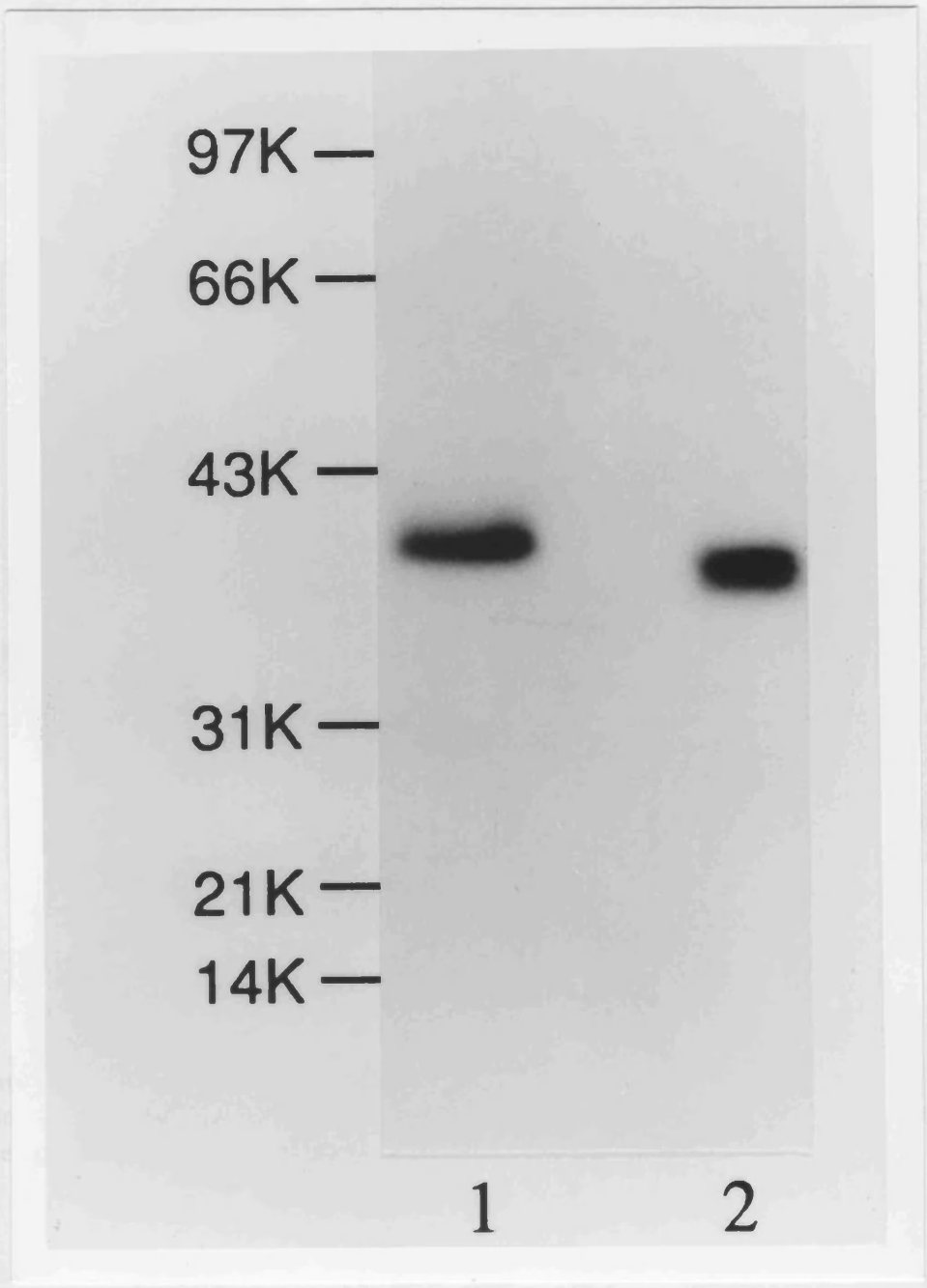


Figure 28. Western blot analysis of substance R and rat submandibular gland kallikrein. Electrophoresis was performed using a linear 7.5-15% gradient gel containing 0.1% SDS, and proteins were electrophoretically transferred to a nitrocellulose sheet. The blots were incubated with sheep anti-rat tissue kallikrein antiserum followed by ^{125}I-labelled rat tissue kallikrein. The antibody-bound protein bands were revealed by autoradiography. Lane 1: substance R (39,000 Da), lane 2: rat submandibular gland kallikrein (38,000 Da).

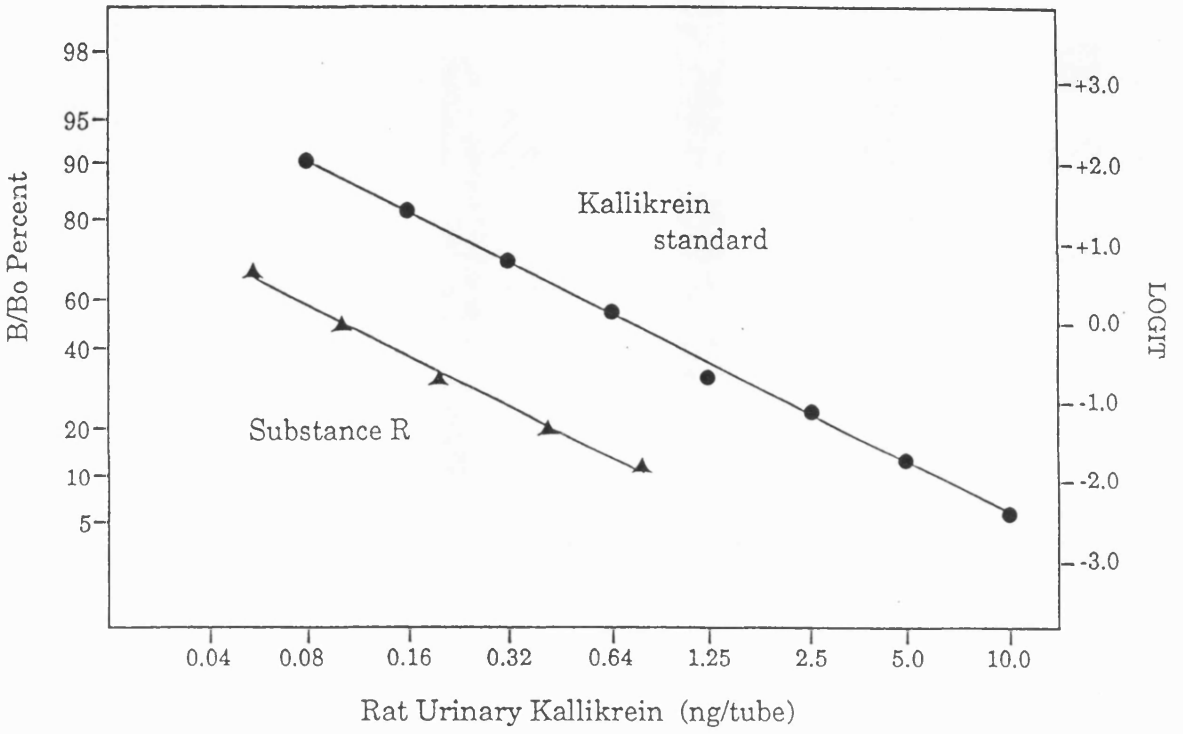


Figure 29. Log-logit transformation of tissue kallikrein radioimmunoassay. A serial diution of a sample of substance R (▲) shows complete parallelism to the standard curve of tissue kallikrein (●).

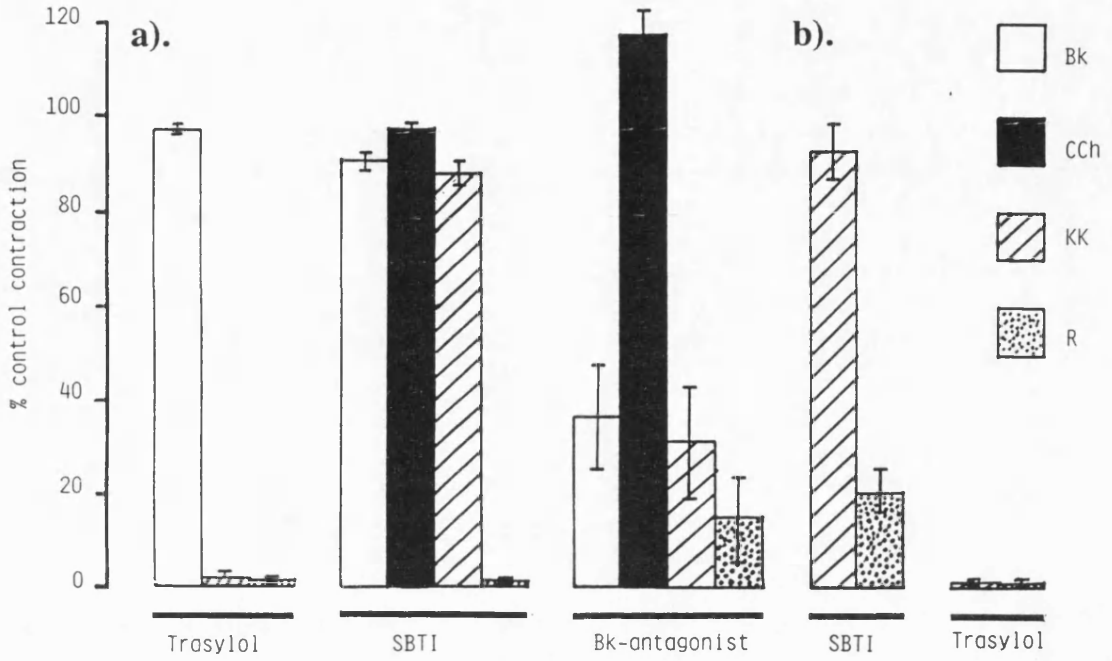


Figure 30. Summary of the effects of the enzyme inhibitors aprotinin (Trasylol, 100 KIU ml^{-1}), SBTI (5 x 10^{-7}M), and the BK_2 receptor antagonist D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin (10^{-7}M) on **a).** the oxytocic activity of bradykinin (Bk), carbachol (CCh), urinary kallikrein (KK) and substance R (R). **b).** The effects of aprotinin (Trasylol, 100 KIU ml^{-1}) and SBTI (5 x 10^{-7}M) on the kinin-releasing activity of urinary kallikrein and substance R. All results are the mean ± s.e. mean (*n* = at least 6 experiments).

SECTION 6

REFERENCES

Adam, H.M., Hardwick, D.C. and Spencer, K.E.V. (1954). Assay of histamine on the isolated guinea-pig ileum by the method of superfusion. *Br. J. Pharmacol. Chemother.*, **9,** 360-366.

Al-Dhahir, H.A.R. and Zeitlin, I.J. (1983). Bile salts activate glandular kallikrein-like activity in rat colon. *Adv. Exp. Med. Biol.*, **156A,** 463-467.

Ames, R.G., Moore, D.H. and van Dyke, H.B. (1950). The excretion of posterior pituitary antidiuretic hormone in the urine and its detection in the blood. *Endocrinol.*, **46,** 215-227.

Amundsen, E. (1966). Kinin formation and destruction in fluid produced by a strangulated segment of ileum in the rat. **In:** *Hypotensive peptides*. ed. Erdös, E. G., Back, N. and Sicuteri, F. pp 474-483. Berlin: Springer-Verlag.

Amundsen, E. and Nustad, K. (1965). Kinin-forming and destroying activities of cell homogenates. *J. Physiol.*, **179,** 479-488.

Amundsen, E., Putter, J., Friberger, P., Knos, M., Larsbraten, M. and Claeson, G. (1979). Methods for the determination of glandular kallikrein by means of a chromogenic tripeptide substrate. *Adv. Exp. Med. Biol.*, **120A,** 83-95.

Araujo, R.L., Dias da Silva, W. and Beraldo, W.T. (1970). Kininogenase and oxytocic properties of rat pancreatic juice. *Adv. Exp. Med. Biol.*, **8,** 77-80.

Back, N. and Steger, R. (1968). Effect of inhibitors on kinin-releasing activity of proteases. *Fed. Proc.*, **27,** 96-99.

Bain, W.A. (1932a). Method of demonstrating humoral transmission of effects of cardiac vagus stimulation in frog. *Q. Jl. Exp. Physiol.*, **22,** 269-274.

Bain, W.A. (1932b). On the mode of action of vasomotor nerves. *J. Physiol.*, **77,** 3P-4P.

Barabé, J. Drouin, J.-N., Regoli, D. and Park, W.K. (1977). Receptors for bradykinin in intestinal and uterine smooth muscle. *Can. J. Physiol. Pharmacol.*, **55,** 1270-1285.

Barabé, J. and Regoli, D. (1988). Kinin antagonists. *Methods Enzymol.*, **163,** 282-292.

Barlas, A., Gao, X. and Greenbaum, L.M. (1987). Isolation of a thiol activated T-kininogenase from the rat submandibular gland. *FEBS Lett.*, **218,** 266-270.

Baumgarten, C.R., Schwarting, R. and Kunkel, G. (1989). Localization of glandular kallikrein in nasal mucosa of allergic and non-allergic individuals. *Adv. Exp. Med. Biol.*, **247B,** 523-528.

Becker, E.L. and Kagen, L. (1964). The permeability globulins of human serum and the biochemical mechanism of hereditary angioneurotic edema. *Ann. N.Y. Acad. Sci.*, **116,** 866-873.

Beraldo, W.T., Araujo, R.L. and Mares-Guia, M. (1966). Oxytocic esterase in rat urine. *Am. J. Physiol.*, **211,** 975-980.

Beraldo, W.T., Araujo, R.L., Siqueira, G., Rodrigues, J.A.A. and Machado, C.S. (1971). Peculiaridades do sistema cinina-cininogenina do rato. *Cience Cult. (Sao Paulo)*, **23,** 475-480; through *Biol. Abstr.*, (1972), **54,** 36662.

Beraldo, W.T., Siqueira, G., Heneine, I.F. and Lauar, N.L. (1975). Peculiarities of the oxytocic action of rat kallikrein. *Life Sci.*, **16,** 790.

Beraldo, W.T., Lauar, N.L., Siqueira, G., Heneine, I.F. and Catanzaro, O.L. (1976). Peculiarities of the oxytocic action of rat urinary kallikrein. **In:** *Chemistry and biology of the kallikrein-kinin system in health and disease.* ed. Pisano, J.J. and Austen, K.F. pp 375-378. Washington D.C.: Government Printing Office.

Berg, T. (1988). Immuncohemical viewing of kallikrein in tissues. *Methods Enzymol.*, **163,** 143-159.

Berg-Ørstravik, T., Nustad, K. and Brandtzaeg, P. (1980). Localization of glandular kallikreins in rat and man. **In:** *Enzymatic release of vasoactive peptides.* ed. Gross, F. and Vogel, G. pp 137-147. New York: Raven Press.

Bertaccini, G. (1976). Active polypeptides of non-mammalian origin. *Pharmacol. Rev.*, **28,** 127-175.

Bertaccini, G. (1982). Peptides: Locally active peptides ("vasoactive peptides"). *Handb. Exp. Pharmacol.*, **59/II,** 175-200.

Bhoola, K.D., Yi, R.M.M., Morley, J. and Schachter, M. (1961). Kinin-releasing enzyme in the accessory sex glands of the guinea-pig. *J. Physiol.*, **159,** 34P-35P.

Bhoola, K.D., Yi, R.M.M., Morley, J. and Schachter, M. (1962). Release of kinin by an enzyme in the accessory sex glands of the guinea-pig. *J. Physiol.*, **163,** 269-280.

Bhoola, K., Lemon, M. And Matthews, R. (1979). Kallikrein in exocrine glands. *Handb. Exp. Pharmacol.*, **25 suppl.,** 289-523.

Boissonnas, R.A. (1962). The synthesis of bradykinin. *Biochem. Pharmacol.*, **10,** 35-38.

Boissonnas, R.A., Guttmann, St., Jaquenoud, P.-A., Pless, J. and Sandrin, E. (1963). The synthesis of bradykinin and of related peptides. *Ann. N.Y. Acad. Sci.*, **104,** 5-14.

Boschcov, P., Paiva, A.C.M., Paiva, T.B. and Shimuta, S.I. (1984). Further evidence

for the existance of two receptor subtypes for bradykinin responsible for the diphasic effect in the rat isolated duodenum. *Br. J. Pharmacol.*, **83,** 591-600.

ten Cate, J. (1924). Sur la question de l'action humorale du nerf vague. *Archs. neerl. Physiol.*, **9,** 588-597.

Chao, J., Buse, J., Shimamoto, K. and Margolius, H.S. (1981). Kallikrein-induced uterine contraction independent of kinin formation. *Proc. Natl. Acad. Sci. USA*, **78,** 6154-6157.

Chao, J., Woodley, C., Chao, L. and Margolius, H.S. (1983). Identification of tissue kallikrein in brain and in cell-free translation product encoded by brain mRNA. *J. Biol. Chem.*, **258,** 15173-15178.

Chao, J., Chao, L. and Margolius, H.S. (1984). Isolation of tissue kallikrein in rat spleen by monoclonal antibody-affinity chromatography. *Biochem. Biophys. Acta*, **801,** 244-249.

Chao, J., Chao, L., Woodley, C.M., Gerald, W. and Margolius, H.S. (1986a). Active kallikrein, preprokallikrein, and kallikrein-inhibitor complex. *Adv. Exp. Med. Biol.*, **198A,** 181-187.

Chao, J., Tillman, D.M., Wang, M., Margolius, H.S. and Chao, L. (1986b). Identification of a new tissue-kallikrein-binding protein. *Biochem. J.*, **239,** 325-331.

Chao, J. and Chao, L. (1987). Identification and expression of kallikrein gene family in rat submandibular and prostate glands using monoclonal antibodies as specific probes. *Biochem. Biophys. Acta*, **910,** 233-239.

Chao, J., Chao, L., Swain, C.C., Tsai, J. and Margolius, H.S. (1987). Tissue kallikrein in rat brain and pituitary: regional distribution and estrogen induction in the anterior pituitary. *Endocrinol.*, **120,** 475-482.

Chao, J. and Chao, L. (1988). Identification of latent tissue kallikrein, prolactin and growth hormone secretion in GH_3 pituitary cells using modified radioimmunoassays. *Mol. Cell. Endocrinol.*, **55,** 21-31.

Chao, J., Chai, K.X., Chen, L.-M., Xion, W., Chao, S., Woodley-Miller, C., Wang, L., Lu, H.S. and Chao, L. (1990). Tissue kallikrein-binding protein is a serpin. I. Purification, characterization, and distribution in normotensive and spontaneously hypertensive rats. *J. Biol. Chem.*, **265,** 16394-16401.

Chung, D.W., Fujikawa, K., McMullen, B.A. and Davie, E.W. (1986). Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. *Biochemistry*, **25,** 2410-2417.

Cohen-Laroque, E.-S., Bény, J.-L. and Huggel, H. (1990). Microvascular effects of bradykinin in the isolated perfused guinea-pig hindbrain: Method and characteriz-

ation of a possible third type of bradykinin receptor. *Microvasc. Res.*, **40,** 55-62.

Collier, H.O.J. and Shorley, P.G. (1960). Analgesic antipyretic drugs as antagonists of bradykinin. *Br. J. Pharmacol. Chemother.*, **15,** 601-610.

Colman, R.W. and Bagdasarian, A. (1976). Human kallikrein and prekallikrein. *Methods Enzymol.*, **45,** 303-322.

Colman, R.W. (1989). Kallikrein-kinin system in infectious diseases. *Adv. Exp. Med. Biol.*, **247A,** 153-157.

Cuthbert, A.W. and Margolius, H.S. (1982). Kinins stimulate net chloride secretion by the rat colon. *Br. J. Pharmacol.*, **75,** 587-598.

Cuthbert, A.W., Halushka, P.V., Margolius, H.S. and Spayne, J.A. (1984). Role of calcium ions in kinin-induced chloride secretion. *Br. J. Pharmacol.*, **82,** 587-595.

Cuthbert, A.W. and MacVinish, L.J. (1989). Diversisty of kinin effects on transporting epithelia. *Adv. Exp. Med. Biol.*, **247A,** 105-111.

Dorer, F.E., Kahn, J.R., Lentz, K.E., Levine, M. and Skeggs, L.T. (1974a). Hydrolysis of bradykinin by angiotensin converting enzyme. *Circ. Res.*, **34,** 824-827.

Dorer, F.E., Ryan, J.W. and Stewart, J.M. (1974b). Hydrolysis of bradykinin and its higher homologues by angiotensin converting enzyme. *Biochem. J.*, **141,** 915-917.

Douglas, G.J., Williams, K.I. and Flower, R.J. (1988). What is Gaddum's "substance R"? *Br. J. Pharmacol.*, **94,** 371P.

Douglas, G.J., Williams, K.I. and Flower, R.J. (1989). Further characterisation of Gaddum's 'substance R'. *Br. J. Pharmacol.*, **96,** 282P.

Douglas, G.J., Williams, K.I. and Flower, R.J. (1991). Characterization of Gaddum's substance R. *Br. J. Pharmacol.*, **102,** 926-930.

van Dyke, H.B., Chow, B.F., Greep, R.O. and Rothen, A. (1942). The isolation of a protein from the pars neuralis of the ox pituitary with constant oxytocic pressor and diuresis-inhibiting activities. *J. Pharmacol. Exp. Ther.*, **74,** 190-209.

Edery, H. (1964). Potentiation of the action of bradykinin on smooth muscle by chymotrypsin, chymotrypsinogen and trypsin. *Br. J. Pharmacol. Chemother.*, **22,** 371-379.

Edery, H. (1965). Further studies of the sensitization of smooth muscle to the action of plasma kinins by proteolytic enzymes. *Br. J. Pharmacol. Chemother.*, **24,** 485-496.

Edery, H. and Grunfeld, Y. (1969). Sensitization of smooth muscle to plasma kinins: effects of enzymes and peptides on various preparations. *Br. J. Pharmacol.*, **35,** 51-61.

Elliott, D.F. (1970). The discovery and characterization of bradykinin. *Handb. Exp. Pharmacol.*, **25,** 7-13.

Elliott, D.F., Lewis, G.P. and Horton, E.W. (1960). The structure of bradykinin - a plasma kinin from ox blood. *Biochem. Biophys. Res. Comm.*, **3,** 87-91.

Erdös, E.G. (1970). Preface. *Handb. Exp. Pharmacol.*, **25,** v-vi.

Erdös, E.G. (1979). Kininases. *Handb. Exp. Pharmacol.*, **25 suppl.,** 427-487.

Erdös, E.G. and Sloane. E.M. (1962). An enzyme in human blood that inactivates bradykinin and kallidins. *Biochem. Pharmacol.*, **11,** 585-592.

Erdös, E.G., Renfrew, A.G., Sloane, E.M. and Wohler, J.R. (1963). Enzymatic studies on bradykinin and similar peptides. *Ann. N.Y. Acad. Sci.*, **104,** 222-234.

Erdös, E.G., Back, N. and Sicuteri, F. (editors) (1966). *Hypotensive peptides.* Berlin: Springer-verlag.

Erdös, E.G., Tague, L.L. and Miwa, I. (1968). Kallikrein in the granules of the submaxillary gland. *Biochem. Pharmacol.*, **17,** 667-674.

von Euler, U.S. (1966). In memorium. **In:** *Erdös, E.G. et al. (editors). Hypotensive peptides.* ed. Erdös, E. G., Back, N. and Sicuteri, F. pp xxii-xxiv. Berlin: Springer-Verlag.

von Euler, U.S. and Gaddum, J.H. (1931). An unidentified depressor substance in certain tissue extracts. *J. Physiol.*, **72,** 74-87.

Farmer, S.G., Burch, R.M., Meeker, S.A. and Wilkins, D.E. (1989). Evidence for a pulmonary B_3 bradykinin receptor. *Mol. Pharmacol.*, **36,** 1-8.

Fatsch, S., Hulten, L., Johnson, B.J., Nordgren, S. and Zeitlin, I.J. (1978). Mobilization of colonic kallikrein following pelvic nerve stimulation in the atropinized cat. *J. Physiol.*, **285,** 471-478.

Feldberg, W. (1967). John Henry Gaddum. *Biogr. Mem. Fellows Roy. Soc.*, **13,** 57-77.

Feldberg, W. and Gaddum, J.H. (1934). The chemical transmitter at synapses in a sympathetic ganglion. *J. Physiol.*, **81,** 305-319.

Ferreira, S. and Vane, J.R. (1967). The disappearance of bradykinin and eledoisin in the circulation and vascular beds of the cat. *Br. J. Pharmacol. Chemother.*, **30,** 417-424.

Ferreira, S. and de Souza Costa, F. (1976). A laminar flow superfusion technique with much increased sensitivity for the detection of smooth muscle-stimulating substances. *Eur. J. Pharmacol.*, **39,** 379-381.

Fielder, F. (1979). Enzymology of glandular kallikreins. *Handb. Exp. Pharmacol.*, **25 suppl.,** 103-161.

Fielder, v.F. and Werle, E. (1967). Vorkommen zweier Kallikreinogene im Schweinepankreas und Automation der Kallikrein - und Kallikreinogenbestimmung. *Hoppe-Seyler's Z. Physiol. Chem.*, **348,** 1087-1089.

Figueiredo, A.F.S., Salgado, A.H.I., Siqueira, G.R.T. and Beraldo, W.T. (1989). Kallikrein-induced rat uterus contraction is dependent on kinin release. *Adv. Exp. Med. Biol.*, **247A,** 563-566.

Figueiredo, A.F.S., Salgado, A.H.I., Siqueira, G.R.T., Velloso, C.R. and Beraldo, W.T. (1990). Rat uterine contraction by kallikrein and its dependence on uterine kininogen. *Biochem. Pharmacol.*, **39,** 763-767

Figueroa, C.D. and Bhoola, K.D. (1989). The discovery of a tissue kallikrein in human neutrophils. *Br. J. Pharmacol.*, **97,** 439P.

Figueroa, C.D., MacIver, A.G., Dieppe, P., Mackenzie, J.C. and Bhoola, K.D. (1989). Presence of immunoreactive tissue kallikrein in human polymorphonuclear (PMN) leukocytes. *Adv. Exp. Med. Biol.*, **247B,** 207-210.

Fink, E., Geiger, R., Witte, J., Biedermann, S., Seifert, J. and Fritz, H. (1980). Biochemical, pharmacological, and functional aspects of glandular kallikreins. **In:** *Enzymatic release of vasoactive peptides.* ed. Gross, F. and Vogel, G. pp 101-113. New York: Raven Press.

Fink, E. and Schill, W.-B. (1983). Tissue kallikrein in human seminal plasma. *Adv. Exp. Med. Biol.*, **156B,** 1175-1180.

Fink, E., Feng, X.-C. and Richter, E. (1989). Studies on the intestinal absorption of tissue kallikrein. *Adv. Exp. Med. Biol.*, **247B,** 663-668.

Finkleman, B. (1930). On the nature of inhibition in the intestine. *J. Physiol.*, **70,** 145-157.

Flower, R.J. (1984). Macrocortin and the antiphospholipase proteins. *Adv. Inflamm. Res.*, **8,** 1-34.

Flower, R.J. and Blackwell, G.J. (1979). Anti-inflammatory steroids induce

biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. *Nature*, **278,** 456-459.

Forssmann, W.-G. (1980). Distribution of vasoactive peptides in the gastrointestinal tract and autonomic nervous system. **In:** *Enzymatic release of vasoactive peptides.* ed. Gross, F. and Vogel, G. pp 171-189. New York: Raven Press.

Frankish, N.H. and Zeitlin, I.J. (1977a). The assay of tissue kallikrein in rat intestine. *Br. J. Pharmacol.*, **59,** 517P.

Frankish, N.H. and Zeitlin, I.J. (1977b). Kallikrein release from rat duodenum stimulated by bile acids and other factors. *J. Physiol.*, **273,** 60P-61P.

Frankish, N.H. and Zeitlin, I.J. (1980). The effect of diet on tissue levels of kinin-forming enzyme in blood-free rat gastrointestinal tract. *J. Physiol.*, **298,** 361-370.

Frey, E.K. (1926). Zusammenhange zwischen Herzarbeit und Nierentatigkeit. *Arch. Klin. Chir.*, **142,** 633-669.

Frey, E.K. and Kraut, H. (1928). Ein neues Kreislaufhormon und seine Wirkung. *Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmak.*, **133,** 1-56; through *Physiol. Abstr.*, (1929-30), **14,** 2028.

Frey, E.K., Kraut, H. and Schultz, F. (1930). Über eine neue innersekretorische Funktion des Pankreas. V. Mittelung: Über ein Kreislaufhormon. *Naunyn-Schmiedeberg's Arch. Exp. Path. Pharmak.*, **158,** 334; through *Physiol. Abstr.*, (1931-32), **16,** 1018.

Friberger, A.B., Aurell, L. and Claeson, G. (1981). Chromogenic substrates for kallikreins and related enzymes. *Agents Actions Suppl.*, **9,** 83-90.

Fritz, H. Fink, E. and Truscheit, E. (1979). Kallikrein inhibitors. *Fed. Proc.*, **38,** 2753-2759.

Fritz, H., Dietze, G., Fiedler, F. and Haberland, G.L. (1981). Preface to 'Recent progress on kinins'. *Agents Actions Suppl.*, **9,** 5.

Fujikawa, K., Chung, D.W., Hendrickson, L.E. and Davie, E.W. (1986). Amino acid sequence of human factor XI, a blood coagulation factor with four tandem repeats that are highly homologous with plasma prekallikrein. *Biochemistry*, **25,** 2417-2424.

Fujimori, H., Levison, P.R. and Schachter, M. (1986a). Purification and partial characterization of cat colon and submandibular gland kallikreins. *Adv. Exp. Med. Biol.*, **198A,** 211-218.

Fujimori, H., Levison, P.R. and Schachter, M. (1986b). Purification and partial characterization of cat pancreatic and urinary kallikreins - comparison with other cat

tissue kallikreins and related proteases. *Adv. Exp. Med. Biol.*, **198A,** 219-229.

Fukushima, D., Kitamura, N. and Nakashini, S. (1985). Nucleotide sequence of cloned cDNA for human pancreatic kallikrein. *Biochemistry*, **24,** 8037-8043.

Gaddum, J.H. (1953). The technique of superfusion. *Br. J. Pharmacol. Chemother.*, **8,** 321-326.

Gaddum, J.H. (editor) (1955). *Polypeptides which stimulate plain muscle.* Edinburgh: E & S Livingstone.

Gaddum, J.H. (1955). The pharmacological analysis of tissue extracts. **In:** *Polypeptides which stimulate plain muscle.* ed. Gaddum, J.H. pp 1-6. Edinburgh: E & S Livingstone.

Gaddum, J.H., Jang, C.S. and Kwiatkowski, H. (1939). The effect on the intestine of the substance liberated by adrenergic nerves in a rabbit's ear. *J. Physiol.*, **96,** 104-108.

Gaddum, J.H. and Horton, E.W. (1959). The extraction of human urinary kinin (substance Z). *Br. J. Pharmacol. Chemother.*, **14,** 117-124.

Gaddum, J.H. and Guth, P.S. (1960). A comparison of the kallikrein-kinin system in sheep and dogs. *Br. J. Pharmacol. Chemother.*, **15,** 181-184.

Garrett, J.R., Harrison, J.D., Kidd, A., Kyriacou, K. and Smith, R.E. (1982). Kallikrein-like activity in human salivary glands and colon, including mast cells. *J. Physiol.*, **334,** 78P.

Gautvik, K.M., Berg-Oerstradvik, T. and Nustad, K. (1976). Role of the kallikrein-kinin system in glandular secretion. **In:** *Chemistry and biology of the kallikrein-kinin system in health and disease.* ed. Pisano, J.J. and Austen, K.F. pp 335-357. Washington D.C.: Government Printing Office.

Gautvik, K.M., Ørstradvik, T.B. and Svindahl, K. (1980). Release of glandular kallikrein. **In:** *Enzymatic release of vasoactive peptides.* ed. Gross, F. and Vogel, G. pp 287-298. New York: Raven Press.

Geiger, R., Feifel, G. and Haberland, G.L. (1977). A precursor of kinins in human gastric mucus. **In:** *Kininogenases: Kallikrein 4.* ed. Haberland, G.L., Rohen, J.W. and Suzuki, T. pp 73-77. Stuttgart-New York: F.K. Schauttauer.

Gerald, W.L., Chao, J. and Chao, L. (1986). Immunological identification of rat tissue kallikrein cDNA and characterization of the kallikrein gene family. *Biochem. Biophys. Acta*, **866,** 1-14.

Gilfoil, T.M. and Kelly, C.A. (1966). Mechanism of action of chymotrypsin on plain muscle. *Br. J. Pharmacol. Chemother.*, **27,** 120-130.

Greenbaum, L.M. (1979). Kininogenases of blood cells. (Alternate kinin generating system). *Handb. Exp. Pharm.*, **25 suppl.,** 91-102.

Greenbaum, L.M., Semente, G., Prakash, A. and Roffman, S. (1978). Leukokinins; their pharmacological properties and role in pathology of fluid (ascites) accumulation). *Agents actions*, **8,** 80-84.

Greenbaum, L.M. (1984). T-kinin and T-kininogen - an historical overview. *Adv. Exp. Med. Biol.*, **198A,** 55-59.

Greenbaum, L.M. and Okamoto, H. (1988). T-kinin and T-kininogen. *Methods Enzymol.*, **163,** 272-282.

Gryglewski, R.J., Moncada, S. and Palmer, R.M.J. (1986). Bioassay of prostacyclin and endothelium-derived relaxing factor (EDRF) from porcine aortic endothelial cells. *Br. J. Pharmacol.*, **87,** 685-694.

Guimarães, J.A., Borges, D.A., Prado, E.S. and Prado, J.L. (1973). Kinin-converting aminopeptidase from human serum. *Biochem. Pharmacol.*, **22,** 3157-3172.

Gutman, N., Moreau, T., Alhenc-Gelas, F., Baussant, T., El Moujahed, A., Akpona, S. and Gauthier, F. (1988). T-kinin release from T-kininogen by rat-submaxillary-gland endopeptidase K. *Eur. J. Biochem.*, **171,** 577-582.

Habermann, E. (1966). Enzymatic kinin release from kininogen and from low-molecular compounds. **In:** *Hypotensive peptides*. ed. Erdös, E. G., Back, N. and Sicuteri, F. pp 116-128. Berlin: Springer-Verlag.

Hamberg, U. (1978). Current and past nomenclature of the kininogen-kinin system. *Agents Actions*, **8,** 165-166.

Han, Y.N., Komiya, S., Iwanaga, S. and Suzuki, T. (1975). Studies on the primary structure of bovine high-molecular-weight kininogen. Amino acid sequence of a fragment ("histidine-rich peptide") released by plasma kallikrein. *J. Biochem. (Tokyo)*, **77,** 55-68.

Hardcastle, J., Hardcastle, P.T., Flower, R.J. and Sanford, P.A. (1978). The effect of bradykinin on the electrical activity of rat jejenum. *Experientia*, **34,** 617-618.

Harpel, P.C., Mosesson, M.W. and Cooper, N.R. (1975). Studies on the structure and function of alpha-2-macroglobulin and C_1 inactivator. **In:** *Proteases and biological control.* ed. Reich, E., Rifkin, D.B. and Shaw, E. pp 387-404. Cold Spring Harbor Laboratory.

Heimark, R.L. and Davie, E.W. (1981). Bovine and human plasma prekallikrein. *Methods Enzymol.*, **80,** 157-172.

Hirata, Y., Takata, S. and Takaichi, S. (1989). Specific binding sites for bradykinin and its degradation process in cultured rat vascular smooth muscle cells. *Adv. Exp. Med. Biol.*, **247A,** 415-420.

Hojima, Y., Moriwaki, C. and Moriya, H. (1975). Isolation of dog, rat and hog pancreatic kallikreins by preparative disc electrophoresis and their properties. *Chem. Pharm. Bull.*, **23,** 1128-1136.

Holmstedt, B. and Liljestrand, G. (1981). *Readings in pharmacology.* 2nd edition. New York: Raven Press. pp 225-227.

Horton, E.W. (1959). Human urinary kinin excretion. *Br. J. Pharmacol. Chemother.*, **14,** 125-132.

Innis, R.B., Manning, D.C., Stewart, J.M. and Snyder, S.H. (1981). [^{3}H]Bradykinin receptor binding in mammalian tissue membranes. *Proc. Natl. Acad. Sci. USA.*, **78,** 2630-2634.

Jacobsen, S. (1966a). Substrates for plasma kinin-forming enzymes in human, dog and rabbit plasmas. *Br. J. Pharmacol. Chemother.*, **26,** 403-411.

Jacobsen, S. (1966b). Substrates for kinin-forming enzymes in rat and guinea-pig plasma. *Br. J. Pharmacol. Chemother.*, **28,** 64-72.

Jendrassik, I. (1924). Humorale Übertragbarkeit von Nervenreizen beim Warmblüter. *Biochem. Z.*, **144,** 520-535.

Johnson, A.R. (1979). Effects of kinins on organ systems. *Handb. Exp. Pharmacol.*, **25 suppl.,** 357-399.

Jose, P.J., Forrest, M.J. and Williams, T. J. (1981). Human C_{5a} des Arg increases vascular permeability. *J. Immunol.*, **127,** 2376-2380.

Kagen, L.J., Leddy, J.S. and Becker, E.L. (1963). The presence of two permeability globulins in human serum. *J. Clin. Invest.*, **42,** 1353-1361.

Kaplan, A.P. and Silverberg, M. (1988). Mediators of inflammation: An overview. *Methods Enzymol.*, **163,** 3-23.

Kaplan, A.P., Silverberg, M., Ghebrehiwet, B., Atkins, P. and Zweiman, B. (1989). The kallikrein-kinin system in inflammation. *Adv. Exp. Med. Biol.*, **247A,** 125-136.

Kato, H., Nagasawa, S. and Iwanaga, S. (1981). HMW and LMW kininogens. *Methods Enzymol.*, **80,** 172-198.

Kimura, M., Sueyoshi, T., Morita, T., Tanaka, K. and Iwanaga, S. (1989). Ornitho-kininogen and ornitho-kinin: isolation, characterization and chemical structure. *Adv. Exp. Med. Biol.*, **247A,** 359-367.

Kizuki, K., Shimamoto, Y., Ikekita, M. and Moriya, H. (1986). An inactive form of kallikrein in human urine. *Adv. Exp. Med. Biol.*, **198A,** 329-337.

Kraut, H., Frey, E.K. and Bauer, E. (1928). Über ein neues Kreislaufhormon. II. Mitteilung. *Z. Physiol. Chemie*, **175,** 97-114; through *Biol. Abstr.*, (1929), **3,** 1414; *Physiol. Abstr.*, (1928-29), **13,** 2599.

Kraut, H., Frey, E.K. and Werle, E. (1930a). Der Nachweis eines Kreislaufhormons in der Pankreadrüse. *Z. Physiol. Chemie*, **189,** 97-106: through *Physiol. Abstr.*, (1930-31), **15,** 3178.

Kraut, H., Frey, E.K. and Werle, E. (1930b). Über die Inaktivierung des Kallikreins. *Z. Physiol. Chemie*, **192,** 1-21; through *Physiol. Abstr.*, (1931-32), **16,** 529.

Kwiatkowski, H. (1941). Observations on the relation of histamine to reactive hyperaemia. *J. Physiol.*, **100,** 147-158.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227,** 680-665.

Lembeck, F., Griesbacher, T., Eckhadt, M., Henke, S., Breiphohl, G. and Knolle, J. (1991). New, long-acting, potent bradykinin antagonists. *Br. J. Pharmacol.*, **102,** 297-304.

Loewi, O. (1921). Über humorale übertragbarkeit der herznervenwirkung. *Pfleugers Arch. Ges. Physiol.*, **189,** 239-242.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagents. *J. Biol. Chem.*, **193,** 265-275.

Lu, H.S., Lin, F.-K., Chao, L. and Chao, J. (1989). Human urinary kallikrein. Complete amino acid sequence and sites of glycosylation. *Int. J. Peptide Protein Res.*, **33,** 237-249.

MacDonald, R.J., Margolius, H.S. and Erdös, E.G. (1988). Molecular biology of tissue kallikrein. *Biochem. J.*, **253,** 313-321.

MacIntosh, F.C. (1986). Notes. **In:** Gaddum, J.H. *Vasodilator substances of the tissues*. 50th Anniversary edition. 1986. Cambridge: Cambridge University Press. Originally published in German as *"Gefasserweiternde Stoff der Gewebe"*. 1936. Leipzig. pp 210-217.

Magnus, R. (1904). Verusche am überlebenden Dünndarm von Säugethieren. Erste Mitth. *Pflügers Arch. Ges. Physiol.*, **102,** 123-151.

Mandle, R.J., Colman, R.W. and Kaplan, A.P. (1976). Identification of prekallikrein and high-molecular-weight kininogen as a complex in human plasma. *Proc. Natl. Acad. Sci. U.S.A.*, **73,** 4179-4183.

Mandle, R., Jr. and Kaplan, A.P. (1977). Hageman factor substrates. Human plasma kallikrein: Mechanism of activation in Hageman factor-dependent fibrinolysis. *J. Biol. Chem.*, **252,** 6097-6104.

Manning, D.C., Snyder, S.H., Kachur, J.F., Miller, R.J. and Field, M. (1982). Bradykinin receptor-mediated chloride secretion in intestinal function. *Nature*, **299,** 256-259.

Manning, D.C. and Snyder, S.H. (1986). ^{3}H-bradykinin binding site localization in guinea-pig urinary system. *Adv. Exp. Med. Biol.*, **198A,** 563-569.

Marceau, F., Ussier, A., Regoli, D. and Giroud, J.P. (1983). Pharmacology of kinins: their relevance to tissue injury and inflammation. *Gen. Pharmacol.*, **14,** 209-229.

Margolius, H.S. (1989). Tissue kallikreins and kinins: Roles in human disease and target for new drug development. *Adv. Exp. Med. Biol.*, **247A,** 1-6.

Marin-Grez, M., Schaechtelin, G. and Hermann, K. (1982). Kininogenase activity in rat uterus homogenates. *Hoppe-Seyler's Z. Physiol. Chem.*, **363,** 1359-1364.

Mathias, A.P. and Schachter, M. (1958). The chromatographic behaviour of wasp venom kinin, kallidin and bradykinin. *Br. J. Pharmacol. Chemother.*, **13,** 326-329.

Miwa, I., Erdös, E.G. and Seki, T. (1969). Seperation of peptide components of urinary kinin (substance Z). *Proc. Soc. Exp. Biol. Med.*, **131,** 768-772.

Moncada, S., Gryglewski, R.J., Bunting, S. and Vane, J.R. (1976). An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature*, **263,** 663-665.

Morichi, S., Sako, E., Hasewaga, E., Suyama, T. and Moriya, H. (1984). Large scale purification and characterization of human urinary kallikrein. *Chem. Pharm. Bull.*, **32,** 1152-1162.

Moriwaki, C. and Fujimori, H. (1979). Effect of kallikrein-kinin system on ion transport across rat small intestine. *Adv. Exp. Med. Biol.*, **120A,** 461-471.

Moriwaki, C., Fujimori, H., Moriya, H. and Kizuki, K. (1977a). Studies on kallikreins. IV. Enhancement of valine transport across the rat small intestine. *Chem. Pharm. Bull.*, **25,** 1174-1178.

Moriwaki, C., Fujimori, H. and Toyono, Y. (1977b). Intestinal kallikrein and the influence of kinin on the intestinal transport. **In:** *Kininogenases: Kallikrein 4.* ed. Haberland, G.L., Rohen, J.W. and Suzuki, T. pp 283-290. Stuttgart-New York: F.K. Schauttauer.

Moriya, H., Ikekita, M. and Kizuki, K. (1983). Some aspects of carbohydrate

contents in glandular kallikrein. *Adv. Exp. Med. Biol.*, **156A,** 309-316.

Movat, H.Z. (1978). The kinin system: Its relationship to blood coagulation, fibrinolysis and the formed elelments of the blood. *Rev. Physiol. Biochem. Pharmacol.*, **84,** 143-202.

Movat, H.Z. (1979). The plasma kallikrein-kinin system and its interrelationship with other components of blood. *Handb. Exp. Pharmacol.*, **25 suppl.,** 1-89.

Mulholland, F., Ashford, A. and Baily, G.S. (1986). Initial observations of a rat kallikrein-like enzyme associated with the plasma membranes of rat adipocytes. *Adv. Exp. Med. Biol.*, **198A,** 41-46.

Müller-Esterl, W. and Fritz, H. (1984). Human kininogens and their function in the kallikrein-kinin systems. *Adv. Exp. Med. Biol.*, **167,** 41-62.

Müller-Esterl, W., Iwanaga, S. and Nakanishi, S. (1986). Kininogens revisited. *Trends Biochem. Sci.*, **11,** 336-339.

Musch, M.W., Miller, R.J., Field, M. and Siegel, M. (1982). Stimulation of colonic secretion by lipoxygenase metabolites of arachidonic acid. *Science*, **217,** 1255-1256.

Musch, M.W., Kachur, J.F., Miller, R.J., Field, M. and Stoff, J.S. (1983). Bradykinin-stimulated electrolyte secretion in rabbit and guinea-pig intestine. Involvement of arachidonic acid metabolites. *J. Clin. Invest.*, **71,** 1073-1083.

Nasjletti, A. and Malik, K.U. (1980). Interrelationship of the renal kallikrein-kinin and prostaglandin systems. **In:** *Enzymatic release of vasoactive peptides.* ed. Gross, F. and Vogel, G. pp 323-333. New York: Raven Press.

Nolly, H. and Lama, M.C. (1982). `Vascular kallikrein': a kallikrein-like enzyme present in vascular tissue of the rat. *Clin. Sci.*, **63,** 249s-251s.

Nolly, H., Scicli, A.G., Scicli, G. and Carretero, O.A. (1985). Characterization of a kininogenase from rat vascular tissue resembling tissue kallikrein. *Circ. Res.*, **56,** 816-821.

Nolly, H., Scicli, A.G., Scicli, G., Lama, M.C., Guercio, A.M. and Carretero, O.A. (1986). Kininogenase from rat vascular tissue. *Adv. Exp. Med. Biol.*, **198A,** 11-17.

Nustad, K. and Pierce, J.V. (1974). Purification of rat urinary kallikreins and their specific antibody. *Biochemistry*, **13,** 2312-2319.

Nustad, K., Gautvik, K.M. and Pierce, J.V. (1976). Glandular kallikreins: Purification, characterization, and biosynthesis. **In:** *Chemistry and biology of the kallikrein-kinin system in health and disease.* ed. Pisano, J.J. and Austen, K.F. pp 77-92. Washington D.C.: Government Printing Office.

Nustad, K., Johansen, L., Østravik, T.B. and Pierce, J.V. (1980). Submandibular and kidney kallikreins: Biochemistry, origin, and function. **In:** *Enzymatic release of vasoactive peptides*. ed. Gross, F. and Vogel, G. pp 89-98. New York: Raven Press.

Odya, C.E., Goodfriend, T.L. and Peña, C. (1980). Bradykinin receptor-like binding studied with iodinated analogues. *Biochem. Pharmacol.*, **29,** 175-185.

Okamoto, H. and Greenbaum, L.M. (1983). Isolation and structure of T-kinin. *Biochem. Biophys. Res. Comm.*, **112,** 621-628.

Okamoto, H. and Greenbaum, L.M. (1984). Isolation and properties of two rat plasma T-kininogens. *Adv. Exp. Med. Biol.*, **198A,** 69-75.

Orce, C.G., Carretero, O.A., Scicli, G. and Scicli, A.G. (1989). Kinins contribute to the contractile effects of rat glandular kallikrein on the isolated rat uterus. *J. Pharmacol. Exp. Ther.*, **249,** 470-475.

Overlack, A., Scicli, A.G. and Carretero, O.A. (1983). Intestinal absorption of glandular kallikrein in the rat. *Am. J. Physiol.*, **244,** G689-G694.

Oxford English Dictionary. (1989). Prepared by Simpson, J.A. and Weiner, E.S.C.. 2nd edition. Oxford: Clarendon Press.

Pernow, B. (1983). Substance P. *Pharmacol. Revs.*, **35,** 85-141.

Piper, P.J. and Vane, J.R. (1969). Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature (London)*, **233,** 29-35.

Pisano, J.J. (1970). Kinins of non-mammalian origin. *Handb. Exp. Pharmacol.*, **25,** 589-595.

Pisano, J.J. (1975). Chemistry and biology of the kallikrein-kinin system. **In:** *Proteases and biological control.* ed. Reich, E., Rifkin, D.B. and Shaw, E. pp 199-222. Cold Spring Harbor Laboratory.

Pisano, J.J. (1979). Kinins in nature. *Handb. Exp. Pharmacol.*, **25 suppl.,** 273-285.

Polivka, E., Maier, M. and Binder, B.R. (1981). Purification and characterization of a kallikrein-like kininogenase from pig pituitary glands. *Agents Actions Suppl.*, **9,** 153-156.

Rabito, S.F., Scicli, A.G. and Carretero, O.A. (1980). Immunoreactive glandular kallikrein in plasma. **In:** *Enzymatic release of vasoactive peptides*. ed. Gross, F. and Vogel, G. pp 247-256. New York: Raven Press.

Rabito, S.F., Scicli, A.G., Kher, V. and Carretero, O.A. (1982). Immunoreactive glandular kallikrein in rat plasma: a radioimmunoassay for its determination. *Am. J. Physiol.*, **242,** H602-H610.

Regoli, D., Barabe, J. and Park, W.K. (1977). Receptors for bradykinin in rabbit aortae. *Can. J. Physiol. Pharmacol.*, **55,** 855-867.

Regoli, D., Marceau, F. and Barabé, J. (1978). *De novo* formation of vascular receptors for bradykinin. *Can. J. Physiol. Pharmacol.*, **56,** 674-677.

Regoli, D. and Barabé, J. (1980). Pharmacology of bradykinin and related kinins. *Pharmacol. Rev.*, **32,** 1-46.

Regoli, D. and Barabé, J. (1988). Kinin receptors. *Methods Enzymol.*, **163,** 210-230.

Regoli, D., Drapeau, G., Rovero, P., D'Orleans-Juste, P. and Barabé, J. (1986a). The actions of kinin antagonists on B_1 and B_2 receptor systems. *Eur. J. Pharmacol.*, **123,** 61-65.

Regoli, D., Drapeau, G., Rovero, P., Dion, S., Rhaleb, N.-E., Barabé, J., D'Orleans-Juste, P. and Ward, P. (1986b). Conversion of kinins and their antagonists into B_1 receptor activators or blockers in isolated vessels. *Eur. J. Pharmacol.*, **127,** 219-224.

Regoli, D., Rhaleb, N.-E., Dion, S. and Drapeau, G. (1990). New selective receptor antagonists and bradykinin B_2 receptor characterization. *Trends Pharmacol. Sci.*, **11,** 156-161.

Rocha e Silva, M. (1939). The pharmacology of trypsin. *Arquivos do Instituto Biologico*, **10,** 93-128.

Rocha e Silva, M. (1960). Biochemistry and pharmacology of bradykinin. **In:** *Polypeptides which affect smooth muscles and blood vessels.* ed. Schachter, M. pp 210-231. London: Pergamon Press.

Rocha e Silva, M. (1975). Bradykinin and bradykininogen. Introductory remarks. *Life Sci.*, **16,** 782.

Rocha e Silva, M. (1976a). Past, present and future of kinins. Introductory remarks. *Adv. Exp. Med. Biol.*, **70,** 1-3

Rocha e Silva, M. (1976b). Bradykinin and bradykininogen - introductory remarks. **In:** *Chemistry and biology of the kallikrein-kinin system in health and disease.* ed. Pisano, J.J. and Austen, K.F. pp 7-15. Washington D.C.: Government Printing Office.

Rocha e Silva, M., Beraldo, W.T. and Rosenfeld, G. (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and trypsin. *Am. J. Physiol.*, **156,** 261-273.

Roffman, S. and Greenbaum, L.M. (1979). Properties of leukokininogen isolated from human neoplastic ascites. *Biochem. Pharmacol.*, **28,** 1043-1050.

Roscher, A.A., Maganiello, V.C., Jalsema, C.L. and Moss, J. (1983). Receptors for bradykinin in intact cultured human fibroblasts: Identification and characterization by direct binding study. *J. Clin. Invest.* **72,** 626-635.

Sakamoto, W., Satoh, F., Nagasawa, S., Handa, H., Suzuki, S., Uehara, S. and Hirayama, A. (1989). A novel kinin, Met-Ile-Ser-Bradykinin (Met-T-kinin) is released from T-kininogen by an acid protease of granulatomous tissues in rats. *Adv. Exp. Med. Biol.*, **247B,** 103-108.

Schachter, M. (editor) (1960). *Polypeptides which affect smooth muscles and blood vessels.* London: Pergamon Press.

Schachter, M. (1969). Kallikreins and kinins. *Physiol. Rev.*, **49,** 509-547.

Schachter, M. (1979). Biological and physiological considerations arising from cellular localization and other recent observations on kallikreins. *Adv. Exp. Med. Biol.*, **120B,** 249-254.

Schachter, M. (1980a). Kallikreins (kininogenases) - A group of serine proteases with bioregulatory actions. *Pharmacol. Rev.*, **31,** 1-17.

Schachter, M. (1980b). Kallikrein localization and its significance. **In:** *Enzymatic release of vasoactive peptides.* ed. Gross, F. and Vogel, G. pp 151-160. New York: Raven Press.

Schachter, M. and Thain, E.M. (1954). Chemical and pharmacological properties of the potent, slow contracting substance (kinin) in wasp venom. *Br. J. Pharmacol. Chemother.*, **9,** 352-359.

Schachter, M., Peret, W., Billing, A.G. and Wheeler, G.D. (1983). Immunological localization of the protease, kallikrein, in the colon. *J. Histochem. Cytochem.*, **28,** 1255-1260.

Schachter, M., Peret, M.W., Moriwaki, C., Wheeler, G.D., Matthews, R.W., Mehta, J.G. and Labedz, T. (1986). The varied localization and functional significance of kallikrein-like enzymes in salivary glands, pancreas, colon, sex glands and spermatozoa, including evidence for the presence of nerve growth factor (NGF) in bull sperm acrosome. *Adv. Exp. Med. Biol.*, **198A,** 1-10.

Schachter, M., Uchida, Y., Longridge, D.J., Labedz, T., Whalley, E.T., Vavrek, R.J. and Stewart, J.M. (1987). New synthetic antagonists of bradykinin. *Br. J. Pharmacol.*, **92,** 851-855.

Schröder, E. (1970). Structure-activity relationships of kinins. *Handb. Exp. Pharmacol.*, **25,** 324-350.

Scicli, G., Nolly, H., Carretero, O.A. and Scicli, A.G. (1989). Glandular kallikrein-like enzyme in adrenal glands. *Adv. Exp. Med. Biol.*, **247B,** 217-222.

Sealy, J.E (1980). Prorenin activation by renal and plasma kallikreins. **In:** *Enzymatic release of vasoactive peptides.* ed. Gross, F. and Vogel, G. pp 117-133. New York: Raven Press.

Seki, T. Nakajima, T. and Erdös, E.G. (1972). Colon kallikrein, its relationship to the plasma enzyme. *Biochem. Pharmacol.*, **21,** 1227-1235.

Shimamoto, K., Tanaka, S., Nakao, T., And, T., Nakahashi, Y., Sakuma, M. and Miyahara, M. (1979). Measurement of urinary kallikrein activity by kinin radioimmunoassay. *Jap. Circ. J.*, **43,** 147-152.

Shimojo, N., Chao, J., Chao, L., Margolius, H.S. and Mayfield, R.K. (1987). Identification and characterization of a tissue kallikrein in rat skeletal muscles. *Biochem. J.*, **243,** 773-778.

Silverberg, M. and Kaplan, A.P. (1988). Prekallikrein. *Methods Enzymol.*, **163,** 85-95.

Siqueira, G., Dias da Silva, W. and Beraldo, W.T. (1970). A kinin-like forming enzyme in saliva presenting a potent oxytocic activity. *Adv. Exp. Med. Biol.*, **8,** 137-140.

Spragg, J. (1983). Characterization of purified human latent kallikrein. *Adv. Exp. Med. Biol.*, **156A,** 393-398.

Spragg, J. (1988). Latent kallikrein from human urine. *Methods Enzymol.*, **163,** 96-102.

Stewart, J.M. (1979). Chemistry and biologic activity of peptides related to bradykinin. *Handb. Exp. Pharmacol.*, **25,** 227-272.

Sugo, T., Ikari, N., Iwanaga, S. and Fuji, S. (1980). Functional sites of bovine high molecular weight kininogen as a co-factor in kaolin mediated activation of factor XII (Hageman factor). *Biochemistry*, **19,** 3215-3220.

Takaoka, M., Okamura, H., Iwamoto, T. and Morimoto, S. (1986). Purification of inactive kallikrein from rat urine. *Adv. Exp. Med. Biol.*, **198A,** 339-345.

Terragno, N.A. and Terragno, A. (1979). Release of vasoactive substances by kinins. *Handb. Exp. Pharmacol.*, **25 suppl.,** 401-426.

Trautschold, I., Werle, E. and Zickgraf-Rudel, G. (1967). Trasylol. *Biochem. Pharmacol.*, **16,** 59-72.

Tschesche, H., Mair, G., Godec, G., Fielder, F., Ehret, W., Hirschauer, C., Lemon, M., Fritz, H., Schmidt-Kastner, G. and Kutzbach, C. (1979). The primary structure of porcine glandular kallikrein. *Adv. Exp. Med. Biol.*, **120A,** 245-260.

Uchida, K., Kushiro, H., Hitomi, Y., Niinobe, M. and Fujii, S. (1979). Rat stomach kallikrein: Its purification and properties. *Adv. Exp. Med. Biol.*, **120A,** 291-303.

Uchida, K., Kushiro, H., Hitomi, Y., Niinobe, M. and Fujii, S. (1981). Rat thyroid gland kallikrein: Its purification and properties. *Agents Actions Suppl.*, **9,** 167-172.

Uetsuji, S., Yamamura, M., Yamamoto, M., Uchida, K., Kushiro, H., Kodama, J. and Fujii, S. (1981). Human stomach kallikrein. *Agents Actions Suppl.*, **9,** 137-142.

Vane, J.R. (1957). A sensitive method for the assay of 5-hydoxytryptamine. *Br. J. Pharmacol. Chemother.*, **12,** 344-349.

Vane, J.R. (1958). The blood-bathed isolated organ: a method of testing the circulating blood for active substances. *J. Physiol.*, **143,** 75P-76P.

Vane, J.R. (1964). The use of isolated organs for detecting active substances in the circulating blood. *Br. J. Pharmacol. Chemother.*, **23,** 360-373.

Vane, J.R. (1966). The estimation of catecholamines by biological assay. *Pharmacol. Revs.*, **18,** 317-324.

Vane, J.R. (1969). The release and fate of vaso-active hormones in the circulation. *Br. J. Pharmacol.*, **35,** 209-242.

Vane, J.R. (1983). Adventures and excursions in bioassay - the stepping stones to prostacyclin. *Postgrad. Med. J.*, **59,** 743-758.

Vio, C.P., Silva, R.H. and Powers, C.A. (1989). Immunoctochemical identification of glandular kallikreins in the rat anterior pituitary. *Adv. Exp. Med. Biol.*, **247B,** 183-188.

Vogel, R. (1979). Kallikrein inhibitors. *Handb. Exp. Pharmacol.*, **25 suppl.,** 163-225.

Vogel, R. and Werle, E. (1970). Kallikrein inhibitors. *Handb. Exp. Pharmacol.*, **25,** 213-249.

Vogt, W. (1966). The presence of two separate kinin-forming systems in human and other plasma. **In:** *Hypotensive peptides.* ed. Erdös, E. G., Back, N. and Sicuteri, F. pp 184-192. Berlin: Springer-Verlag.

Vogt, M. (1970). John Henry Gaddum, 1900-1965. *Rev. Physiol. Biochem. Pharmacol.*, **82,** 1-5.

Walker, J.M. (1967). Oxytocin. **In:** *Hormones in blood. Volume 1.* ed. Gray, C.H. and Bacharach, A.L. pp 129-144. 2nd edition. London: Academic Press.

Ward, P.E. and Margolius, H.S. (1979). Renal and urinary kallikreins. *Handb. Exp. Pharmacol.*, **25 suppl.,** 525-548.

Webster, M.E. (1966). Report of the committee on nomenclature for hypotensive peptides. **In:** *Hypotensive peptides.* ed. Erdös, E. G., Back, N. and Sicuteri, F. pp 648-653. Berlin: Springer-Verlag.

Webster, M.E. (1970a). Kallikreins in glandular tissues. *Handb. Exp. Pharmacol.*, **25,** 131-155.

Webster, M.E. (1970b). Recommendations for the nomenclature and units. *Handb. Exp. Pharmacol.*, **25,** 659-665.

Webster, M.E. and Pierce, J.V. (1963). The nature of the kallidins released from human plasma by kallikreins and other enzymes. *Ann. N.Y. Acad. Sci.*, **104,** 91-105.

Werle, E. (1936a). Über die Wirkung einiger Kreislaufstoffe beim Huhn und über eine neue Kreislaufaktive Substanz aus Hühnerkot. *Klin. Wschr.*, **15,** 164-167; through *Physiol. Abstr.*, (1936-37), **21, 2623.**

Werle, E. (1936b). Über Kallikrein aus Blut. *Biochem. Z.*, **287,** 235-261; through *Physiol. Abstr.*, (1937-38), **22,** 216.

Werle, E. (1936c). Die Wirkung des Kallikrein (Padutin) auf den isolierten Darm. *Klin. Wschr.*, **15,** 848-849; through *Physiol. Abstr.*, (1936-37), **21,** 2623.

Werle, E. (1937). Über die Wirkung des kallikreins auf den Darm und uber eine darmkontrahierende Substanz. *Biochem. Z.*, **281,** 217-233.

Werle, E. (1955). The chemistry and pharmacology of kallikrein and kallidin. **In:** *Polypeptides which stimulate plain muscle.* ed. Gaddum, J.H. pp 20-27. Edinburgh: E&S Livingstone.

Werle, E. (1970). Discovery of the most important kallikreins and kallikrein inhibitors. *Handb. Exp. Pharmacol.*, **25,** 1-6.

Werle, E. (1975). A short history of the kallikrein-kinin system. *Life Sci.*, **16,** 781-782.

Werle, E. (1976). A short history of the kallikrein-kinin system. **In:** *Chemistry and biology of the kallikrein-kinin system in health and disease.* ed. Pisano, J.J. and Austen, K.F. pp 1-6. Washington D.C.: Government Printing Office.

Werle, E. and Hürter, J. (1936). Über Ornitho-kallikrein. I. Mitteilung über kreislaufaktive Substanzen der Vogel. *Biochem. Z.*, **285,** 175-191; through *Physiol. Abstr.*, (1936-37), **21,** 3238.

Werle, E. and von Roden, P. (1936). Über das Vorkommen von Kallikrein in den Speicheldrüsen und im Mundspeichel. *Biochem. Z.*, **286,** 213-219; through *Physiol. Abstr.*, (1937-38), **22,** 2805.

Werle, E., Götze, W. and Keppler, A. (1936). Über die Wirkung des Kallikreins auf den isolierten Darm und über eine neue darmkontrahierenden Substanz. *Biochem. Z.*, **289,** 217-233; through *Physiol. Abstr.*, (1937-38), **22,** 3998.

Werle, E. and Berek, U. (1948). Zur kenntnis des kallikreins. *Agnew. Chem.*, **60(A),** 53.

Werle, E. and Berek, U. (1950). Über kallidin. *Biochem. Z.*, **320,** 136-145.

Werle, E. and Maier, L. (1952). Uber die chemische und pharmakologische unterscheidung von kallikrein verschiedener herkunft. *Biochem. Z.*, **323,** 279-283.

Werle, E. and Vogel, R. (1961). Über die Freisetzung einer Kallikreinartigen Substanz aus Extraken verschiedener Organe. *Arch. Int. Pharmacodyn.*, **131,** 257-261.

Werle, E. and Trautschold, I. (1963). Kallikrein, kallidin, kallikrein inhibitors. *Ann. N.Y. Acad. Sci.*, **104,** 117-129.

Werle, E., Hochstrausser, K. and Trautschold, I. (1966). Studies of bovine plasma kininogen, ornitho-kallikrein, and ornitho-kinin. **In:** *Hypotensive peptides.* ed. Erdös, E. G., Back, N. and Sicuteri, F. pp 105-115. Berlin: Springer-Verlag.

Whalley, E.T. (1979). Action of kallikrein and trypsin on isolated rat uterus. *Acta Endocr. Suppl.*, **225,** 118.

Williams, T.J. (1979). Prostaglandin E_2, prostaglandin I_2 and the vascular changes in inflammation. *Br. J. Pharmacol.*, **65,** 517-524.

Woodbury, R.G., Gruzenski, G.M. and Lagunoff, D. (1978). Immunofluorescent localization of a serine protease in rat small intestine. *Proc. Natl. Acad. Sci. USA*, **75,** 2785-2789.

Woodley, C.M., Chao, J., Margolius, H.S. and Chao, L. (1985). Specific identification of tissue kallikrein in exocrine tissues and in cell-free translation products with monoclonal antibodies. *Biochem. J.*, **231,** 721-728.

Yakada, Y, Skidgel, R.A. and Erdös, E.G. (1985). Purification of human urinary prokallikrein. Identification of the site of activation by the metalloproteinase thermolysin. *Biochem. J.*, **232,** 851-858.

Yanaihara, N., Okaru, Y., Mochizuki, T., Yanihara, C., Iwanaga,T. and Fujita, T. (1983). Comparative immunochemical characterization of various kallikreins. *Adv. Exp. Med. Biol.*, **156A,** 661-670.

Yang, H.Y.T. and Erdös, E.G. (1967). Second kininase in blood plasma. *Nature (London)*, **215,** 1402-1403.

Zeitlin, I.J. (1971). Pharmacological characteristics of kinin-forming activity in rat intestinal tissue. *Br. J. Pharmacol.*, **42,** 648P-649P.

Zeitlin, I.J. (1972). Rat intestinal kallikrein. *Adv. Exp. Med. Biol.*, **21,** 289-296.

Zeitlin, I.J., Singh, Y.N., Lembeck, F. and Theiler, M. (1976). The molecular weight of plasma and intestinal kallikreins in rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **293,** 159-161.

Zeitlin, I.J., Al-Dhahir, H.A.R., Cook, S., Currie, A. and Donovan, K. (1986). Bile acids and the intestinal kallikrein-kinin system. *Adv. Exp. Med. Biol.*, **198A,** 47-53.

APPENDIX

Publication: Characterization of Gaddum's substance R

British Journal of Pharmacology (1991), **102,** 926-930

Br. J. Pharmacol. (1991), 102, 926–930

Characterization of Gaddum's substance R

[1]G.J. Douglas, K.I. Williams & [2]R.J. Flower

Pharmacology Group, School of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY

1 When the isolated small intestine of the rat is perfused via the mesenteric artery, an oxytocic principle (Gaddum's substance R) is released which is detectable in the perfusate after 30 min and is present in samples collected 8 h later.

2 The oxytocic activity of substance R is lost after boiling but is unaffected by treatment with thioglycolate. Furthermore, atropine, methysergide and indomethacin failed to antagonize uterine contractions to substance R.

3 Neither substance R nor urinary kallikrein alone induce a contraction of the guinea-pig isolated ileum. However, in the presence of kininogen both substance R and urinary kallikrein produce a slow and prolonged contraction of the guinea-pig ileum.

4 The oxytocic and kininogenase properties of both substance R and urinary kallikrein are inhibited by Trasylol.

5 Soy bean trypsin inhibitor (SBTI) selectively inhibited both the oxytocic and the kininogenase activities of substance R but not those of urinary kallikrein.

6 Gel filtration of substance R resolved a single peak of oxytocic activity with an estimated molecular weight of 40 kDa.

7 We conclude that substance R is a kininogenase enzyme which may be distinguished from plasma kallikrein by its molecular weight and from urinary kallikrein by its susceptibility to SBTI. The exact nature of this enzyme remains to be elucidated.

Keywords: Substance R; kallikrein; perfused small intestine; kininogenase

Introduction

In 1953 Gaddum published a highly cited paper in which he described for the first time, the superfusion technique. Less well-known is that in this paper Gaddum also reported the production by the perfused rat intestine of a factor with oxytocic activity. The results he obtained suggested that this factor was a protein. We have recently confirmed these observations (Douglas *et al.*, 1988). Furthermore, we observed that the spectrum of pharmacological actions of 'substance R' was similar, but not identical, to those of a tissue kallikrein.

Methods

Collection of substance R

Male Wistar rats (200–300 g) were first anaesthetized with ether, and heparin (1000 u) was administered by a tail vein to prevent clotting during the perfusion. Following laparotomy the small intestine was ligated at the duodenal level, distal to the pancreas and also at the ileo-caecal junction. The superior mesenteric artery was cannulated and all other vessels, with the exception of the hepatic portal vein, were tied off. The intestine was sectioned anterior to the duodenal ligature and distal to the caecal ligature. The intact mesenteric bed was then removed, care being taken to exclude all pancreatic tissue, suspended in a heated jacket and perfused with oxygenated Tyrode solution (37°C, 0.4 ml min^{-1}) through the mesenteric artery. Effluent from the hepatic portal vein was either collected for consecutive 20 min periods over 6 h and aliquots of each fraction were assayed for oxytocic activity, or, after a 30 min equilibration period, perfusate, collected over 6 h, was pooled, lyophylized and stored at −20°C. For preparative purposes pooled perfusates from three experiments were used.

The pooled perfusates from three such preparations were then dialysed overnight against 10 volumes of ammonium bicarbonate (50 mM, pH 7.8). The high molecular weight residue was freeze-dried and stored at −20°C. The lyophylized perfusate from nine intestinal preparations was redissolved in 6 ml of Trisbuffer (50 mM, pH 7.5) and filtered before application to the ion-exchange column.

Assay of oxytocic activity

Segments of stilboestrol-primed rat uterus superfused with de Jalon's solution (30°C, 5 ml min^{-1}) were used for the assay of oxytocic activity of samples. Volumes of between 10 and 150 μl were injected into the stream of superfusion fluid above the tissue.

Assay of kinin-releasing activity

The kinin-releasing activity of substance R and kallikrein was assayed on the guinea-pig isolated ileum. In these experiments a 2 cm length of ileum was suspended in an 8 ml organ bath and bathed in oxygenated Tyrode solution containing captopril (100 μg ml^{-1} at 37°C). Bradykinin was used as a standard.

Ion-exchange chromatography

Ion-exchange chromatography of the soluble intestinal extract was performed by use of DEAE-Sepharose. The sample was applied to the column (2.6 × 30 cm), equilibrated in Tris buffer (50 mM, pH 7.5). Fractions of 5 ml were eluted at a flow rate of 0.4 ml min^{-1} by a linear salt gradient of 0–1 M sodium chloride and assayed for oxytocic activity before the absorbance at 280 nm was measured. Fractions containing oxytocic activity were then pooled, dialysed against 50 volumes of ammonium bicarbonate (50 mM, pH 7.8) for 12–18 h and lyophilized.

Gel filtration

Gel filtration of the oxytocic principle was carried out routinely on a calibrated Sephacryl S-300 column (2.6 × 90 cm). The column was equilibrated with phosphate-buffered saline

[1] Author for correspondence.
[2] Present address: Department of Biochemical Pharmacology, St Bartholomew's Hospital Medical College, Charterhouse Square, London EC1M 6BQ.

(50 mM, pH 7.5 (PBS) and 5 ml of soluble intestinal extract was applied to the column. The sample was eluted at a flow rate of 0.4 ml min^{-1} and 5 ml fractions were collected. Fractions were then assayed for oxytocic activity and the absorbance at 280 nm measured.

Estimation of the molecular weight of the oxytocic principle was made on a Sephacryl S-200 column (2.6 × 90 cm), calibrated with molecular weight markers, under the conditions described above and the fraction containing the peak oxytocic activity was determined by assay on the superfused isolated uterus of the rat.

Urinary kallikrein

Initially, crude preparations of rat urinary kallikrein were made by desalting rat urine on a Sephadex G-25 (PD-10) column equilibrated with PBS. Later, for comparative purposes, rat urinary kallikrein was prepared from urine collected overnight from male Wistar rats. The urine from individual rats was pooled and filtered before an overnight dialysis against PBS. The dialysed sample was then stored at −20°C. A partially purified preparation or urinary kallikrein was obtained by the same preparative and assay techniques employed in the isolation of substance R.

Materials

Reagents and drugs were obtained from the following sources: aprotinin (Trasylol), (Bayer UK Ltd); H-D-Val-L-Leu-L-Arg-*p*-nitroanilide dichloride (S-2266), (Kabi Vitrum), D-Arg-[Hyp^3, $Thi^{5,8}$, D-Phe^7]-bradykinin, (Novabiochem); PD-10 columns, Sephacryl S-200, Sephacryl S-300 and DEAE-Sepharose, (Pharmacia Ltd); methysergide hydrogen maleate, (Sandoz UK Ltd), atropine sulphate, bradykinin, carbamylcholine, α- and δ-chymotrypsin, diethylstilboestrol, heparin sulphate, indomethacin, soy bean trypsin inhibitor (SBTI), thioglycolic acid, Trizma base, Trizma hydrochloride, and trypsin, (Sigma); captopril, (Squibb). Human plasma kininogen was kindly donated by Dr K.D. Bhoola, University of Bristol.

Statistical analysis

Data are expressed as mean ± s.e.mean. Statistical significance was computed by the paired *t* test or analysis of variance (ANOVAR) where appropriate.

Results

The effluent from perfused rat small intestines was found to contract the superfused rat uterus in a dose-dependent manner. Contractions were delayed in onset (30 s) when compared to those produced by carbachol or bradykinin. When consecutive fractions of perfusate were collected, at least 30 min elapsed before oxytocic activity was detectable (Figure 1). However, oxytocic activity was released in increasing amounts thereafter, reaching a maximum concentration at 3–4 h, which was sustained for a further 2–3 h. Protein concentration, estimated by the Lowry-Folin method (Lowry *et al.*, 1951), remained between 1–2 mg ml^{-1} throughout the period of perfusion with no correlation between protein concentration and oxytocic activity.

The characteristics of the oxytocic principle isolated are very similar to those described by Gaddum (1953) for substance R. As Gaddum himself observed, these properties are similar to those of (then recently described) kallikrein. To compare the activities of the two substances further, we used crude preparations of rat urinary kallikrein as a source of tissue kallikrein, and compared its properties with those of substance R.

The oxytocic principle from the intestinal perfusate was non-dialysable (mol. wt. cut off 10–15 kDa) and oxytocic activity was not inhibited by addition of atropine (10^{-6} M), methysergide (5.7×10^{-7} M) or indomethacin (1×10^{-5} M) to the superfusate. Samples of perfusate possessing substantial oxytocic activity were inactive when tested on the isolated superfused guinea-pig ileum or rat fundic strip preparations. The perfusate lost oxytocic activity on heating but was unaffected by treatment with thioglycolate which inactivates oxytocin (Ames *et al.*, 1950). The material was stable when frozen at −20°C for at least 8 weeks.

Trypsin and chymotrypsin contract the superfused rat uterus in a similar way to substance R (Gaddum, 1953). Gaddum (1953) found that a preparation of substance R was more active, weight for weight, on the rat uterus than crystalline preparations of trypsin or chymotrypsin. It has since been shown that both trypsin and chymotrypsin sensitize certain smooth muscle preparations to bradykinin (Aarsen, 1968; Edery & Grumfeld, 1969). In the present study it was found that trypsin, α-chymotrypsin and δ-chymotrypsin induced sensitization to bradykinin of both the superfused rat uterus and guinea-pig ileum. However, in each of three experiments, substance R and urinary kallikrein failed to induce sensitization to bradykinin in either of these preparations.

To investigate further the similarity between substance R and tissue kallikrein, the effect of inhibitors of kallikrein on the oxytocic activities of substance R and urinary kallikrein were studied. Addition of the dual plasma and tissue kallikrein inhibitor aprotinin (Trasylol, 50–100 Kiu ml^{-1}), to the fluid superfusing the isolated uterus completely inhibited the activity of both substance R and urinary kallikrein in six experiments (Figure 2). However, the plasma kallikrein inhibitor, soy bean trypsin inhibitor (SBTI, 100 μg ml^{-1}), completely inhibited the action of substance R on the isolated uterus (99.4 ± 0.3%) whereas the oxytocic activity of urinary kallikrein was only reduced by 12.6 ± 2.5% ($n = 6$) (Figure 3). A reduction of 9.9 ± 1.8% and 3.0 ± 0.8% was seen in response to bradykinin and carbachol respectively ($n = 4$). The inhibition of the oxytocic activity of substance R by SBTI was significant ($P < 0.01$, ANOVAR).

Both urinary kallikrein and substance R were found to contract the guinea-pig isolated ileum in the presence of crude human plasma kininogen. Use of the selective bradykinin receptor antagonist, D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin (Regoli *et al.*, 1986; Schachter *et al.*, 1987) inhibited the contractions induced by substance R plus kininogen indicating that the generated factor was a kinin. The kinin releasing activity was assayed on the guinea-pig isolated ileum in the

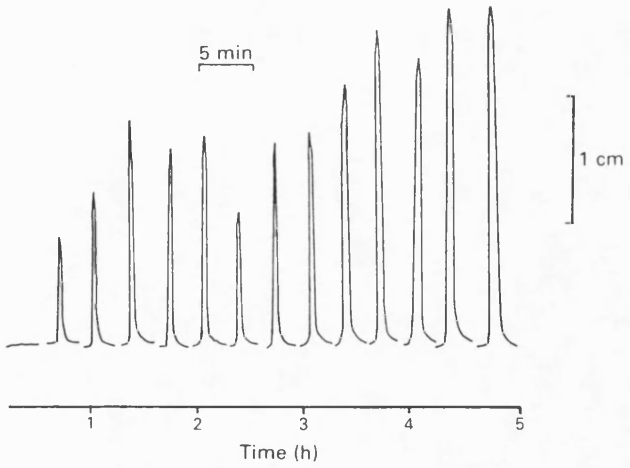


Figure 1 Appearance of oxytocic activity in perfusate from the mesenteric bed. The contractions of the superfused rat uterus induced by 100 μl aliquots of intestinal perfusate from samples collected over consecutive 20 min periods. No oxytocic activity was detectable during the first 20 min of perfusion, but thereafter the samples contained increasing amounts of activity, reaching a maximum concentration at 3–4 h. The lower time scale refers to the time of collection of the perfusate.

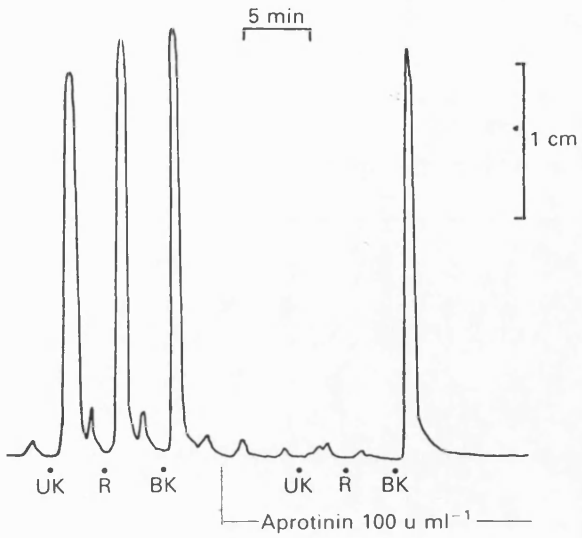


Figure 2 The effect of aprotinin on the oxytocic activities of urinary kallikrein (UK), substance R (R) and bradykinin (BK). Aprotinin completely inhibited uterine contraction to both kallikrein and substance R but did not affect responses to bradykinin.

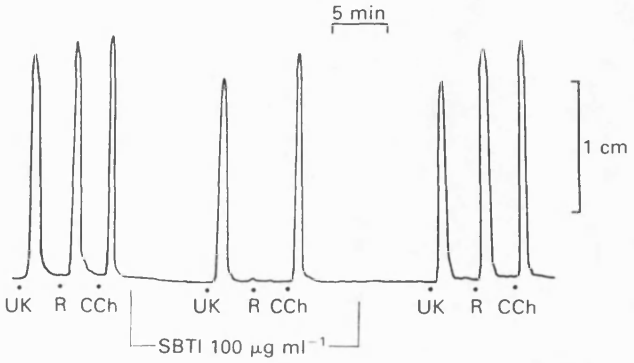


Figure 3 The effect of soy bean trypsin inhibitor (SBTI) on the oxytocic activities of urinary kallikrein (UK), substance R (R) and carbachol (CCh). SBTI reversibly inhibited the response to substance R without a significant reduction in the response to kallikrein or carbachol.

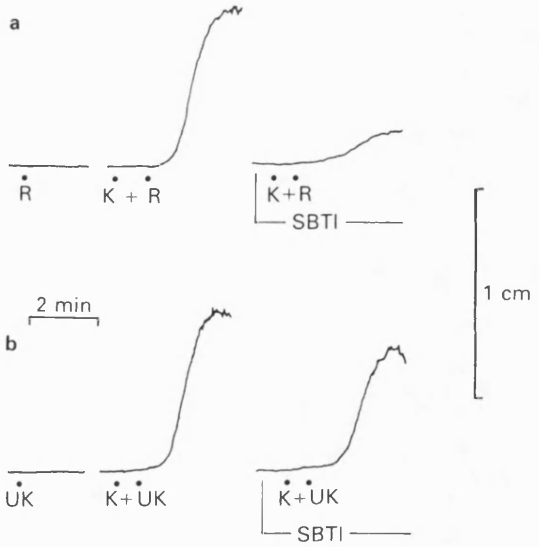


Figure 4 The kininogenase activity of substance R. (a) Addition of substance R (R) alone to guinea-pig isolated ileum elicited no response. However, when kininogen (K) was added, followed by substance R, a slow contraction was seen which was blocked by the presence of soy bean trypsin inhibitor (SBTI). (b) Urinary kallikrein (UK) induced no effect unless kininogen was also present. The contraction induced by kininogen plus urinary kallikrein was not markedly inhibited by SBTI.

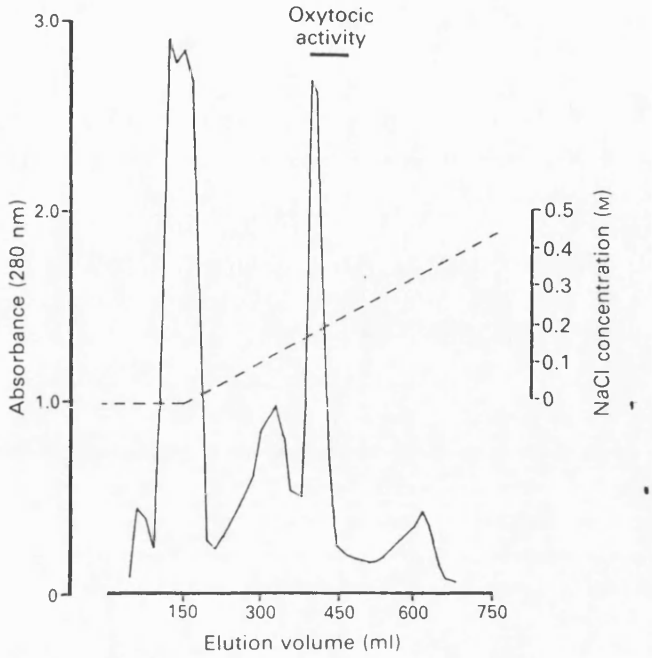


Figure 5 Ion-exchange chromatography of crude intestinal perfusate on DEAE-Sepharose. The sample was applied at V = 0 and was washed with 100 ml Tris buffer (50 mM, pH 7.5). Substance R was eluted with a linear salt gradient at approximately 0.2 M NaCl indicated by the solid bar above the elution profile. The absorbance of fractions was measured at 280 nm and oxytocic activity determined on the superfused rat uterus.

presence of captopril (4.6×10^{-4} M). The ileum was unresponsive to either kallikrein, substance R, or kininogen alone (Figure 4a and b). However, in the presence of kininogen, addition of kallikrein or substance R to the bath resulted in kinin liberation (Figure 4a). Aprotinin (100 Kiu ml^{-1}) completely inhibited the kinin-releasing activity of both urinary kallikrein and substance R ($n = 3$). In contrast, SBTI (100 $\mu g\, ml^{-1}$) produced a small inhibition of the kinin liberation by urinary kallikrein of 13.2 ± 6.3% but significantly ($P < 0.001$, paired t test) reduced the release of kinin by substance R by 80.4 ± 5.0% ($n = 6$) (Figure 4b).

When the selective BK_2 bradykinin receptor antagonist D-Arg-[Hyp^3,$Thi^{5,8}$,D-Phe^7]-bradykinin was added to the superfusate (1×10^{-7} M), oxytocic responses to bradykinin, kallikrein and substance R were reduced by 63.8 ± 10.7%, 69.5 ± 11.7%, 85.9 ± 8.6%, respectively ($P < 0.01$, ANOVAR) ($n = 8$). Responses to carbachol were not reduced in the presence of the antagonist.

Chromatography of crude intestinal perfusate preparations on DEAE-sepharose resulted in elution of a single peak of activity with approximately 0.2 M NaCl (Figure 5). When the active ion-exchange fractions were applied to the Sephacryl S-200 column, a single peak of oxytocic activity was resolved with a molecular weight of approximately 40 kDa (Figure 6). There was no detectable oxytocic activity in fractions either side of this peak. Preliminary studies with SDS-PAGE confirm this estimate of molecular weight. Urinary kallikrein was found to have very similar chromatographic properties to substance R.

The kininogenase activity of fractions eluted from the Sephacryl column was determined by hydrolysis of the chromogenic substrate S-2266 using the endpoint method. A single peak of kininogenase activity was found which coincided with that of oxytocic activity.

Discussion

Perfusion of the rat isolated small intestine resulted in the liberation of a non-dialysable oxytocic principle which Gaddum

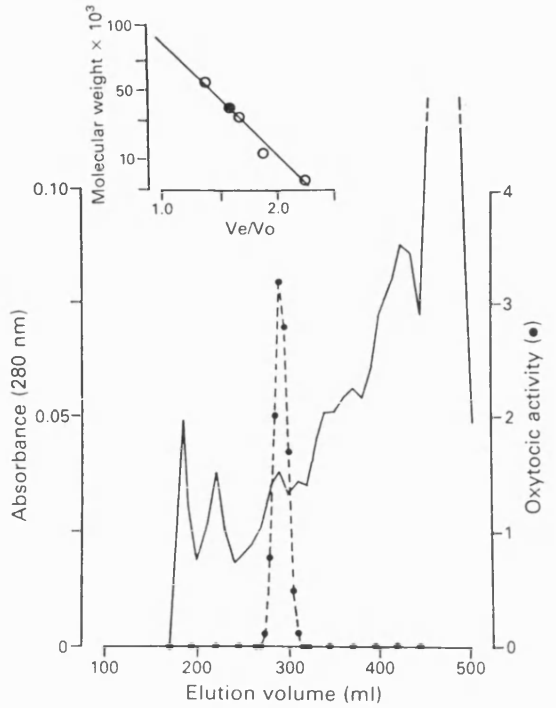


Figure 6 Gel filtration on Sephacryl S-200 of pooled oxytocic fractions previously subjected to ion-exchange chromatography. The sample was applied at V = 0 and was eluted with 50 mM PBS, pH 7.5. The absorbance of fractions was measured at 280 nm and the elution profile is shown by the solid line. Oxytocic activity of the eluted fractions was determined on the isolated superfused uterus of the rat, indicated by the dashed line, and is expressed as % maximum contraction per μl eluate. The molecular weight of the substance R peak (●) corresponded to 40 kD when compared to calibrating proteins (○).

(1953) named substance R. Release of substance R from the perfused intestine increased with time from 20 min to approximately 3 h after which the oxytocic activity of samples collected remained high and fairly constant. Contraction of the rat uterus by substance R was unaffected by atropine, methysergide or by treatment with thioglycolate. However, boiling samples of substance R for 3–4 min resulted in a substantial loss of activity. These results are in agreement with the observations of Gaddum (1953).

Substance R and tissue kallikrein share a number of properties, as Gaddum himself remarked (1953). Our results with the guinea-pig isolated ileum support this, as kallikrein and substance R only induce contraction of the ileum when kininogen is also present, resulting in the generation of a kinin.

The polyvalent protease inhibitor, aprotonin (Trautschold *et al.*, 1967) was found to inhibit the oxytocic responses of the rat uterus to both substance R and urinary kallikrein. However, SBTI, which selectively inhibits plasma kallikrein (Werle & Maier, 1952; Back & Steger, 1968) blocked responses of the uterus to substance R without affecting those to urinary kallikrein. The release of kinin from kininogen by substance R is likewise inhibited by SBTI and aprotinin, whereas kinin release by urinary kallikrein was inhibited by the latter only. These results suggest that substance R possesses kininogenase activity but is unlikely to be a tissue kallikrein. An attempt was therefore made to purify and further characterise substance R.

Gel filtration chromatography of the oxytocic principle obtained from ion exchange chromatography of crude intestinal perfusate resolved a single peak of biological activity with a molecular weight corresponding to approximately 40 kDa. Preliminary experiments with SDS-PAGE confirm this estimate. As plasma kallikreins have been reported to possess a molecular mass of over 100 kDa (see Movat, 1979; Schachter, 1980) it is unlikely that substance R is a plasma kallikrein. The site of release of substance R from the perfused intestine is at present unknown. The presence of kininogenase in rat vascular tissues has previously been reported (Nolly & Lama, 1982; Nolly *et al.*, 1985; 1986). However, when the arterial vasculature of the mesentery alone was perfused, none of the 20 min fractions collected over 6 h contained oxytocic activity. The kininogenase reported by Nolly and colleagues was not inhibited by SBTI. It thus seems probable that substance R is released from the intestinal tissue during perfusion, rather than the arterial vasculature (unpublished data). The physiological significance of substance R in the gut is not fully understood but is the subject of further investigation.

It has recently been demonstrated that contraction of the rat isolated uterus by glandular kallikrein is dependent on the presence of kininogen within the uterine tissue (Figueiredo *et al.*, 1990). This supports the evidence presented by Orce *et al.* (1989) who suggested that the oxytocic activity of glandular kallikrein is mediated, at least in part, by kinins generated from uterine kininogen. In the present study uterine contractile responses to both urinary kallikrein and substance R were inhibited by the kinin antagonist D-Arg-[Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin. There was no significant difference between the inhibition of responses to bradykinin, urinary kallikrein or substance R by this antagonist, suggesting that the oxytocic activity of each is mediated by the BK_2 kinin receptor. It is proposed that substance R shares with glandular kallikrein the same mechanism in the contraction of the rat isolated uterus.

We conclude that substance R is a proteolytic enzyme with a molecular weight of approximately 40 kDa which is biochemically related to the tissue kallikrein family of enzymes but has an anomalous sensitivity to SBTI. The reasons for this discrepancy require further elucidation.

G.J.D. is a SERC student.

We thank Dr M. Schachter for valuable advice, Dr Julie Chao for helpful discussion and Mrs Helen Lynch for secretarial assistance.

References

AARSEN, P.N. (1968). Sensitization of guinea-pig ileum to the action of bradykinin by trypsin hydrolysate of ox and rabbit plasma. *Br. J. Pharmacol.*, **32**, 453–465.

AMES, R.G., MOORE, D.H. & VAN DYKE, H.B. (1950). The excretion of posterior pituitary antidiuretic hormone in the urine and its detection in the blood. *Endocrinol.*, **46**, 215–227.

BACK, N. & STEGER, R. (1968). Effect of inhibitors on kinin-releasing activity of proteases. *Fed. Proc.*, **27**, 96–99.

DOUGLAS, G.J., WILLIAMS, K.I. & FLOWER, R.J. (1988). What is Gaddum's 'Substance R'? *Br. J. Pharmacol.*, **94**, 371P.

EDERY, H. & GRUNFELD, Y. (1969). Sensitization of smooth muscle to plasma kinins: effect of enzymes and peptides on various preparations. *Br. J. Pharmacol.*, **35**, 51–61.

FIGUEIREDO, A.F.S., SALGADO, A.H.I., SIQUEIRA, G.R.T., VELLOSO, C.R. & BERALDO, W.T. (1990). Rat uterine contraction by kallikrein and its dependence on uterine kininogen. *Biochem. Pharmacol.*, **39**, 763–767.

GADDUM, J.H. (1953). The technique of superfusion. *Br. J. Pharmacol. Chemother.*, **8**, 321–326.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagents. *J. Biol. Chem.*, **193**, 265–275.

MOVAT, H.Z. (1978). The kinin-system: its relationship to blood coagulation, fibrinolysis and the formed elements of the blood. *Rev. Physiol. Biochem. Pharmacol.*, **84**, 143–202.

NOLLY, H. & LAMA, M.C. (1982). 'Vascular kallikrein': a kallikrein-like

enzyme present in vascular tissue of the rat. *Clin. Sci.*, **63**, 249s–251s.

NOLLY, H., SCICLI, A.G., SCICLI, G. & CARRETERO, O.A. (1985). Characterisation of a kininogenase from rat vascular tissue resembling tissue kallikrein. *Circ. Res.*, **56**, 816–821.

NOLLY, H., SCICLI, A.G., SCICLI, G., LAMA, M.C., GUERCIO, A.M. & CARRETERO, O.A. (1986). Kininogenase from rat vascular tissue. *Adv. Exp. Med. Biol.*, **198A**, 11–17.

ORCE, G.G., CARRETERO, O.A., SCICLI, G. & SCICLI, A.G. (1989). Kinins contribute to the contractile effects of rat glandular kallikrein on the isolated rat uterus. *J. Pharmacol. Exp. Ther.*, **249**, 470–475.

REGOLI, D., DRAPEAU, G., ROVERO, P., DION, S., D'ORLEANS-JUSTE, P. & BARABE, J. (1986). The actions of kinin antagonists on B_1 and B_2 receptor systems. *Eur. J. Pharmacol.*, **123**, 61–65.

SCHACHTER, M. (1980). Kallikreins (kininogenases) – a group of serine proteases with bioregulatory actions. *Pharmacol. Rev.*, **31**, 1–17.

SCHACHTER, M., UCHIDA, Y., LONGRIDGE, D.J., LABEDZ, T., WHALLEY, E.T., VAVREK, R.J. & STEWART, J.M. (1987). New synthetic antagonists of bradykinin. *Br. J. Pharmacol.*, **92**, 851–855.

TRAUTSCHOLD, I., WERLE, E. & ZICKGRAF-RUDEL, G. (1967). Trasylol. *Biochem. Pharmacol.*, **16**, 59–72.

WERLE, E. & MAIER, L. (1952). Uber die chemische und pharmakologische unterscheidung von kallikrein verschiedener herkunft. *Biochem. Z.*, **323**, 279–283.

(Received February 22, 1990
Revised October 2, 1990
Accepted November 11, 1990)