STUDIES ON BRADYKININ

IN

HEALTH AND DISEASE

Thesis
Presented for the Degree of
Doctor of Philosophy
in the University of Edinburgh

by

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September 1968

STATEMENT IN TERMS OF Ph.D. REGULATION 13

OF THE UNIVERSITY OF EDINBURGH

Some of the studies described in this thesis have been described in the following publications:-

Brocklehurst, W. E. and Zeitlin, I. J. (1967), J. Physiol., 191, 417.

Smith, A. N. and Zeitlin, I. J. (1966), Br. J. Surg., 53, 867.

Zeitlin, I. J. and Smith, A. N. (1966), Lancet ii, 986.

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Infusion of 5-HT and Adrenaline, and Physiological Stress

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SYNOPSIS

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The present work describes the development of a method for the estimation of plasma kinin and kininogen suitable for use with clinical blood samples. This method has been used to study kinin-release in two clinical conditions, the carcinoid and post-gastrectomy (dumping) syndromes, and its possible relationship to physiological stress. The experimental study is divided into two sections, (i) Methodology and (ii) Application of Method.

I. Methodology

Plasma kinins and their precursors are difficult to assay owing to the ease with which inadvertent formation and destruction of kinin may take place during blood sampling and purification procedures. These difficulties probably account for the wide variation in published estimates of the normal levels of these substances in human blood. Moreover, the methods previously described have required separate samples for the estimation of free plasma kinin and kininogen, and immediate processing of the samples. Most methods for free kinin estimation have needed large volumes of blood, give low recovery, or are relatively unspecific. For these reasons, there is little undisputed evidence for the release of plasma kinins in clinical conditions.

The method developed during the present work (Appendix 2) substantially avoids these failings. The blood (6 ml.) was sampled using standard disposable equipment, 1 ml. was taken for haematocrit estimation while the remainder was rapidly ejected into chilled ethanol (15 ml.). This step temporarily inactivated plasma enzymes and precipitated proteins. The kininogen was precipitated with the protein, while free kinin was extracted into the ethanolic supernatant. The fractions were separated and the enzymes were completely inactivated by heating in boiling ethanol. The ethanolic extract from normal blood, evaporated to dryness, was found to contain levels of K^+ sufficient to interfere with the bio-assay of the low levels of free kinin. Furthermore, pathological conditions of interest for kinin analysis would probably contain raised levels of the biologically active amines, histamine, adrenaline and 5-HT.

The separation of bradykinin from K^+ and small molecules was attempted using various small-column chromatographic procedures, including filtration on Sephadex G-25 eluted with water or 75% ethanol and separation on "ion retardation" resin. None of these gave satisfactory recovery of kinin nor adequate separation. The method finally adopted was the extraction of kinin from acidified (pH 1.5) saturated saline into butanol. This procedure gave a recovery of 81.5% (s.d. = 8.0%) for bradykinin (50 - 250 ng.) added to the ethanol at the same time as 5 ml. of whole blood, even when stored for at least 8 hours.

Potassium is very insoluble in butanol and the procedure reduced the K^+ content of the ethanolic extracts by at least 90%. In acid solution bases with high pK_a are highly ionized and unlikely to pass into butanol. The recovery of 100 µg. of 5-HT (pK_a = 10.0) per ml. of blood was 3.2% and that of 10 µg. of histamine (pK_a = 9.7) was 0.4%. The recovery of adrenaline, with a pK_a (9.9) intermediate between those of 5-HT and histamine, was not tested, however, this should be of the same order.

The kininogen-containing precipitate, which possessed no kinin-forming nor destroying activity, was homogenized in saline, and aliquots were incubated with trypsin (200 µg./ml.) in phosphate buffer at pH 7.35. The activated plasma kinin was compared with synthetic bradykinin using the isolated oestrus rat uterus. Isolated blood cells contained no detectable kininogen, thus the whole-blood estimate was corrected using the haematocrit to give the plasma kininogen content. The values for plasma kininogen calculated from whole blood were lower (88.7%) than those determined directly from plasma according to the method of Lahiri.

In normal subjects the mean value for free kinin was 2.8 ng. (bradykinin equivalent) per ml. of plasma and 6.1 µg. per ml. of kininogen, and these values were within the range of those found by the majority of workers.

II. Application of the Method

The vasomotor changes of the carcinoid syndrome are characterized by episodic or continuous flushing, with often a fall in blood pressure at the commencement of flush. The dramatic crimson colouration may be provoked by ingestion of alcohol or food, emotion, or intravenous injection of adrenaline or noradrenaline. Raised blood levels of 5-HT and other indoles and a raised urinary excretion of 5-HIAA and other indoles are diagnostic of the condition. However, the appearance of raised levels of 5-HT in the blood stream does not correlate well with the vasomotor symptoms, nor does intravenous infusion of 5-HT in carcinoid patients always produce the typical vasomotor changes. Earlier workers had detected raised levels of a kinin-like polypeptide in hepatic venous blood of patients during carcinoid flush. The present work supported and extended these findings. Raised levels of bradykinin-like activity, characterized by assay on rat uterus and rat duodenum, were found in peripheral venous blood during flushing in carcinoid patients. The increase in free kinin was accompanied by a fall in plasma kininogen. The levels of free kinin were more closely related to the severity of flushing than were levels of whole blood 5-HT or urinary 5-HIAA.

Many features of the dumping syndrome are similar to those of the carcinoid syndrome. Both are often provoked by similar types of stimulus and are characterized by similar intestinal and vasomotor symptoms. Although release of 5-HT into portal venous blood has been found during experimental dumping in dogs, no similar relationship between symptoms and indole metabolism has been demonstrated in man. In the present studies, raised levels of bradykinin-like activity have been found in the blood of patients during the hypotensive flushing episodes of a dumping syndrome provoked by ingesting hypertonic glucose. The activity was characterized by parallel bio-assay on rat uterus, rat duodenum and guinea pig ileum, and chymotryptic degradation. The rise in free kinin level was concomitant with a fall in plasma kininogen. The levels of free kinin correlated well with the severity of symptoms. Administration of a hypotonic placebo solution to the dumpers and a hypertonic glucose solution to normal subjects provoked neither symptoms nor changes in plasma kinin. In both the carcinoid and dumping syndromes the release of plasma kinin could reasonably account for the vasomotor symptoms.

The hypotensive fainting often seen during the dumping syndrome is very like vasovagal syncope. Abnormally raised levels of free plasma kinin were detected in the blood of two healthy but apprehensive subjects, who fainted during otherwise normal venepunctures.

It seemed possible that the plasma kinin release detected in the carcinoid and dumping syndromes was secondary to the release of 5-HT. To test this hypothesis, 5-HT (20 - 80 µg./kg./minute) was infused intravenously in anaesthetized dogs. Although cutaneous vasodilatation, hypotension, and a fall in plasma kininogen were detected, no increase in free kinin occurred. Possible explanations of this phenomenon are discussed.

Extensive sympathetic stimulation is known to occur during emotional fainting and undoubtedly occurs too during the stress of the carcinoid and dumping syndromes. Furthermore, many types of physiological stress and intravenous adrenaline are known to stimulate fibrinolysis, and in theory should also provoke plasma kinin release. The possibility that plasma kinin release was secondary to raised circulating levels of adrenaline was therefore tested. Adrenaline (2 - 4 µg./kg./minute) infused intravenously in anaesthetized dogs provoked the release of large quantities of free kinin in two dogs out of five. Although adrenaline normally produced marked cutaneous vasoconstriction, cutaneous vasodilatation occurred in the presence of raised kinin levels. These findings indicate that, under certain conditions, kinin release may be secondary to raised circulating levels of adrenaline. However, in the conditions under investigation it is clear that other factors must also be involved.

Adrenaline infusion in the dogs caused an increase in the plasma kininogen level. A similar increase was seen in two human subjects undergoing mild exercise. The results would be explained by the existence of two pools of kininogen in the body.

The significance of the release of 5-HT and kinin in the carcinoid and dumping syndromes is considered in terms of the normal physiological mechanism.

INTRODUCTION

During the last ten years there has been growing awareness that bradykinin may play an important part in many physiological events. Lack of a convenient but reliable method for taking samples of human blood for kinin assay has resulted in few clinical studies. Proximity and related interests between the gastro-intestinal surgeons and the pharmacologists in Edinburgh provided an unusual opportunity for such studies.

It was necessary to improve Lahiri's method for the extraction of plasma kinin, and it was logical to evaluate the kininogen precipitated during the process. Solvent extraction proved to be the best method for removing interfering potassium ions and concentrating kinins in one operation. Extraction at acid pH permitted the substantial removal of biologically active bases. Biological assay was retained because it alone would detect and characterize the extremely small amounts of kinin in normal blood.

The method has been used to study kinin release in two clinical conditions, the carcinoid syndrome and the post-gastrectomy syndrome, and its possible relationship to physiological stress. Although the whole forms a coherent and related investigation, the backgrounds to the three subjects - the carcinoid and post-gastrectomy syndromes and stress - are so widely diverse that, for the sake of clarity, each is presented in a separate section with its own introductory review. The pharmacology and assay of plasma kinins is reviewed in the General Introduction.

THE KININS

1. Historical Perspective

Physiological actions produced by certain body fluids and proteolytic enzymes, probably as the result of their kinin content or their kinin-releasing activity, were described long before bradykinin had been shown to exist as a separate entity. The presence of a hypotensive substance in urine was reported in 1909 (Abelous and Bardier, 1909); in 1922, Dale and Kellaway showed that a smooth-muscle-stimulating principle was generated in guinea pig serum by contact with starch or bacterial suspensions; while in 1935, Feldberg and Guimarais showed that saliva contained a depressor substance which was destroyed by boiling.

Investigations into the kinin-releasing system may be said to date from a series of papers published by Frey, Kraut, Schulz, and Werle from 1926 onwards (Frey, 1926; Frey and Kraut, 1928; Frey, Kraut and Schulz, 1930; Frey and Werle, 1933). They showed that intravenous injection in the dog of extracts of pancreas, of pancreatic secretion itself, and of urine produced a fall in arterial blood pressure. They called the active principle "Kallikrein" from the Greek work meaning pancreas, since they believed it to be a pancreatic hormone. It was not until some years later, at the time when it was first recognised

that renin owed its hypertensive action to the release of a secondary vaso-active substance from a plasma globulin (Pickering and Prinzmetal, 1937-38; Muñoz, Braun-Menendez, Fasciolo and Leloir, 1939), that these early workers showed that kallikrein similarly owed its activity to the enzymic release of an active substance from a plasma precursor (Werle, Götze and Keppler, 1937; Werle and Gruntz, 1939). These workers recognised the analogy between the two systems (Werle and Hambuechen, 1943). The active substance released by kallikrein was shown to have all the properties of kallikrein in vivo, and it was named Darm Kontrahierende Substanz (intestine contracting substance) or substance DK, since, unlike kallikrein it also contracted many isolated smooth muscle preparations in vitro. Werle and Berek, in 1948, suggested that substance DK should be called more simply "kallidin", and its precursor, "kallidinogen".

In 1949, Rocha e Silva, Beraldo and Rosenfeld described the release of a hypotensive peptide from plasma globulin by the action of snake venoms and trypsin. They named the substance "bradykinin", because it caused a relatively slow contraction of the isolated guinea pig ileum (Rocha e Silva et al, 1949). These workers distinguished bradykinin from acetyl choline, histamine, adenosine, 'hypertensin', and

other active substances including kallikrein. They were unaware (Rocha e Silva, 1960) that kallikrein had already been shown to release a smooth-muscle-stimulating substance, kallidin, from a plasma precursor. It is now known that the original 'kallidin' was probably a mixture of two polypeptides, kallidin I and kallidin II (Webster and Pierce, 1963). Kallidin I is identical with bradykinin, the polypeptide released by trypsin from plasma globulin, shown by Elliott, Lewis and Horton (1960) to be a nonapeptide; while kallidin II is a decapeptide, lysyl-bradykinin. These substances, together with some other very closely related peptides, have been given the generic name of kinins (Rocha e Silva, 1962, 1963; Webster, 1966). The Committee on Nomenclature for Hypotensive Peptides recently ruled that bradykinin should refer specifically to the nonapeptide, and kallidin to the decapeptide (Webster, 1966).

2. Chemistry

Bradykinin (Kallidin I, Kallidin-9)

H-Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg-OH

Structure

Rocha e Silva, Beraldo and Rosenfeld (1949) observed that the incubation of pseudoglobulin with trypsin produced the hypotensive, smooth-muscle-stimulating polypeptide, bradykinin. Bradykinin, formed by the action of trypsin on

acid-treated ox pseudoglobulin, was isolated by Elliott, Horton and Lewis (1960 a) after ethanol extraction, counter current distribution, CM-cellulose chromatography, and preparative paper electrophoresis. The same group established the amino acid composition and sequence (Elliott, Lewis and Horton, 1960).

As the result of close collaboration with Elliott and his colleagues in London, synthesis was achieved by a Swiss group led by Boissonas. The synthetic peptide was identical in biological activity to the pure ox bradykinin (Boissonas, Guttman and Jaquenoud, 1960; Boissonas, Guttmann, Jaquenoud, Konzett and Stürmer, 1960; Konzett and Stürmer, 1960; Konzett and Boissonas, 1960).

The molecular weight of bradykinin dihydrochloride is 1131 (Elliott, Horton and Lewis, 1960 b).

Kallidin (Lysyl-bradykinin, Kallidin II, Kallidin-10)

H-Lys.Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg-OH

Structure

Kallidin was isolated and characterized independently by Pierce and Webster (1961), and by Werle, Trautschold and Leysath (1961). Pierce and Webster showed that when acid-treated human plasma was incubated with human urinary kallikrein, two pharmacologically active peptides were formed, kallidins I and

II (being the order in which they were eluted from a column of CM-cellulose). Kallidin I was identical with the nonapeptide bradykinin, while kallidin II was a decapeptide differing from kallidin I by having an additional terminal lysine residue. Werle et al (1961) similarly, showed that bradykinin and lysyl-bradykinin were formed by the action of bovine submaxillary kallikrein on acid-treated ox plasma. Lysyl-bradykinin was synthesized by Nicolaides, DeWald and McCarthy (1961).

Methionyl-lysyl-bradykinin

H-Meth.Lys.Arg.Pro.Pro.Gly.Phe.Ser.Pro.Phe.Arg-OH

Structure

Pseudoglobulin prepared from ox serum, kept at acid pH for several days, and then neutralised and incubated at 37°C, released two smooth-muscle-stimulating polypeptides. Only one of these could be isolated, this was the hendecapeptide, methionyl-lysyl-bradykinin (Elliott, Lewis and Smyth, 1963; Elliott and Lewis, 1965). Methionyl-lysyl-bradykinin was synthesised by Schröder (1964). The hendecapeptide differs from bradykinin and kallidin in that it is much more slowly attacked by kininases, a 15 minute incubation causing little or no inactivation. It does not appear to be formed naturally in plasma, and cannot be detected following glass or

acid activation of plasma kallikreins (Habermann, 1966), only bradykinin being formed.

Other Kinins

The euglobulin fraction of human plasma, precipitated by 33% saturation with ammonium sulphate, formed kinins F and S spontaneously on incubation. They were separated and distinguished from bradykinin and lysyl-bradykinin by paper chromatography (Armstrong and Mills, 1963). A kinin-forming enzyme from the euglobulin fraction acted on fresh plasma to form kinin E, similarly chromatographically distinguished. These substances have not been further characterized.

A peptide having bradykinin-like biological activity is formed by the action of calf or human saliva on acid-treated bovine colostrum (Guth, 1959; Moriya, Moriwaki, Yamazaki, Akimoto and Fukushima, 1966). This "colostrokinin" (Werle, 1959) may be distinguished from bradykinin by parallel bio-assay, and its precursor is present only in colostrum and not normal milk (Guth, 1959; Moriya et al, 1966).

In addition to the kinins so far described, which are of the most direct relevance to the present work, it should be mentioned that the bradykinin homologues and bradykinin-like activity are found throughout the animal kingdom. Peptides with bradykinin-like activity are found in wasp venom

(Schachter and Thain, 1954; Prado, Tamura, Pisano and Udenfriend, 1966) (from which the new kinin, glycyl-bradykinin was isolated), in scorpion venom (Diniz and Moura Goncalves, 1960), and in the skin of frogs and other amphibians (Erspamer and Anastasi, 1966). Fowl pancreatic kallikrein liberates a kinin named ornithokinin from fowl plasma, that is inactive on mammalian blood pressure, but is hypotensive in the fowl, and also contracts the hen ileum on which mammalian kinins are without influence. The molecular weight of ornithokinin (6184) is much higher than that of bradykinin, (Werle, and Hürter, 1936; Werle, Hochstrasser and Trautschold, 1966; Werle and Leysath, 1967). Kinins have also been detected in the plasma of reptiles (Erdös, Miwa and Graham, 1967).

3. Pharmacological Actions of Bradykinin

Plasma kinins stimulate certain smooth muscles, produce hypotension and vasodilatation in several regions of the systemic circulation, increase capillary permeability, evoke pain from an exposed blister base, and may cause chemotaxis of leucocytes although this is in dispute. Kinins also stimulate sympathetic ganglia. The pharmacological actions of pure bradykinin were first described by Elliott, Horton and Lewis (1960 b).

3(a). Actions on Smooth Muscle

(i) In vitro: The isolated guinea pig ileum gives a characteristic slow contraction with 1 ng. per ml. of bradykinin, while the oestrus rat uterus contracts to 0.1 ng. per ml. The dose-response curve of bradykinin on the isolated oestrus rat uterus is very steep (see Fig. 22). Certain smooth muscles which show rhythmic activity and a high resting tone are inhibited by bradykinin. The rabbit duodenum and rat colon show inhibition followed by stimulation, while rat duodenum shows only relaxation to a concentration of 8 ng. bradykinin per ml. (Elliott et al, 1960). Less sensitive than these is the isolated guinea pig seminal vesical which contracts at a concentration of 0.5 µg. per ml. (Konzett and Stürmer, 1960), while isolated human uterine muscle taken at Caesarian operation contracts to bradykinin concentrations of 1 - 10 µg. per ml. (Berde and Saamelli, 1961).

Bradykinin contracts only longitudinal muscle strips from the guinea pig ileum and has no

action on circular muscle strips (Brownlee and Harry, 1963). On isolated whole muscle preparations in which longitudinal movements and volume changes can be recorded, bradykinin contracts the longitudinal muscle of the guinea pig ileum but inhibits peristalsis (Beleslin, Bogdanović and Radmanović, 1964). In man, bradykinin contracts longitudinal muscle strips from the jejunum and ileum (0.1 - 1.0 ng./ml.). It has very little effect on the circular muscle of the jejunum (1 µg./ml.), and relaxes that of the ileum (0.1 - 0.5 ng./ml.) (Fishlock, 1966). Bradykinin relaxes the longitudinal muscle of the human colon in low concentrations (5 - 100 ng./ml.) and produces relaxation followed by contraction in high concentration (0.5 - 1.0 µg./ml.) while it relaxes colonic circular muscle at all concentrations.

The actions of bradykinin on the isolated rat uterus and guinea pig ileum are not affected by the presence of atropine, morphine, cholinesterase inhibitors, and ganglion blocking agents other than tetra-ethylammonium chloride (which has a non-specific action on the smooth muscle itself, and potentiates

the response to bradykinin) (Khairallah and Page, 1963). The peptide thus appears to act directly on the smooth muscle.

(ii) In vivo: The intravenous injection of bradykinin in the anaesthetised guinea pig causes bronchoconstriction (Elliott et al, 1960; Collier, Holgate, Schachter and Shorley, 1960), demonstrated by the method of Konzett and Rössler (1940), and on a molar basis bradykinin is some ten times more active than histamine. The bronchoconstriction produced by bradykinin is inhibited fairly specifically by many of the analgesic anti-pyretics, including acetylsalicylic acid, phenylbutazone, amidopyrine, phenazone, and mefenamic and flufenamic acids, which act with high potency. Paracetamol, cinchophen, sodium salicylate and acetanilide are moderately potent antagonists of this bronchoconstriction, while cortisone, hydrocortisone, and morphine are inactive or unspecific (Collier and Shorley, 1960, 1963). Intravenous bradykinin also produces bronchoconstriction in the cat, although at a level some fifty times that in the guinea pig (Konzett and Stürmer, 1960).

In man, Herxheimer and Stresemann (1961) showed that most normal men are unaffected by inhalation of aerosols of 2% bradykinin in saline, whereas 13 out of 15 asthmatic patients developed dyspnoea, and a 10 - 30% reduction in vital capacity.

Although, *in vitro*, bradykinin is almost as active as oxytocin in contracting the rat uterus, this is not the case *in vivo*, and in the rat, cat, and rabbit, intravenous bradykinin produces contractions, but is some 10 - 1,000 times less active than oxytocin (Berde and Saameli, 1961). Single injections of bradykinin up to 2 µg. per kg. or infusions of up to 0.56 ug. per kg. per minute in women are without effect on uterine contraction. Single intravenous injections of bradykinin or kallidin (2 - 16 µg./kg.) produce a biphasic change in intestinal motility in the anaesthetized cat and rabbit, a primary inhibition followed by stimulation; intravenous infusion produces only inhibition (Bauer, Gmeiner and Winkler, 1966). Intravenous bradykinin infusion in man (20 µg./minute) produces inhibition of the motility of the distal colon (Murrell and Deller, 1967).

3(b). Actions on the Circulation

(i) Hypotension: In all animals which have so far been investigated, intravenous bradykinin causes a fall in arterial blood pressure; these include the cat, dog, rabbit, rat, guinea pig (0.05 - 0.5 µg./kg.) and, in very high dose, the chicken (40 - 80 µg./kg.) (Elliott, Horton and Lewis, 1960 b; Konzett and Stürmer, 1960); monkey, chimpanzee (Paratt, 1964) and man (Gersmeyer and Spitzbarth, 1961; Bishop, Harris and Segel, 1963; Mason and Melmon, 1965). The hypotension produced by a single injection is short-lived, reflecting the very short half-life of bradykinin in the circulation (0.5 min.) (Saameli and Eskes, 1962; McCarthy, Potter and Nicolaides, 1965). Intravenous infusion causes an initial sharp drop in pressure followed by a rise probably as the result of reflex mechanisms; it stabilizes at a new hypotensive level depending on the rate of infusion (Page and Olmsted, 1961; Bishop, Harris, and Segel, 1963).

(ii) Vasodilatation: Bradykinin is a very potent vasodilator in most areas of the circulation, and causes a fall in total peripheral resistance (Page and Olmsted, 1961; Maxwell, Elliott and Kneebone, 1962; Montague, Rosas and Bohr, 1963;

Anguissola, Feruglio, Campus, Chiandussi, Pandolfo and Bert, 1966). It increases limb and muscle blood flow in animals and man (Elliott et al, 1960b; Fox, Goldsmith, Kidd and Lewis, 1961; Burch and DePasquale, 1962; Paldino, Hyman and Lenthall, 1962; Mason and Melmon, 1965). Bradykinin dilates cutaneous vessels with very great potency, and in man causes a characteristic flush and increase in cutaneous flow in doses too small to produce detectable changes in the rest of the circulation (Fox et al, 1961; Javett and Coffman, 1962). In both dog (Carpi and Corrado, 1961) and man (Sicuteri, Franchi and Michelacci, 1961) bradykinin produces increased cerebral blood flow. Bradykinin lowers myocardial vascular resistance in the rabbit, cat, dog, monkey and chimpanzee (Maxwell et al, 1962; Paratt, 1964; Anguissola et al, 1966), and dilates coronary vessels in the isolated hearts of rat, guinea pig, rabbit, cat, and dog (Antonio and Rocha e Silva, 1962).

(iii) Vasoconstriction: Bradykinin is not, however, a universal vasodilator. Vasoconstriction in the isolated rabbit lung has been demonstrated (Lecomte

and Troquet, 1960) in the absence of broncho-motor effects (Hauge, Lunde, and Waaler, 1964). Similar observations have been made in the intact human (Gersmeyer and Spitzbarth, 1961; Bishop, Harris and Segel, 1963), and in the cat and guinea pig (Klupp and Konzett, 1963; 1965). This vasoconstrictor effect is not limited to pulmonary vessels. The results of Burch and DePasquale (1962) indicate that in the human finger, bradykinin produces either constriction of arterio-venous anastomoses or venoconstriction, causing reduced flow and passive capillary dilatation. Rowley (1964) and Guth, Bobbin, Cano and Amaro (1966) have shown that bradykinin causes a similar venoconstriction in the rat and rabbit respectively.

3(c). Capillary Permeability Changes

Intradermal injection of bradykinin in the guinea pig, rat and rabbit causes a marked local increase in capillary permeability, visualized by prior treatment with intravascular dye (Elliott, Horton and Lewis, 1960b; Konzett and Stürmer, 1960; Carr and Wilhelm, 1965). Doses as low as 1 ng. per 0.1 ml. produce this effect in the guinea pig, and on a molar basis bradykinin is some fifteen times more active than histamine. In man, intradermal

injection of 0.5 - 200 µg. bradykinin per ml. in 0.05 or 0.1 ml. doses causes a weal and flare (Herxheimer, 1963).

Although it has been widely accepted that bradykinin produces permeability increases by a direct action on the capillaries, Rowley (1964) has elegantly shown that in the rat at least, the permeability increase produced by bradykinin, histamine, and 5-HT is apparently the result of venoconstriction. He found that venular dilatation produced by these substances was passive and secondary to the venoconstriction, while retrograde infusion of the veins with bradykinin, histamine or 5-HT did not cause an increase in permeability. Rowley concluded that these agents provoked a venoconstriction which caused a raised back-pressure, resulting in passive dilatation of the venules and an apparent increase in the capillary permeability.

3(d). Stimulation of Pain Fibres

Application of 0.1 - 1.0 µg. per ml. of bradykinin to an exposed blister base on the human forearm caused transient pain (Elliott, Horton and Lewis, 1960 b). In this, bradykinin was less active on a molar basis than 5-HT, and produced sensations qualitatively different from those of histamine - a burning instead of an itching sensation. After repeated doses of bradykinin, higher concentrations were needed to produce a comparable response. The densensitized area was unchanged in its sensitivity to

histamine or 5-HT. Pain has also been produced by intra-arterial injections of bradykinin in man (Burch and DePasquale, 1962) and in the dog (Braun, Guzman, Horton, Lim and Potter, 1961; Guzman, Braun, Lim, Horton and Potter, 1961).

3(e). Leucotaxis

Lewis (1962) found that when 0.5 ml. of 5 µg./ml. bradykinin was injected intradermally into the guinea pig one hour before killing it, histological examination showed infiltration of leucocytes into the connective tissue surrounding the site of injection. It was also found that flooding the mesentery of the anaesthetized guinea pig, rat or rabbit with solutions of 10 - 100 µg./ml. of bradykinin caused accumulation of leucocytes in the venules of the affected area. Chemotaxis of leucocytes thus became generally accepted as part of the pharmacological profile of bradykinin. However, attempts by Spector and Willoughby (1964) and Gowland (1964) to confirm this finding in the rat and man were unsuccessful. Spector and Willoughby tested bradykinin (10 - 100 µg./ml.) amongst a number of other agents, by intradermal injection in the rat and subsequent excision of the area of skin, after 45 minutes or 24 hours, for histological examination. They concluded that bradykinin and a number of other substances, including histamine and 5-HT, did not produce significant leucotaxis.

Gowland (1964) prepared "skin windows" on the human forearm by scraping the skin with a scalpel blade until a moist red area, 3 mm. square, was produced, in which there was no punctate bleeding. This was covered with a screw-capped perspex cover which was stuck to the surrounding skin with adhesive. The "window" was filled with the test solution. After gentle mixing, a drop was removed at intervals, for up to seven hours, for histological examination. The window was kept topped up with fresh test solution. Gowland, too, concluded that bradykinin caused no significant leucotaxis.

These findings have been supported by those of Keller and Sorkin (1965) and Ward, Cochrane and Muller-Eberhard (1966) using the less physiological technique of Boyden. They used a perspex chamber separated into two compartments by a filter membrane of such pore size that leucocytes could not pass through it except by active migration. The lower chamber contained the substance under test, while the upper chamber contained the cell suspension. The unit was incubated at 37°C. for three hours and the filter was removed for histological staining. Bradykinin and kallidin (10^{-5} - 10^{-9}), 5-HT and histamine did not cause significant migration of rabbit polymorphonuclear leucocytes through the membrane.

3(f). Actions on Ganglia

As a result of the demonstration that bradykinin (Lecomte, Troquet and Dresse, 1961; Feldberg and Lewis, 1964) and angiotensin (Feldberg and Lewis, 1964) release catecholamines from the adrenal medulla, Lewis and Reit (1965) investigated the action of these peptides on sympathetic ganglia. When the peptides were delivered to the superior cervical ganglion of the spinal cat by rapid retrograde injection into the external carotid while the blood flow from this artery to the nictitating membrane was interrupted, the membrane contracted. Contractions occurred only on the side of the injection and were thus not due to the peptides reaching the general circulation. Removal of the ganglion, or cutting the post-ganglionic nerves abolished the contraction. The stimulant effect of the peptides was resistant to the actions of hexamathonium and atropine. The fact that angiotensin is vasoconstrictor and bradykinin is vasodilator made it unlikely that their excitatory activity on the ganglion was due to a local circulatory effect.

The excitatory action of the peptides on the superior cervical ganglion was confirmed by Haefely, Hürlimann and Thoenen (1966), who recorded the post-ganglionic nerve activity. Both peptides, when applied by close-arterial injection to the ganglion, initiated an asynchronous firing of the post-ganglionic

nerves simultaneously with the increase in tone of the nictitating membrane. Injection of the peptides during sub-maximal electrical stimulation of the ganglion caused marked facilitation of symaptic transmission (Haefely et al, 1966).

In the cat, the threshold dose of angiotensin was 0.1 - 0.3 µg. and that of bradykinin was 0.5 - 10.0 µg. (Lewis and Reit, 1965), while the dog and rabbit were far less sensitive to both peptides, doses of 30 - 50 µg. of bradykinin producing very small responses (Lewis and Reit, 1966). Most of these dose-levels are very high, and seem unlikely to be reached in the physiological situation.

The receptors for the peptides appear to be fairly specific, and desensitization of the ganglion (Lewis and Reit, 1965) or the adrenal medulla (Staszewska-Barczak and Vane, 1967) to one peptide does not result in desensitization to the other.

4. Pharmacological Activity of Bradykinin Homologues

The pharmacological properties of lysyl-bradykinin and methionyl-lysyl-bradykinin are qualitatively identical to those of bradykinin. There are, however, quantitative differences between the three kinins (See table 1).

Table 1

The relative potencies of the plasma kinins

Bradykinin = 1

Assay	Lysyl-Bradykinin	Methionyl-Lysyl-Bradykinin
In vitro:		
Guinea pig ileum	1/3 (a)	1/4 (b)
Rat uterus	2/3 (a); 1 (c)	1/4 (b)
Rat duodenum	1/2 (a)	1/4 (b)
Rabbit duodenum	2 (a)	
Hen rectal caecum	2 (a)	
In vivo:		
Bronchoconstriction (Guinea Pig)	1/3 (d)	1/4 (b)
Vasodilatation (Dog)	2 (c)	
Vasodilatation (Cat)		1/4 (b)
Blood Pressure (Rabbit)	1.9 (a)	2 (e)
Blood Pressure (Cat)	2/3 (a)	
Capillary Permeability Increase (Guinea Pig)	1 (a)	1 (b)
Pain Production (Man)		1/4 (b)

Reference (a) Stürmer and Berde, 1963
(b) Elliott, Lewis and Smyth, 1963
(c) Webster and Pierce, 1963
(d) Nicoliades, deWald and Croft, 1963
(e) Schröder, 1964

PLASMA KININ PRECURSORS

1. Initial Experiments

Bradykinin was shown by Rocha e Silva, Beraldo and Rosenfeld (1949) to be released by the action of trypsin, or the venom of the snake Bothrops jararaca, on dog or ox blood and serum. They further found that the total potency of blood for bradykinin release could be localised in the globulin fraction precipitated by 30 - 45% saturation with ammonium sulphate. This was the classical pseudoglobulin fraction, which contains all of the plasma proteins but with relatively small quantities of albumin and γ-globulin. Rocha e Silva and his colleagues called the bradykinin precursor protein precipitated with this fraction, bradykininogen.

Van Arman (1952) studied the bradykininogen content of bovine plasma fractions prepared by Cohn method 10. The bradykinin precursor was found in fraction IV-4, and amongst the subfractions of IV-4, IV-6 was the richest in substrate (Van Arman, 1955). "Kallidinogen", the substrate for glandular kallikrein, was similarly concentrated in IV-6. Werle (1955) further specified the origin of kininogen when he found, using paper-electrophoresis, that the substrates for both trypsin and kallikrein migrated as α_2-globulins. It seemed that there was only one kininogen, and until relatively recently this was

believed to be the case, despite several paradoxical experimental results.

2. Evidence for Two Plasma Kininogens

Margolis and Bishop (1963) found that activation of human plasma kallikrein by allowing plasma to come in contact with glass beads (See following sections) caused depletion of no more than 40% of the total plasma kininogen. The unused kininogen was still susceptible to the action of trypsin and glandular kallikreins, but not to plasma kallikrein however activated or concentrated. These workers visualised a single kininogen molecule or complex, having two radicals susceptible to the two types of enzyme, the plasma and glandular kallikreins. Similar findings have been noted by several workers including, most recently, Armstrong (1966).

An explanation of these phenomena was advanced by Vogt and his colleague (John and Vogt, 1965; Vogt, 1966). They found that, following glass activation of human plasma until no further free kinin was formed, kininogen, which could be activated by incubation with glandular (pancreatic) kallikrein in the presence of kininase inhibitors, still remained in the plasma. The residual kininogen represented more than 50% of the total. Thus it appeared that only a proportion of the total

kininogen in plasma was available to glass-activatable plasma kallikrein. Vogt (1966) also performed the complementary experiment. Human plasma was incubated with pancreatic kallikrein until no further kininogen depletion took place. Further additions of kallikrein in the presence of kininase inhibitors caused no further release of kinin. The plasma did, however, still liberate kinin when activated by contact with glass. From these experiments, Vogt concluded that plasma contained two separate kininogens; one, kininogen I, was a specific substrate for glandular kallikreins, the second, kininogen II, was the substrate for glass-activated plasma kallikrein.

Vogt further found that if kininogen I was added to glass-activated, kininogen II-depleted plasma, no kinin was formed. If this same plasma was first acid-treated by incubation at pH 2 - 3 for 30 minutes at 37°C and then neutralised, an enzyme was activated which digested the added kininogen. This enzyme Vogt called kininogenase I, while the glass-activatable enzyme was kininogenase II. He thus suggested that human plasma contained two complete kinin releasing systems, I and II, each containing a kininogenase and a kininogen (See Fig. 1).

The earliest indication that there may be two distinct

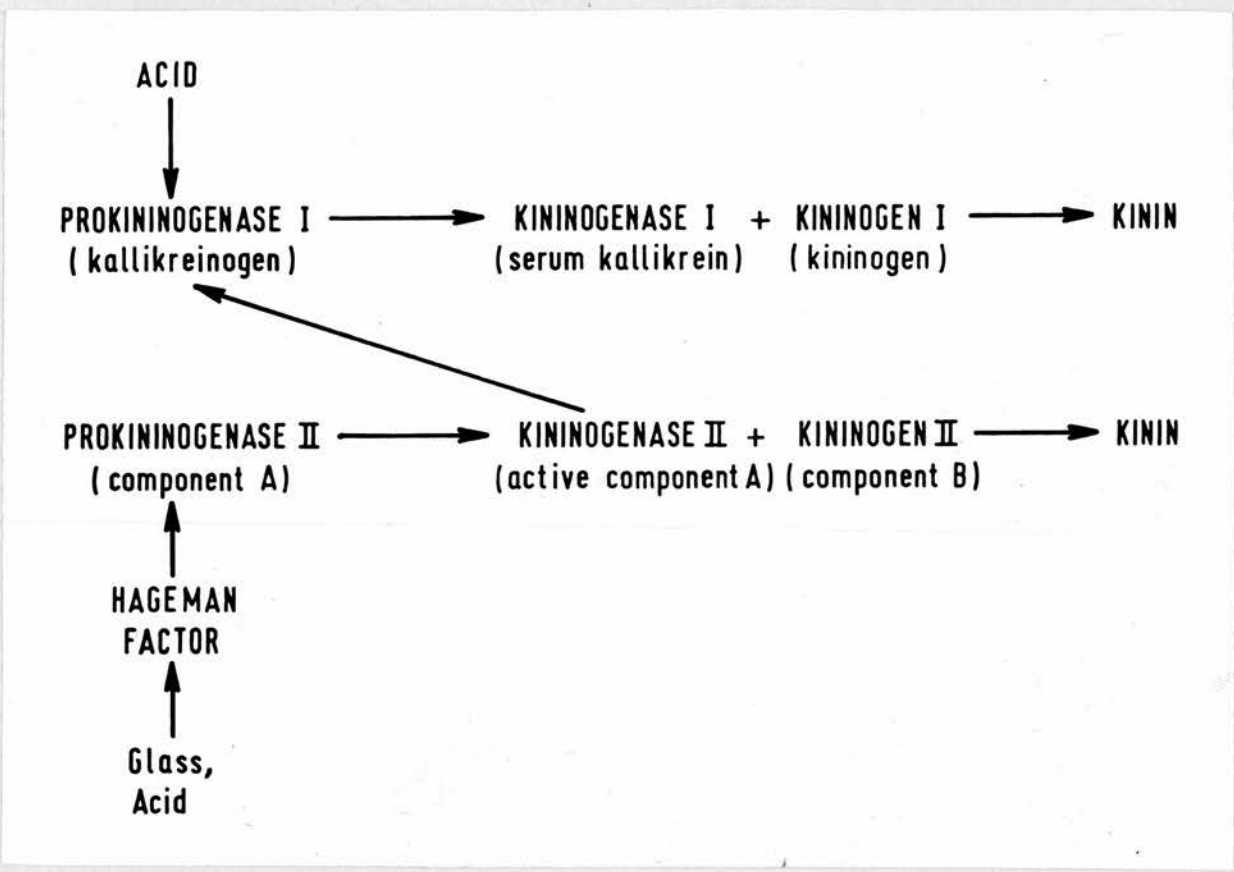

Fig. 1: The kinin-releasing systems as visualized by Vogt (1966).

kininogen protein fractions in plasma was the finding by Brocklehurst and Mawer (Brocklehurst and Mawer, 1963; Mawer, 1963) that filtration of a purified fraction of kininogen on Sephadex G-200, gave two peaks of activity. From the elution characteristics of these peaks, Brocklehurst and Mawer (1966) suggested that they were probably due to two separate kininogens. The separation of two kininogen proteins from human plasma was first achieved by Pierce and Webster (1966). Using DEAE-cellulose, followed by hydroxylapitite chromatography, they obtained two kininogen-containing peaks. These were further purified on Sephadex G-200 and additional hydroxylapatite chromatography. The two proteins were found to be immunologically identical, but were clearly distinguished by various chemical procedures, including enzymic and chemical degradation.

The findings of Pierce and Webster were fully confirmed in a series of papers by Jacobsen and his colleague (Jacobsen, 1966 (a), (b), (c); Jacobsen and Kriz, 1967). Jacobsen found that the plasmas of man, dog, rabbit, rat and guinea pig all contained two kininogens. He found that one kininogen gave rapid kinin formation only with glandular kallikrein and a very slow formation with plasma kallikrein. The other kininogen formed kinin with both plasma and glandular kallikreins, but more rapidly with the plasma kallikrein. The substrate for glandular kallikrein

obtained from human plasma, equivalent to Vogt's kininogen I, had a molecular weight of 57,000; the other kininogen had a molecular weight of 197,000. These findings were in accord with the conclusions of Pierce and Webster (1966), who reported that the molecular weight of the smaller molecule was 50,000 - 52,000. The larger molecule was a polymer of this.

3. The Nature of the Bond split in Kinin Formation

The kinin-releasing enzymes have, in common with some other proteolytic enzymes, esterolytic activity directed towards certain specific synthetic substrates (e.g. Webster and Pierce, 1961). Hamberg and Rocha e Silva (1957) showed that heating of snake venom brought about a rapid destruction of the proteolytic activity towards casein, whereas the bradykinin-releasing activity and the esterolytic action on benzoyl-arginine methyl ester decreased much more slowly and in parallel. Holtz, Raudonat and Contzen (1960) found that on dialysis of snake venom, the total proteolytic activity was retained by the membrane, while material possessing both esterolytic and bradykinin-forming activity passed through. This and similar circumstantial evidence led Elliot (1963) to suggest that an ester bond was the most likely link between the polypeptides and the rest of the kininogen molecule.

Habermann and his colleagues (Habermann and Blennemann, 1964 (a), (b); Habermann and Müller, 1966) have since shown that,

in bovine plasma kininogen at least, it is a peptide link that is cleaved in kinin formation. Habermann et al divided kinin-forming enzymes into two groups, the K-9 enzymes, including crotalus venom, trypsin and pig plasma kallikrein, forming the nonapeptide; the K-10 enzymes, including pig pancreatic kallikrein, forming the decapeptide. They concluded that bradykinin and lysyl-bradykinin came from the same kinin-yielding group of the kininogen molecule, since K-9 enzymes incubated with purified kininogen also utilised the substrate for K-10 enzymes, and vice versa. They found that if the kininogen was partially degraded with pepsin, not only could the total potential kinin still be released by subsequent incubation with K-9 enzymes, but that the potential kinin resided in only two peptides formed by the peptic degradation, "PKFL" and "PKFS". Further analysis showed that PKFL has the structure:

NH_2

Met.Lys-Bradykinin-Ser.Val.Glu.OH

and this structure was confirmed by synthesis of the peptide. PKFS was not a homogeneous peptide, but in fact two peptides, one probably having the structure:

NH_2

Met.Lys-Bradykinin-Ser.Val.Glu.Val.

and the other:

NH_2

Met.Lys-Bradykinin-Ser.Val.Glu.Val.Met.

Synthetic PKFL was shown to be a substrate for all kinin-releasing enzymes tested. Further studies showed that met.lys.bradykinin is not an intermediate in the formation of lysyl-bradykinin by K-10 enzymes and is unlikely to occur naturally. Kinin release by K-10 enzymes is the result of their endopeptidase activity. Likewise, lysyl-bradykinin is not an intermediate in kinin release by the K-9 enzymes. Habermann and his colleagues concluded that in the intact kininogen molecule (Fig. 2) the K-10 enzymes act at peptide bonds (a) and (c). Although in small peptide fragments bonds (a) and (b) are relatively resistant to K-9 enzymes, in the intact molecule, K-9 enzymes act directly at peptide bonds (b) and (c).

It should be added that mixtures of both the nona- and deca-peptides are formed when K-10 enzymes - including pancreatic, salivary and urinary kallikreins - are incubated with pseudoglobulins or with plasma (Elliott, 1963; Webster and Pierce, 1963); while trypsin and snake venoms release only the nonapeptide. These findings are accounted for by the fact that although most glandular kallikreins are capable of forming only the decapeptide, plasma and pseudoglobulin preparations contain an aminopeptidase, capable of converting the decapeptide to the nonapeptide by removal of the N-terminal lysine (Erdös, Renfrew, Sloane and Wohler, 1963; Webster and

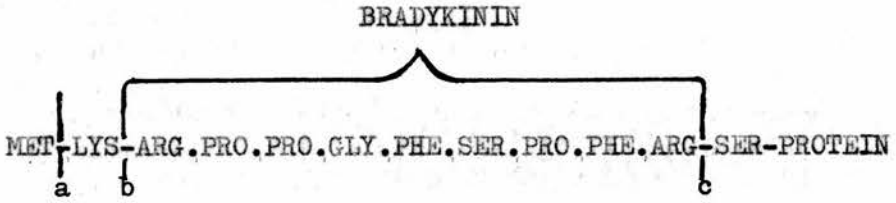

FIG. 2

The kininogen molecule showing points of enzymic attack

Pierce, 1963; Oates, Pettinger and Doctor, 1966). Trypsin and snake venoms normally act on the lysyl-arginyl bond to form the nonapeptide directly, they are themselves capable of converting lysyl-bradykinin to bradykinin (Webster and Pierce, 1963; Habermann and Blennemann, 1964 (a) and (b)).

KININ-RELEASING ENZYMES

1. Kallikreins

Many aspects of the kallikreins have already been considered in other sections. The kinin-releasing enzyme, kallikrein, was originally thought to be a single enzyme, formed only in the pancreas, released into the bloodstream and excreted in the urine (Frey, Kraut and Schultz, 1930). It is now known that this is not the case. As has already been discussed, plasma contains at least two types of kinin-forming activity. Kinin-releasing activity is also found in many other body fluids and secretions, including saliva (Hilton and Lewis, 1955), tears (Lewis, 1959), sweat (Fox and Hilton, 1958), lymph (Edery and Lewis, 1963) and cerebrospinal fluid (Chapman and Wolff, 1958).

Not only are there many sites of occurrence of activity, but there is more than one distinct kinin-forming enzyme in any one species so far examined. Thus in man, urinary and pancreatic kallikreins have molecular weights of 40,500 and 31,200 respectively, and migrate at different rates during electrophoresis in cyanogum gel (Moriya, Pierce and Webster, 1963). Furthermore, the enzyme in human plasma is inhibited by soybean trypsin inhibitor (SBTI) which does not affect the kinin-forming enzymes in human pancreas or urine; while egg-white trypsin inhibitor inhibits the urinary enzyme while being ineffective on

that of the pancreas (Webster and Pierce, 1963). Similar findings in the pig are reported by Trautschold, Fritz and Werle (1966), who conclude that the four kallikreins from pancreas, submandibulary gland, urine and serum are different as regards:-

(i) behaviour during purification;

(ii) electrophoretic mobility;

(iii) stability;

(iv) proteolytic activity;

(v) kinin-forming activity;

(vi) esterolytic activity;

(vii) biological activity and destruction *in vivo* and *in vitro*;

(viii) immunological properties;

(ix) inactivation by natural inhibitors.

2. Plasma Kallikreins

Normal plasma contains little or no detectable kinin-forming activity. Plasma kallikreins occur as inactive precursors or kallikreinogens. Activation of plasma kallikreinogens has been described after the following treatments: acidification of serum with HCl to pH 2.0 at 0°C (Kraut, Frey and Werle, 1933); incubation with trypsin or papain (Werle, 1955); exposure of serum

to 20% acetone for four hours (Webster and Pierce, 1960); treatment with chloroform (Lewis, 1958); contact with glass (Armstrong, Jepson, Keele and Stewart, 1957); and dilution with physiological saline (Schachter, 1956).

The exact mechanism of kallikrein activation and the nature of kallikreinogen have not yet been finally elucidated. It is probable that normally, the plasma kallikreins are inhibited by specific inhibitors. Frey, Kraut and Bauer in 1927, found an inhibitor of kallikrein hypotension present in human blood (Frey, 1927; Kraut, Frey and Bauer, 1928). Human blood has since been shown to contain several proteinase inhibitors, inhibiting a number of proteolytic enzymes with varying degrees of specificity (Trautschold, Werle and Zickgraf-Rüdel, 1967) including trypsin, chymotrypsin, plasmin, and the kallikreins found in plasma, glands and urine. These inhibitors are all polypeptides of molecular weight 6,000 - 14,000, and have been found at most of the sites where proteolytic enzymes occur. In mammalian plasma, at least two specific kallikrein inhibitors have been found (Trautschold, Fritz and Werle, 1966). Both inhibitors migrate with the α-globulins during electrophoresis, one a little faster than the other. Kinetic studies on inhibitor-kallikrein complexes show that they readily dissociate at acid pH (Trautschold et al, 1966), possibly

a factor in acid-activation of kallikreins. It may be that dissociation of such complexes is also effected by the presence of certain organic solvents and by hydrolytic enzymes such as plasmin and trypsin.

3. Activation of Plasma Kallikreins

Activation of plasma kinin release by contact with glass (Armstrong et al, 1957) has been studied in detail (Keele, 1960; Eisen, 1963; Margolis, 1963; Margolis and Bishop, 1963; Vogt, 1966). Margolis noted the similarities between the action of glass in initiating blood-clotting and activating plasma kinin release. He suggested (Margolis, 1957) that glass contact initiates clotting by activating a heat-labile precursor, component A, which then interacts with plasma thromboplastin antecedent which reacts with other factors to form thromboplastin. He considered that similarly activated component A also reacted with a postulated component B, a cofactor necessary for kinin-release, and with kininogen to form kinin. When component A was activated, component B became used up. Activated component A appeared to be a protease.

Margolis found that plasma from patients with Hageman trait, which does not clot normally on contact with glass, also did not release kinin. He found that Hageman trait plasma contained both components A and B. He concluded that a further cofactor,

the Hageman factor, was missing from this plasma. Glass carrying adsorbed Hageman factor from normal blood was capable of activating Hageman-deficient plasma. Margolis' studies showed (1963) that Hageman factor was adsorbed by negatively charged surfaces (e.g. glass, kaolin, alumina) and its physical state was altered so that it became capable of activating component A. From further studies (Margolis and Bishop, 1963) it seems probable that component A is a plasma kallikrein and component B is a plasma kininogen, identical with the kininogenase II and kininogen II of Vogt (1966) (see Fig. 1).

Activation of plasma by dilution to form plasma kinin (Schachter, 1956) also depends on the presence of activated Hageman factor and the component B kininogen (Keele, 1960), and appears to be due to the same enzyme as glass activation (Eisen, 1961). Dilution of plasma without contact with a suitable glass surface does not produce activation (Margolis, 1963). Dilution seems merely to render plasma more sensitive to other activating factors.

The circumstantial evidence put forward by Vogt (1966) for the existence of two plasma kallikrein-kininogen systems has already been mentioned. More direct evidence for the existence of two plasma kallikreins has been provided by the demonstration that human plasma contains two permeability globulins (Kagen, Leddy and Becker, 1963; Kagen, 1964).

Both globulins cause increased vascular permeability on intradermal injection; both are esterases, splitting the synthetic ester TAMe (p-toluene sulphonyl-L-arginine methyl-ester); both can be inhibited by proteinase inhibitors; and both can act as kinin-releasing enzymes. One of the proteins travels during electrophoresis as a γ-globulin, while the other travels as a slow β- globulin. The properties of the γ-globulin permeability factor have been confirmed in guinea pig plasma by Davies and Lowe (1963, 1967). They further found that the γ-globulin kallikrein could be isolated in the inactive form by DEAE-cellulose chromatography, and could then be activated by contact with Hageman factor coated glass beads.

4. Plasmin

Plasminogen, the enzyme precursor of the blood fibrinolytic agent, plasmin, has been identified as a β-globulin (Robbins and Summaria, 1963), with a molecular weight of 143,000 (Shulman, Alkjaersig and Sherry, 1958). It is precipitated in Cohn fraction III (Cohn, Strong, Hughes, Mulford, Ashworth, Melin and Taylor, 1946). Plasma kallikrein activity shows, in some respects, more resemblance to plasmin than to the glandular and urinary kallikreins. Unlike the latter, both plasmin and plasma kallikrein are inhibited by SBTI, and are extremely resistant to acidity (Eisen, 1963). Plasminogen is converted

to plasmin spontaneously (Mole, 1948) and by a variety of agents. These include trypsin, urokinase, and streptokinase (Alkjaersig, Fletcher and Sherry, 1958); activators in many mammalian tissues (Astrup and Sterndorf, 1956 (a), (b)); incubation with chloroform and other organic solvents (Denys and Marbaix, 1889; Christensen and Macleod, 1945); and contact with wettable surfaces (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961).

Activation of plasminogen is thought to occur through the activation of Hageman factor (Iatridis and Ferguson, 1962). Haanen, Hommes, Benroad and Morselt (1960) and Mackay, Maycock and Combridge (1962) showed that this factor could be adsorbed on glass powder or celite and eluted with concentrated saline, or alkaline saline. The eluate activated purified plasminogen (Mackay et al, 1962).

The release of plasma kinin by plasmin was first described by Beraldo (1950) who found that crude preparations of bovine "fibrinolysin" released kinin from plasma. Human plasmin released kinin from dog pseudoglobulin (Lewis and Work, 1957; Lewis, 1958) and pre-active human plasma (Eisen, 1961). This was prevented by an "antiplasmin" and SBTI (Lewis, 1958; Schachter, 1960).

The demonstration of differences between plasmin and plasma kallikrein has been attempted in various ways. Webster and Pierce

(1960) showed that the enzymes are inhibited to a differing extent by the same inhibitors, acetone treatment of plasma in fact destroying plasma fibrinolytic activity whilst activating kinin release. Similar results were obtained by Eisen (1963), who compared different methods of activation and inhibition on the two types of activity. Thus in whole plasma, silica surfaces greatly promoted kinin formation and only slightly increased fibrinolysis. Fibrinolytic activity was precipitated mainly in the euglobulin fraction, while most of the kinin-forming activity remained in the supernatant. Acid and acetone treatment destroyed fibrinolytic and casein-digesting activity and stimulated kinin formation. When both the substrate and kinin-forming activity were depleted in plasma by massive contact with glass, fibrinolytic activity was not affected. Streptokinase promoted greater fibrinolysis and less kinin release than did contact activation. Even when maximum plasminogen activation had been achieved, silica contact could promote further kinin formation. Finally, the inhibitor of plasminogen activation, -amino caproic acid (EACA) inhibited fibrinolysis but not contact kinin formation.

The two enzymes cannot be completely distinguished however. Although some workers have found that a plasmin preparation, whilst highly fibrinolytic, did not release kinin from kininogen <u>in vitro</u> (Bhoola, Calle and Schachter, 1960), more recently other

workers have shown that plasmin can release kinin from kininogen without the presence of kallikrein even as an impurity (Eisen, 1964; Henriques, Lavras, Fichman and Picarelli, 1966). Vogt (1964) has shown that plasmin is capable of activating plasma kallikrein, and it at present seems most probable that, while plasmin can release plasma kinin directly, its main plasma kinin-releasing action is through the activation of plasma kallikrein.

5. Trypsin

Trypsin, a pancreatic proteolytic enzyme, was obtained in crystalline form by Kunitz and Northrop (Northrop, Kunitz and Herriott, 1948); its molecular weight is about 24,000 (Cunningham, 1954). The enzymic attack on proteins such as casein is optimal near pH 8, but the enzyme remains active at pH values as low as 6. Trypsin is an endopeptidase which will break peptide bonds in simple synthetic substrates. Bergmann and his colleague (Hofmann and Bergmann, 1939, 1941) concluded that the specificity of trypsin is directed to the hydrolysis of peptide bonds to which an L-arginine or L-lysine residue contributes the carbonyl group. Suitable synthetic substrates are benzoyl-L-argininamide and benzoyl-L-lysinamide. Trypsin can also act at ester linkages provided that the other specificity requirements of the enzyme are met (Neurath and

Schwert, 1950). Thus trypsin hydrolyses benzoyl-L-arginine methyl ester (BAMe). The specificity of trypsin for bonds involving an arginine or lysine residue applies also to protein substrates.

Trypsin has been used as a model for kinin-releasing enzymes since the discovery of bradykinin by Rocha e Silva, Beraldo and Rosenfeld (1949). The incubation of trypsin with human plasma (Webster and Pierce, 1963), or ox pseudoglobulin (Elliott, Horton and Lewis, 1961) releases only bradykinin. When trypsin acts on the kininogen molecule, it breaks the lysine-arginine and arginine-serine bonds of the bradykinin containing sequence (Fig. 2), to form bradykinin directly. It acts at the lysine-arginine link to convert methionyl-lysyl-(Habermann, 1966) and lysyl-bradykinin (Webster and Pierce, 1963; Habermann, 1966) to bradykinin. However, this process takes place at a very much slower rate with the peptides than with the kininogen molecule, and for this reason these peptides cannot function as major intermediates in the formation by trypsin of kinin from kininogen.

Glandular kallikrein and contact-activated plasma kallikrein each activate only part of the total plasma kininogen, while trypsin appears to act on the substrates for both kallikreins and thus to release the total potential kinin (Margolis and Bishop, 1963; Habermann, 1966; Vogt, 1966). Heat denaturation of kininogen does not alter the total amount of kinin which can

be released by trypsin (Van Arman, 1955; Briseid, Dyrud and Rinvick, 1966), and this forms the basis of a method for the assay of plasma kininogen (Diniz, Carvalho, Ryan and Rocha e Silva, 1961; Diniz and Carvalho, 1963) discussed more fully in a following section (P. 56).

6. Snake Venoms

Bradykinin was discovered in the course of experiments on the physiological action of the venom of Bothrops jararaca (Rocha e Silva et al, 1949). Rocha e Silva and his colleagues found that blood samples taken from a dog after injection of minute doses of the venom had a stimulating effect upon the isolated guinea pig ileum. Their subsequent work led to the characterization of the principle which they called bradykinin. The venoms of two other serpents, Denisonia superba and Naia naia, were much less potent than Bothrops venom in releasing bradykinin, and this activity paralleled their relative proteolytic activities on casein and globulin.

However, when the kinin-forming enzyme from Bothrops was purified by selective denaturation and ammonium sulphate fractionation, it was found that non-specific proteolytic activity decreased with purification (Henriques, Fichman and Beraldo, 1960). A similar finding was also reported by Holtz, Raudonat and Contzen (1960), who found that on dialysis of snake

venom, the total proteolytic activity was retained by the membrane, while material possessing both esterolytic and bradykinin-forming activity passed through.

Using the venom of Agkistrodon halys blomhoffii, Suzuki and his colleagues (Sato, Iwanaga, Mizushima and Suzuki, 1965) were able to further fractionate its arginine-ester-hydrolase activity into three distinct enzymic entities, bradykinin-releasing, blood clotting and capillary permeability increasing. The bradykinin-releasing activity was associated with only five per cent of the total arginine-esterolytic activity of the venom, and was not inhibited by Trasylol nor the soybean or egg white trypsin inhibitors (Suzuki, Iwanaga, Nagasawa and Sato, 1966).

KININ DESTROYING ENZYMES

1. Chymotrypsin

During investigations on endopeptidases, Bergmann and Fruton (1941) observed that chymotrypsin hydrolyses peptide or ester linkages from the carboxyl groups of phenylalanine or tyrosine. Subsequent studies (reviewed by Desneulle, 1960) showed that chymotrypsin not only splits the carboxyl links of aromatic amino-acids, but also of leucine, methionine, asparagine and glutamine. Chymotrypsin occupies an intermediate position between the non-specific pepsin and trypsin which splits exclusively the bonds of basic amino acids.

In the presence of chymotrypsin, bradykinin loses its biological activity (Rocha e Silva, 1955) and is split into three parts. These have been identified as Arg.Pro.Pro.Gly.Phe.Ala, Ser.Pro.Phe.Ala and arginine (Elliott, Lewis and Horton, 1960).

2. Trypsin

Trypsin is a highly specific endopeptidase, splitting peptide or ester links formed from the carboxyl groups of L-arginine or L-lysine (Bergmann and Fruton, 1951). Trypsin slowly converts methionyl-lysyl-bradykinin and lysyl-bradykinin to bradykinin (Elliott, Lewis and Smyth, 1963; Erdös, Renfrew, Sloane and Wohler, 1963; Nicolaides, DeWald and Craft, 1963; Habermann, 1966). This results in an increase of activity in most of the

biological actions. Bradykinin itself is stable in the presence of trypsin (Rocha e Silva, 1955) unless a high concentration of trypsin is used (Diniz and Carvalho, 1963). Inactivation by high concentrations of trypsin found by some workers was probably due to contamination with carboxypeptidase or chymotrypsin (Desnuelle and Rovery, 1961).

3. Carboxypeptidase B.

Two carboxypeptidases have been isolated from pancreatic secretions, carboxypeptidase A (Anson, 1937, (a), (b)) and carboxypeptidase B (Folk, 1956; Folk and Gladner, 1958 (a), (b)). Carboxypeptidase A acts on peptides containing carboxyl-terminal aromatic amino acids such as phenylalanine, tyrosine and tryptophan, or branched aliphatic amino acids such as leucine or isoleucine. The enzyme is completely inactive towards substrates containing C-terminal arginine or lysine (Smith, 1954).

Carboxypeptidase B acts uniquely on peptides containing arginine, lysine or orthnithine as the C-terminal group (Folk and Gladner, 1958). The enzyme is very effective in destroying the biological activity of bradykinin and lysyl-bradykinin, and is some 50 - 100 times more active than chymotrypsin (Erdös et al, 1963). Methionyl-lysyl-bradykinin is more resistant to the action of carboxypeptidase B, and higher concentrations of the enzyme are required for inactivation (Erdös and Yang, 1966).

Because of its specificity, carboxypeptidase B is particularly useful for characterizing naturally occurring bradykinin (Erdös et al, 1963).

4. Plasma Kininases

Plasma fractions containing kininogen are usually contaminated with kininase activity (Werle, 1955; Lewis, 1960; Habermann, 1963). Horton (1959) found that dog plasma kininase was inactivated by incubation at pH 2 and 37°C for twenty minutes.

Erdös and Sloane (1962) found kininase activity in the Cohn IV-1 fraction of human plasma. This enzyme is similar to pancreatic carboxypeptidase B, in that it hydrolyses synthetic esters and peptides containing arginine or lysine (Erdös and Yang, 1966), and can split the C-terminal arginyl bond common to both bradykinin and lysyl-bradykinin (Erdös and Sloane, 1962). However, plasma kininase is distinguished from carboxypeptidase B by the different susceptibilities of the two enzymes to inhibitors. Thus EDTA inhibits the plasma enzyme but not that from the pancreas, 6 M-urea similarly has no effect on the pancreatic enzyme but inhibits plasma kininase, while n-butanol potentiates the activity of the pancreatic enzyme while having no effect on the plasma enzyme (Erdös and Sloane, 1962; Erdös et al, 1963). Erdös and Sloane (1962) suggested the name "carboxypeptidase N" for the plasma enzyme.

Inhibition of human plasma carboxypeptidase with Mn^{++} ions reveals additional proteolytic activity (Erdös et al, 1963). This is due to an aminopeptidase which splits the N-terminal bond of lysyl-bradykinin, to form bradykinin and lysine (Erdös et al, 1963; Webster and Pierce, 1963; Oates, Pettinger and Doctor, 1966).

5. Intracellular Kininases

Kininase activity has been found in cell homogenates from gastric, duodenal, jejunal and colonic mucosa of rats and rabbits and from their striated muscle (Amundsen and Nustad, 1965). Kininase has also been found in hog and rat liver homogenates (Werle, 1955; Amundsen and Nustad, 1965) and in hog kidney homogenate (Hamberg and Rocha e Silva, 1954).

A continuous release of kininase has been noted during perfusion of guinea pig lung with Tyrode solution (Brocklehurst and Lahiri, 1962 (b)). The rate of release was not influenced by antigen-antibody reactions. The activity was destroyed by incubation at $56^{\circ}C$ for thirty minutes, and was not inhibited by SBTI.

A kininase has been described in haemolyzed human red blood cells (Erdös et al, 1963). The enzyme was distinguished from the kininase occurring in human plasma by their different sensitivities to inhibitors. The red cell enzyme is inhibited

by heavy metals including Zn^{++} and Co^{++} while the plasma kininase is not; the plasma enzyme is inhibited by ϵ - amino caproic acid and EDTA while the cellular enzyme is unaffected. The kininase was similarly distinguished from other enzymes which may occur in red cells. The enzyme probably splits off the N-terminal arginine of bradykinin.

ESTIMATION OF PLASMA KININ AND KININOGEN LEVELS IN MAN

Comparable estimates of blood levels of plasma kinin and kininogen have only been possible since a suitable standard became available in 1960 with the preparation of synthetic bradykinin (Boissonas, Guttmann and Jaquenoud, 1960). Since that time, many groups have published procedures for the assay of plasma levels of both kinin and kininogen. In general, groups have favoured estimation of either one or other of kinin or kininogen for the demonstration of kinin-release, and prior to the present work there was no published procedure for the estimation of both principles in a single blood sample.

1. Plasma Kininogen Estimation

The procedure most widely used and quoted is that of Diniz, Carvalho, Ryan and Rocha e Silva (1961; 1963). It is based upon the finding by Van Arman (1955) that denaturation of plasma kininogen in boiling dilute acetic acid does not affect the total amount of kinin that can be released on incubation with trypsin. These workers collected venous blood in a siliconed syringe moistened with heparin. After centrifugation in polythene or siliconed glass, 0.2 ml. of plasma was added to 1.8 ml. of 0.2% acetic acid, and heated at 100°C for thirty minutes. The denatured protein suspension was then neutralized

with N-NaOH and 0.5 ml. of 0.2 M-Tris buffer (pH 7.8) was added. After incubation with trypsin (200 µg./ml.), the bradykinin released was extracted with ethanol and assayed on the isolated guinea pig ileum against a standardized bradykinin preparation. These workers found that 1 ml. of human plasma contained potential kinin equivalent to 10.6 µg. of bradykinin. It should be noted that most other groups, using identical or slightly modified procedures, have found levels of kininogen in man much lower than those quoted by Diniz and his colleagues (see Table 2).

A second method for the estimation of kininogen in heparin treated plasma has been described (Brocklehurst and Lahiri, 1962; Lahiri, 1962). Protein precipitation by 80% ethanol at 0°C was followed by heating at 80 - 90°C for twenty minutes. The washed precipitate was incubated for thirty minutes with trypsin (500 µg./ml.) in 0.1 M-sodium phosphate buffer at pH 7.4. The trypsin was inactivated by heating and the kinin concentration determined by bio-assay. Apart from the values determined in the present work, there are no published values of kininogen levels in man as determined by this method. Guinea pig plasma contained 12.8 $\pm$ 1.9 µg. bradykinin equivalent (BK eq.) per ml. of plasma.

Although values for plasma kininogen levels in man have been

quoted by workers using other procedures, these have not been practicable assay techniques. Values (see Table 2) have been obtained as the result of the isolation of kininogen protein (Jacobsen, 1966), or by activation of plasma kallikreins in native plasma (Margolis, 1966; Vogt, 1966).

2. Plasma Kinin Estimation

All methods for estimation of free plasma kinin involve inactivation of plasma enzymes followed by extraction of the kinin activity for assay (Table 3). Three methods have been used for the inactivation of plasma enzymes; ethanolic denaturation (Brocklehurst and Lahiri, 1962; Allwood and Lewis, 1964; Oates, Melmon, Sjoerdsma, Gillespie and Mason, 1964); acid denaturation (Horton, 1964; Abe, Watanabe, Kumagai, Mouri, Seki and Yoshinaga, 1966); and the use of enzymic inhibitors (Webster and Gilmore, 1965). For bio-assay of the kinin, some workers have used the simple ethanolic extract (Brocklehurst and Lahiri, 1962; Allwood and Lewis, 1964), whilst others have carried out a secondary purification by extraction into butanol (Horton, 1964; Abe et al, 1966) or by ion exchange chromatography on Amberlite IRC-50 (Oates et al, 1964; Webster and Gilmore, 1965). Each of the techniques mentioned has been used on human blood samples.

Allwood and Lewis (1964) collected 10 ml. of venous blood in nylon syringes at 0°C and added it to two volumes of chilled ethanol. The samples were spun, and the supernatant fluid was dried *in vacuo* and assayed on isolated oestrus rat uterus, in the presence of 5-hydroxytryptamine (5-HT) and histamine antagonists. These workers found a mean normal value of 32 ng. BK eq. per ml. of whole blood (s.d. = ± 23). This relatively high value is possibly due to the presence in the extracts of intracellular K^+ (Lahiri, 1962), since Allwood and Lewis made no mention of the possibility of contamination of their extracts by this ion, nor did they characterize their assayed activity as kinin-like.

Using an adaptation of the method of Horton (1964) for the estimation of kinins in dogs' blood, Abe et al (1966) collected 10 ml. of venous blood in a chilled siliconised syringe containing 1 ml. of 0.03 M-EDTA. The sample was placed in a centrifuge tube containing 5 ml. of 0.8 N-HCl, shaken for a few minutes, adjusted to pH 2 with normal NaOH or HCl, and saturated with sodium chloride. After addition of 20 ml. of n-butanol, the mixture was shaken and spun. The butanol was removed and the aqueous phase was re-extracted with a second 20 ml. of butanol. The combined extracts were dried with 10 g. of anhydrous sodium sulphate and stored overnight at -20°C. After spinning, 4 ml.

of petroleum ether was added and the mixture was extracted with 4 ml. and then 2 ml. of distilled water. The combined aqueous extract was evaporated in a graduated tube to 0.6 ml., neutralized with 0.1 N-sodium hydroxide, made up to 2 ml. with water, and assayed on guinea pig ileum. The recovery of bradykinin was 51.3% ($\pm$ 10.5) and the plasma kinin level in ten subjects ranged from 0 - 2 ng. BK eq. per ml. of blood.

The inactivation of blood enzymes by inhibitors and kinin extraction using ion exchange were utilised by Webster and Gilmore (1965). These workers took 15 ml. of blood into a syringe containing 2.25 ml. M-phosphoric acid and 1.5 mg. SBTI giving a pH of 4 - 5. The volume was measured in a measuring cylinder and the sample was added to an equal volume of distilled water containing 100 mg. Amberlite IRC - 50(H^+). The suspension was adjusted to pH 4.0 with 10 M-formic acid and stirred at room temperature for one hour. The resin containing the adsorbed kinin was removed by filtration on sintered glass, washed with water, and placed in 2 - 3 ml. of 0.2 M-ammonium formate at pH 8.2. The mixture was adjusted to pH 8.5 - 9 with 10 M-NH_4OH, filtered, and the filtrate was adjusted to pH 8.2 using 10 M-formic acid. The volume was measured and the sample was assayed on isolated oestrus rat uterus. The bradykinin recovery for the procedure using human blood was 27 - 40%, and venous blood levels

were all less than 2.5 ng. BK eq. per ml.

All procedures for the assay of free kinins in human blood published to date consist of combinations of all or part of each of the three methods described above (Table 3).

3. Criteria for a Method for the Detection of Kinin-Release in Clinical Blood Samples.

Plasma kinin release may be provoked by dilution (Schachter, 1956), cooling (Diniz and Carvalho, 1963; Armstrong, Mills and Sicuteri, 1966; Melman and Cline, 1967), and contact with glass (Armstrong, Jepson, Keele and Stewart, 1957). Free kinin in plasma is rapidly destroyed by plasma peptidases (Erdös and Sloane, 1962), and its half-life in the circulation is less than 0.5 minutes (Saamelli and Eskes, 1962; McCarthy, Potter and Nicolaides, 1965). There is thus a great danger both of inadvertent kinin release and of the loss of free kinin already present in plasma during the preparation of blood samples prior to assay. This is reflected in the wide variation in values for the normal levels of both free plasma kinin and its precursor in man, obtained not only by groups using different methods, but even in many cases by different groups using the same method (see Tables 2 and 3).

Furthermore, the methods previously described for the estimation of plasma kinin and kininogen have required separate samples for the estimation of each substance, and immediate processing of the samples. Most methods for free kinin assay either require large volumes of blood, give low recovery, or are relatively unspecific. Because of these methodological considerations, there is as yet little undisputed evidence for the release of free plasma kinins in clinical situations, and there have been relatively few clinical studies.

It has been the aim of the work described in this thesis to devise and apply a method suitable for the detection of plasma kinin release in clinical situations. Such a method should satisfy certain criteria; these are as follows:

1. Standard sterile equipment must be used for blood sampling.
2. The clinician should not be involved in inconvenient manipulations, and the method should permit storage of the sample immediately after it has been taken until a suitable occasion for processing.
3. The method should permit detection of changes in both free plasma kinin and kinin precursor levels, and ideally should allow estimation of both

substances in a single blood sample.

4. The volume of blood required should be reasonably small.
5. The method should give high recoveries of free kinin and kininogen.
6. Substances present in normal or pathological samples which might interfere with the bio-assay of kinins must be removed as far as possible.

In addition, such a procedure should satisfy the general conditions necessary for the estimation of plasma kinins. These are:

7. After sampling, enzymes must be rapidly inactivated to prevent formation or destruction of free kinins as an artefact.
8. Contact between kinin and solutions in which hydrolysis may take place, should be kept to a minimum, and where storage for any length of time is necessary, it should be in a dry state if possible.
9. Bacterial contamination should be guarded against during any lengthy period in solution.

Authors	Kininogen level Bradykinin Equivalents (µg./ml. Plasma)	Methods and Comments
Diniz et al (1961)	10.6	Plasma denatured in hot dil. acetic acid. Trypsin activation. (10 males, 5 females).
Sicuteri et al (1962)	4.4	Method of Diniz et al (1961). (7 subjects).
Amundsen et al (1963)	1.5 - 6.0	Crude estimate from preparation of a stable kinin-forming substrate.
Fasciolo et al (1963)	6 - 10	Method similar to Diniz et al (1961).
Jacobsen (1966)	Kininogen I:- 0.5 - 1.0 Kininogen II:- 2.8 - 4.5	Crude estimate as result of gel filtration preparation of pure kininogen proteins.
Margolis (1966)	circa 4	Value arrived at during plasma kallikrein activation experiments.
Panuccio et al (1966)	2.9	Method of Diniz et al (1961). (7 subjects).
Periti and Gasparri (1966)	2.77	Method of Diniz et al (1961). (79 women).
Rinvik et al (1966)	5.5	Plasma denatured in hot dil. HCl. Trypsin activation. (11 males).
Trautschold et al (1966)	15 - 20	Method of Diniz et al (1961).
Vogt (1966)	6.2	Value arrived at during plasma kallikrein activation experiments.

Table 2

Values for the Normal Level of Plasma Kininogen in Man

Reference	Plasma Kinin BK Equivalents (ng./ml. whole blood)	Comments
Periti et al, (1963)	1.2	Ethanol extraction only (29 subjects)
Allwood and Lewis, (1964)	32 ± 23	Ethanol extraction only (20 subjects)
Webster and Gilmore, (1965)	< 2.5	Inhibition with SBTI and acid pH. Ion exchange purification.
Abe et al, (1966)	0 - 2	Inhibition with EDTA. Acid precipitation. Butanol extraction (10 subjects)
Oates and Melmon, (1966)	1 - 79 (Av. < 40)	Ethanol extraction. Ion exchange purification. (Oates et al, 1964). (15 subjects).
Zacest and Mashford, (1967)	Venous = 0.64 Arterial = 0.25 ± 0.23	Method of Oates et al (1964). (2 subjects for venous values; 18 subjects for arterial values).

Table 3

Values for the Normal Concentration of Free Plasma Kinin in Human Whole Blood

EXPERIMENTAL

I. EXPERIMENTS TO DEVISE AN ASSAY PROCEDURE FOR PLASMA KININ AND KININOGEN IN CLINICAL CONDITIONS

INTRODUCTION

Rocha e Silva and his colleagues (1949), when they first described bradykinin activity, found that it was soluble in 80 - 90% ethanol. In their first stage of purification of bradykinin from incubates of proteolytic venom or trypsin and ox serum, they used the addition of hot ethanol to precipitate the proteins, stop the enzymic reactions, and extract the released kinin. Stabilization and extraction by ethanol have since been used in free plasma kinin estimations (Brocklehurst and Lahiri, 1962; Allwood and Lewis, 1964). A preliminary consideration of the criteria (Page 61), pointed to the use of ethanol stabilization as the basis of a suitable procedure for the assay of plasma kinin in clinical samples. The blood would be taken into a disposable syringe and added to chilled ($4^{\circ}C$) ethanol (Appendix 2(a)). The resultant mixture would contain kininogen, other proteins, and cell debris in the precipitate, and kinin and other ethanol soluble substances in the supernatant.

At this point the procedure must differ from any previously described if a single sample is to be used for both free kinin and kininogen estimations. Furthermore the kinin must be concentrated and substances interfering with the bio-assay removed.

The experimental work involved in devising methods for the assay of free kinin and kininogen will be considered in separate sections.

EXPERIMENTS TO DEVISE A METHOD FOR THE SEPARATION OF BRADYKININ FROM SUBSTANCES INTERFERING IN THE BIO-ASSAY

1. Preamble

The ethanolic extract prepared from whole blood as described in Appendix 2 (a), will contain any kinin present, and other substances which would interfere with the bio-assay of kinins. K^+, a smooth muscle stimulant, which is released on lysis of blood cells, would be present in quantities sufficient to interfere with the assay of low levels of kinin. In addition, and particularly in pathological conditions, histamine, adrenaline, and 5-HT may be present in quantities large enough to interfere with the bio-assay.

2. Levels of K^+ in whole blood extracts

Lahiri (1962) found that the threshold concentration of K^+ producing contractions in the isolated rat uterus varied between 4 - 8 micro-equivalents (μ.Eq.) per ml. of bath fluid. His whole blood extracts from animal sources - guinea pig, rat, and rabbit - contained mean values of 19.8 μ.Eq., 17 μ.Eq. and 23.4 μ.Eq. K^+ respectively per ml. of blood extracted. Samples had routinely to be diluted to at least five times the original

blood volume before assay. Such a requirement greatly raises the threshold of the assay, and the lower limit of detection of free kinin described by Lahiri was of the order of 5 ng. BK Eq. per ml. of whole blood; assuming similar levels of potassium in man, this is equivalent to about 12 ng. BK Eq. per ml. of human plasma.

In order to compare the K^+ content of ethanolic extracts from human whole blood with those obtained by Lahiri, 5 ml. venous blood samples were taken from seven subjects, and extracts were prepared as described in Appendix 2 (a). The samples were reconstituted in 2 ml. of warm (60°) distilled water, and, suitably diluted, were assayed for K^+ content using the flame photometer. The results are given in Table 4.

A mean value of 136 µ.Eq. ($\pm$ 56 µ.Eq.) of K^+ was found to be contained in the ethanolic extract from 5 ml. of human blood (Lahiri's results give 99.0 µ.Eq., 85 µ.Eq., and 117.0 µ.Eq. per 5 ml. blood from guinea pig, rat, and rabbit respectively).

In addition 50 ng. of standard synthetic bradykinin was added to ethanol at the same time as 5 ml. blood samples, and the extracts were prepared as described. It was not possible to assay the added bradykinim, owing to the rapid contraction produced by the K^+ content of the extract, which generally fused with and masked the contraction produced by the bradykinin.

Sample	Potassium Concentration of Extract (μ.Eq./ml.)	Potassium Extracted from 5 ml. of blood (μ.Eq.)
1	50	100
2	57	114
3	70	140
4	30	60
5	83	166
6	67	134
7	120	240
Mean		136 (s.d.=56)

Table 4

The Potassium Content of Ethanolic Extracts from Human Whole Blood

Dilution of the sample in an attempt to remove the K^+ contraction diluted the bradykinin to sub-threshold levels.

Thus, the ethanol extracts of human blood contained levels of K^+ which interfered with the rat uterus bio-assay to such an extent that levels of free plasma kinin below 10 ng. BK. Eq. per ml. of blood (i.e. .20 ng./ml. of plasma) could not be assayed.

3. Bradykinin purification using column chromatography

A series of preliminary experiments was carried out in an attempt to find a suitable chromatographic procedure for the separation of bradykinin from potassium ions. Freeze dried samples were prepared containing standard synthetic bradykinin, and potassium chloride in the quantities described. These samples were dissolved in de-ionized water as required immediately before use, and used to test the efficiencies of the columns described below. Each column was tested twice unless otherwise stated.

4(a) Gel filtration

Sephadex is a modified dextran (Flodin, 1962). The linear macromolecules of the dextran are cross-linked to give a three dimensional network of polysaccharide chains. The substance is inert to anions and cations, and because it contains multiple hydroxyl groups, is strongly hydrophilic. When it is mixed with water or electrolyte solutions, it swells considerably, and when placed in a chromatographic column, acts as a sieve for molecules of different sizes.

At the time when this work was carried out, the Sephadex G-25 was the smallest pore size available. This gel excludes solutes with a molecular weight greater than c. 5000, and provides fractionation below this limit.

b) Sephadex G-25, and water eluent

Sephadex G-25 had previously been used by Pierce and Webster (1961) in the purification of bradykinin and lysyl-bradykinin from human plasma.

The Sephadex was prepared as recommended by the manufacturer. The gel (G-25, medium) was swollen for twenty-four hours in an excess of de-ionized water. Fine particles were removed by repeated washes in de-ionized water, until, after sedimentation of the gel, a clear supernatant was obtained. The gel was de-gassed under reduced pressure, and then packed in a siliconized glass column, 35 cm. x 5 mm. diameter. The column was run at 4°C. The flow rate was 0.12 ml. per minute, and fractions were collected at forty-five minute intervals (5.4 mls.) and after removal of 0.2 ml. for conductivity measurements, were immediately shell frozen and freeze dried. The column load consisted of 200 ng. bradykinin + 32 µg. KCl in 0.8 mls. de-ionized water, and elution was carried out with de-ionized water. For this, as for all columns in this section, conductivity measurements were taken as an estimate of the K^+ content of the fractions, and the evaporated fractions were dissolved in 1 ml. of de Jalon's solution and assayed on oestrus rat uterus for bradykinin content.

The elution pattern obtained is shown in Fig. 3 (a). The mean recovery of bradykinin was 37.9%, however, no separation was obtained.

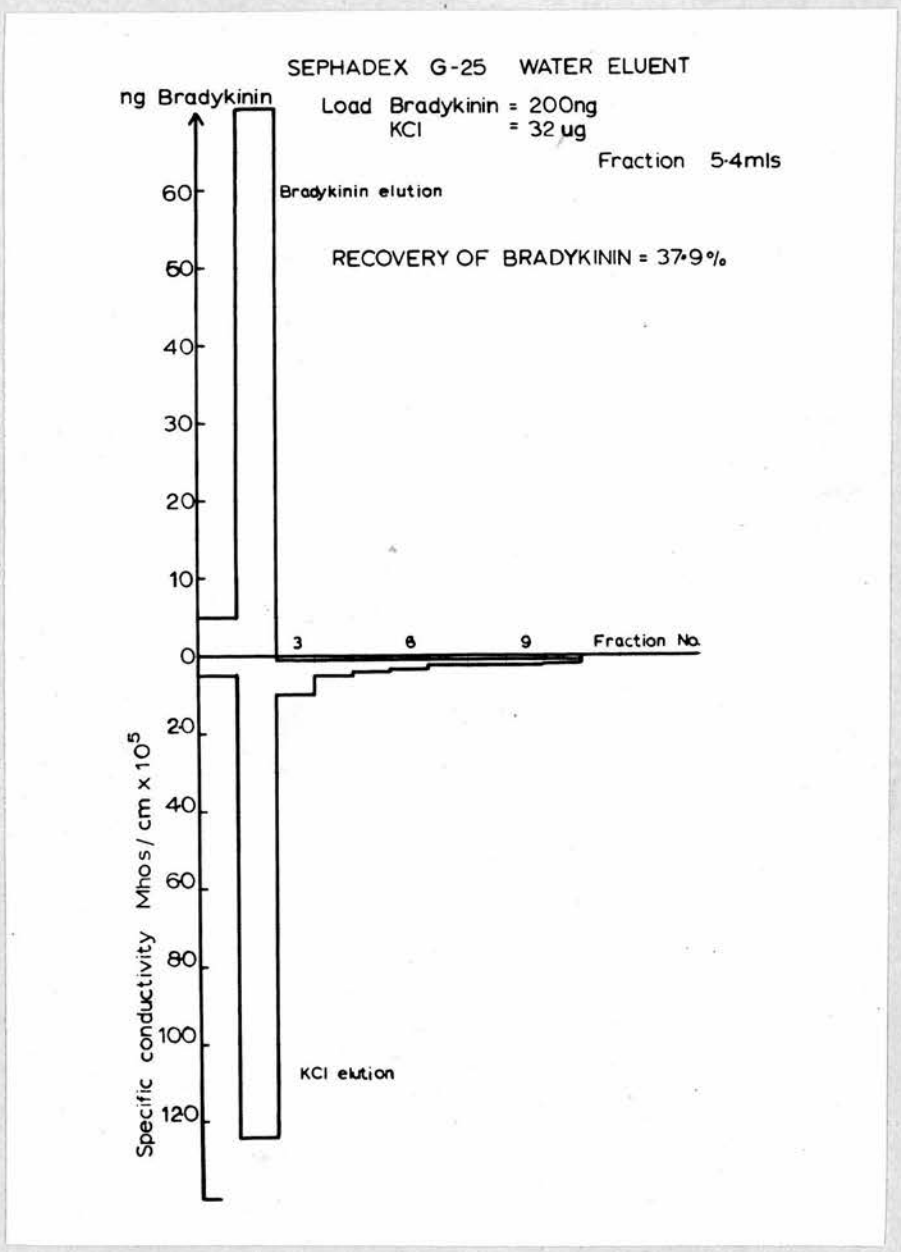

Fig. 3(a): The elution pattern of bradykinin and K^+ on Sephadex G-25 eluted with water.

c) Sephadex G-25 eluted with 75% (v/v) ethanol

The dextran gels are polar. They only swell in the presence of polar solvents such as water, dimethylsulphoxide, formamide, and glycol; while less polar water-miscible solvents such as ethanol and acetone effectively dehydrate water swollen gels (Flodin, 1961). Although Sephadex G-25 has been used to fractionate peptide mixtures containing from di-peptides to peptides with 19 - 22 amino acid residues, its selectivity is not high enough to separate the peptide hormones vasopressin and oxytocin from amino acids. The use of mixed organic/aqueous solvents has been utilized to reduce the swelling of the gel, and thus increase its selectivity for the purification of these hormones (Porath and Lindner, 1961; Porath, 1963).

The eluent chosen in the present experiment was 75% (v/v) glass distilled ethanol. This eluent was used both for its possible effects on the separation of BK from K^+, and also because it is a very powerful bacteriostat, and would thus reduce any bacterial destruction of the bradykinin. In addition, the less polar medium should cut down hydrolysis, if this is a factor in BK loss.

Sephadex G-25 (3 g.) was allowed to swell for twenty-four hours in a large excess of 75% ethanol. The supernatant was

discarded and replaced with fresh 75% ethanol, until, after stirring, no more fine particles appeared in the supernatant. The gel was then packed in a glass column, 20 cm. x 0.6 cm. diameter, which was run at 4°C. The mean flow rate was 0.1 ml./minute, and fractions were collected at twenty minute intervals (2 mls.). As each fraction was obtained, 0.2 mls. were taken for conductivity measurements, and the remainder was immediately evaporated at 25 - 30°C. and 690 mm. Hg. The evaporated fractions were dissolved in 1 ml. de Jalon's solution, and assayed for bradykinin content on oestrus rat uterus.

Of the two column runs, only one was carried out using a siliconized column and siliconized glassware. However, there was little difference in the recoveries, the "non-silicone" run gave a bradykinin recovery of 26%, while the "silicone" run gave a recovery of 27%. The true recovery of these columns was probably greater than that measured, because although separation was obtained (Fig. 3 (b)), there was considerable overlap between the bradykinin and K^+ peaks, and some of the bradykinin containing fractions were not assayable owing to their potassium content.

A siliconized column of double the length (40 cm.) was also set up in an attempt to improve the separation, and thus increase

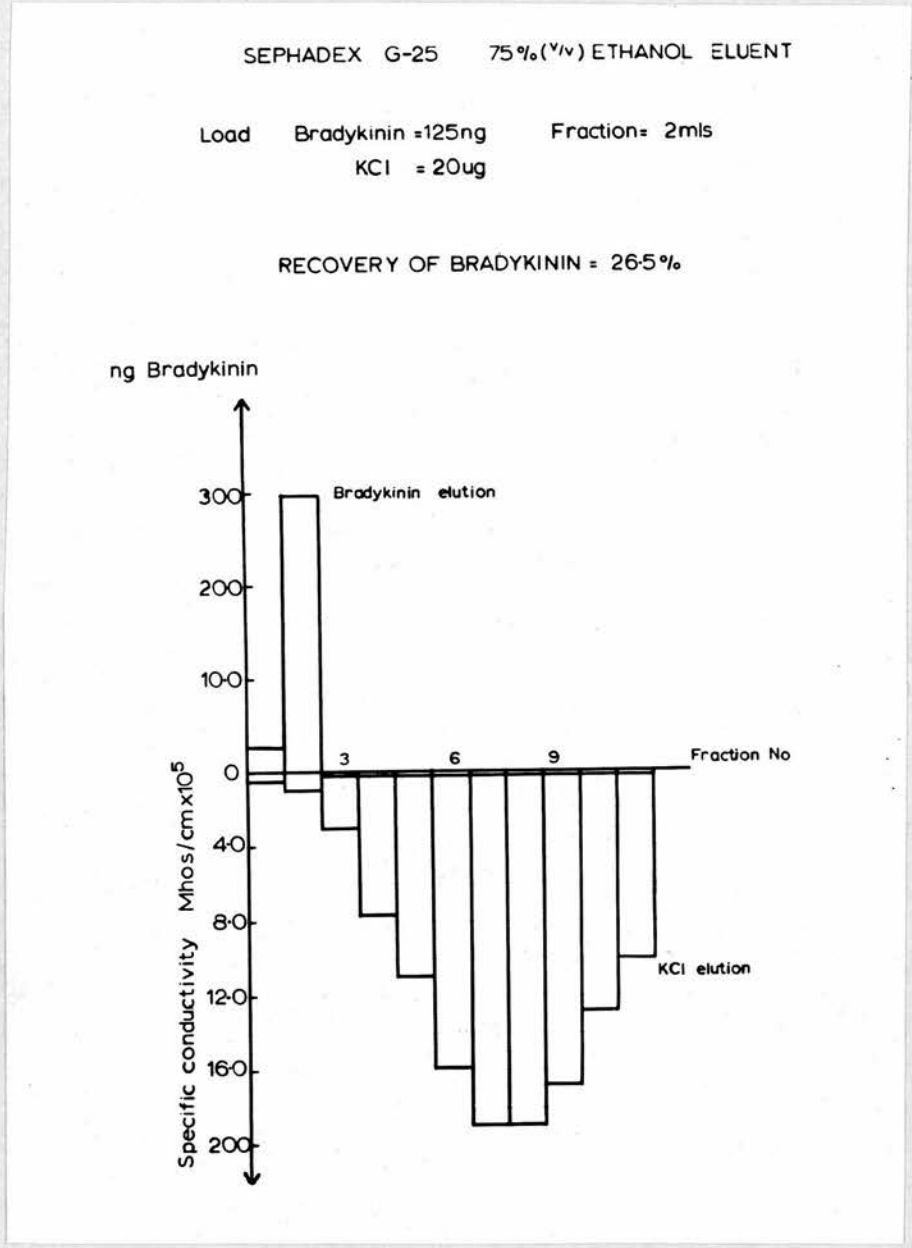

Fig. 3(b): The elution pattern of bradykinin and K^+ on Sephadex G-25 eluted with 75% (v/v) ethanol.

the recovery. The recovery of this column was still only 37%.

5. Ion retardation resin

Ion exchange resins have long been in use for the preparative purification of kinins (e.g. Andrade and Rocha e Silva, 1956; Pierce and Webster, 1961), however the recovery of such procedures has been low. Even in a recently published method for the assay of plasma kinins using the cation exchange resin IRC-50 (Webster and Gilmore, 1965), the recovery was only 27 - 40%, and the authors had to use a blood sample of at least 15 mls. The low recovery from such resins may be due to irreversible adsorption of the peptide, or in many cases it may be the result of increased hydrolysis caused by elution at an alkaline pH.

A recently available ionic resin, an "ion retardation" resin (Rollins, Jensen and Schwartz, 1962) produced commercially as AG11A8, functions on a principle different from that of the conventional ion exchange resins. The resin is made by polymerizing acrylic acid inside Dowex 1. This gives a styrene-divinylbenzene cross-linked polymer lattice with attached quaternary ammonium groups, within which weaves the acrylic acid polymer having carboxyl groups. Each resin bead is a self-neutralized mixture of anion and cation exchangers, and thus associates weakly with both anions and cations in equivalent

amounts. When an aqueous solution containing strongly ionized inorganic salt, and little or non-ionized organic solutes is passed through a column of the resin, the flow of inorganic salt is retarded and appears in a separate fraction following the organic material. The resin is eluted at neutral pH, thus reducing the risk of kinin hydrolysis present with the alkaline elution of cation-exchange resins.

The resin was used as described in the commercial literature. Three grams of resin in de-ionized water was packed in a siliconized glass column, 5 cm. x 1 cm. diameter. The load consisted of 125 ng. BK + 20 µg. KCl in 1 ml. de-ionized water. The column was eluted with de-ionized water at 4°C with a flow rate of 0.33 ml./minute, and fractions were collected at six minute intervals (2 ml.). Conductivity measurements and, after storage at -20°C, assay on rat uterus were carried out as previously described. Separation between ion and bradykinin peaks was not obtained (Fig. 3 (c)). The K^+ level was low enough to allow assay of all fractions. The mean recovery was 2%.

6. Conclusions from chromatography experiments

None of the methods tested was satisfactory. The most promising was gel filtration on Sephadex G-25 eluted with 75% aqueous ethanol. Plasma kinin is obtained in the first instance

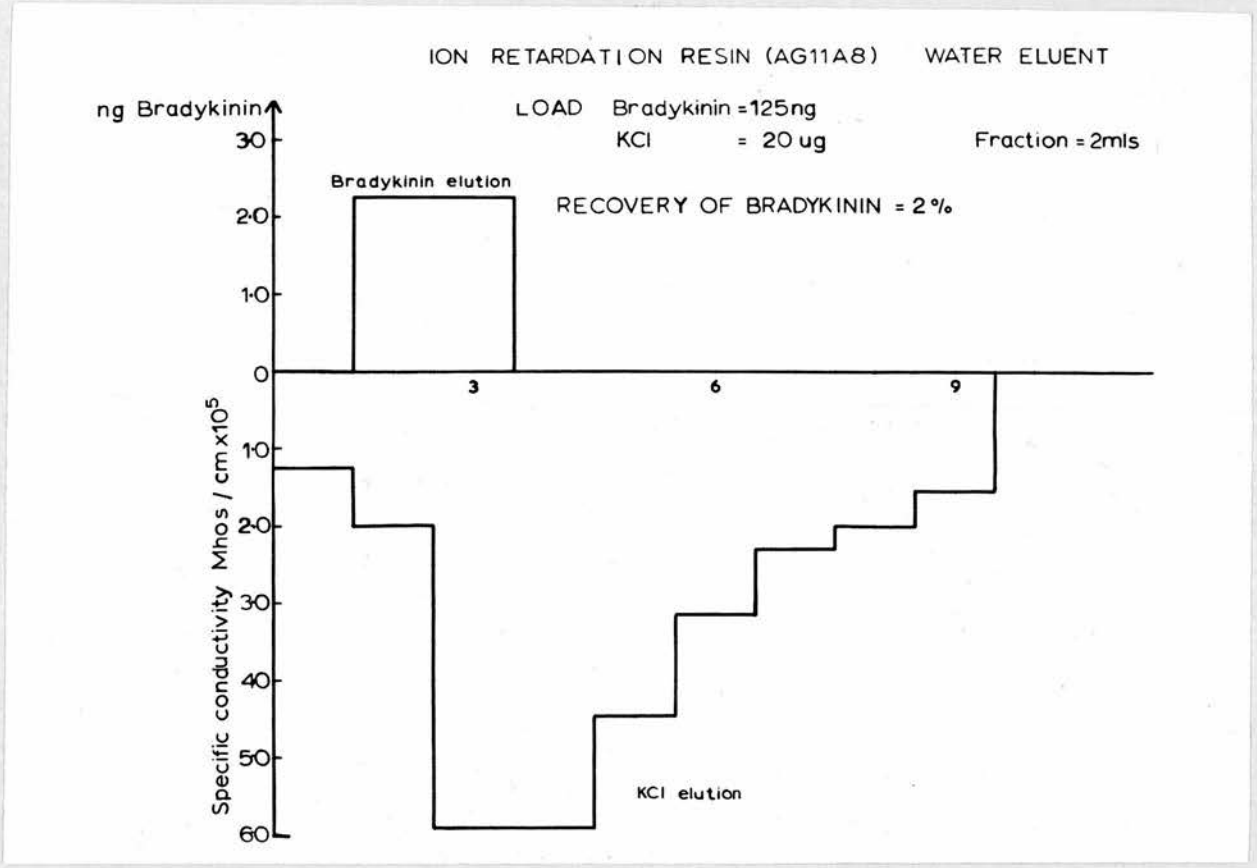

Fig. 3(c): The elution pattern of bradykinin and K^+ on the ion retardation resin, AG11A8, eluted with water.

as an extract in 75 - 80% ethanol, and the use of such a column might have permitted a single step purification. This would have been an asset, since ease of operation and the time involved are vital factors in routine assay procedures. However, the best recovery obtained was only 37%, and fairly large volumes of blood (15 - 20 ml.) would probably be required for the assay of low levels of free kinin.

7. Butanol extraction of free kinin

A butanol extraction procedure was first used for an extraction of kinin from urine by Gomes (1955). Gomes' procedure was a modification of the method employed by Clark et al (1954) for the purification of "hypertensin" by extraction into butanol from a salt saturated acidified (pH 1.5) aqueous solution. Clark's procedure required extraction of the peptide three times with 0.25 vol. butanol, however, Gomes found that this gave only 60% recovery. He found that a single extraction with an equal volume of butanol gave a recovery of practically 100% of the oxytocic activity in urine. Gomes recovered the active material from the butanol extract by precipitation with five volumes of ethyl ether.

Gomes' method for the extraction of kinin peptides from urine was further modified by Gaddum and Horton (1959). Using the method exactly as described by Gomes, they found that the

recovery of oxytocic activity from urine varied from 0.5% to only 10%, in contrast to the 100% reported by Gomes. These workers concluded that ether precipitation was an inefficient method of recovering the activity from butanol. They found that 80 - 90% of the activity could be recovered directly from the butanol by evaporation to dryness under reduced pressure at 50°C.

The conditions of acid pH and salt saturation in the aqueous phase, necessary for quantitative extraction of the peptide into the butanol, were arrived at fairly empirically by Clark and his co-workers (1954). Gomes found that both acidification and salt saturation were necessary for the passage of bradykinin-like activity into the butanol. Neither Gomes, nor Gaddum and Horton discuss the efficiency with which such an extraction might exclude substances interfering with kinin bio-assay, since the nature of their investigations made this unnecessary. Consideration of the conditions of the butanol extraction indicated that it might suit the requirements of the present investigation. Inorganic ions such as K^+ and Na^+ are very insoluble in butanol. Bases with high pK's would tend to be highly ionized at pH 1.5, (see Appendix 5) and may be expected to remain in the aqueous phase. The pK_a of the side chain amino group of histamine is 9.7 (Levy, 1935), that of adrenaline is 9.90 (Barlow, 1964), and that of 5-HT is 10.0 (Vane, 1959). These compounds should therefore remain in

the aqueous phase during the butanol extraction.

In early experiments in which the assay of plasma kinins was attempted, the ethanol extract, prepared as already described (Appendix 2 (a)), was reconstituted in 10 ml. de-ionized water. Extraction of kinin from this solution into butanol was then carried out almost exactly as described by Gaddum and Horton (1959). This involved acidification of the solution to pH 1.5 with hydrochloric acid, and saturation with sodium chloride. This was followed by extraction with 10 ml. freshly distilled n-butanol. The butanol was then evaporated at 60°C at the water pump (60 mm. Hg.). This procedure gave biological assays denoting impracticably variable recoveries (45% - 75%) when synthetic bradykinin (250 ng.) was added to the ethanol at the same time as 5 ml. of blood.

Isolated rat uterus was found to be very sensitive to low concentrations of butanol; a concentration of $1:10^4$ in contact with the tissue for two minutes was enough to reduce contraction size to a given dose of bradykinin by 50%, while continual presence of this concentration in the bath fluid was enough to inactivate the tissue completely. Subsequent experiments indicated that in the extract evaporated as above, there was frequently sufficient butanol or its high boiling oxidation products remaining to interfere with the bio-assay. This trace could be removed by a final five minutes drying, using a vacuum

pump at 0.1 mm. Hg. or less, and 80°C. In addition, it was found that higher recoveries were obtained if the kinin in the aqueous phase was extracted in two stages, first with 10 ml. butanol and then with 5 ml. The final procedure is described in Appendix 2.

8. Recovery of bradykinin

The efficiency of extraction into ethanol, followed by re-extraction into butanol (Appendix 2 (a) and (b)) was estimated by measuring the recovery of added synthetic bradykinin. From each subject, two 6 ml. samples of venous blood were taken through the same needle into two 10 ml. syringes. 5 ml. of each blood sample were inactivated in ethanol, and treated as already described. While one of the two samples was being injected into ethanol, a known amount of bradykinin, contained in 0.25 ml. or less of distilled water, was added to the mixture. The quantities of bradykinin added, ranged from 10 ng. per ml. of blood, to 50 ng. per ml. of blood (See Table 5). The bradykinin solution was not placed in the sample syringe (see for instance Allwood and Lewis 1964; Webster and Gilmore 1965) prior to drawing the blood because of the danger of activation of kinin release by dilution. Although the volume of 0.1 ml. of added solution is small relative to the 5 ml. of blood, the first drops of blood to enter the syringe would be greatly diluted, and as

Subject	Amount of Added BK (ng. per 5 ml. blood)	Kinin Content (ng. BK Eq. per 5 ml. Blood)		Recovery %
		Control Sample	Recovery Sample	
1	50	<1.5	37.7	72.4
2	100	<1.5	75.0	73.5
3	100	<1.6	90.0	88.4
4	200	18.0	200.0	91.0
5	250	4.3	222.2	87.2
6	250	75.0	256.0	72.4
7	250	6.0	226.7	88.3
8	250	5.0	202.0	78.8
Mean				81.5 (s.d. = 8.0%)

Table 5

The Recovery of Added Bradykinin following Ethanol and Butanol Extractions

(Note: Subject (6) fainted while sampling was being carried out)

little as 0.1 ml. of human blood contains quantities of kinin precursors of the order of 300 ng. bradykinin equivalent. Three recovery estimates carried out in this way, in which 50 ng. of bradykinin in 0.1 ml. saline were placed in the syringe prior to drawing the blood, gave absurdly high recoveries (1,500%, 1,800% and 3,010%).

In order to simulate conditions which might arise in routine use, the sample was stored at 4°C for twelve hours, after the blood had been inactivated in ethanol, to await further processing. The samples were otherwise purified and assayed as described in Appendix 2. The total kinin content of the control extract was subtracted from the kinin content of the recovery sample, and the percentage recovery was calculated. When the control sample contained no detectable free kinin, a control value was calculated from the limit of detection of the bio-assay, such a procedure minimizing the danger of calculating a falsely high recovery value.

The results are given in Table 5. The recovery of added BK was found to be 81.5% (s.d. = 8.0%).

9. <u>The exclusion of potassium ions by butanol extraction</u>

Experiments were carried out to estimate the efficiency with which the kinin extraction procedure (Appendix 2 (b)) excludes the

K^+ ions of whole blood, and to give an estimate of the normal range of K^+ concentration in the final butanol extract. Samples were taken from five healthy volunteers. Two 5 ml. samples of venous blood were taken from each individual, and processed as described in Appendix 2 (a), to give dried ethanolic extracts. For each subject, one of the samples was left as the dry ethanol extract, while the other sample was processed further as described in Appendix 2 (b) to give a dried butanol extract.

All of the extracts, both ethanolic and butanolic, were dissolved in 2 ml. of hot (60°C) de-ionized water, and the K^+ content was estimated using a flame photometer. The results are given in Table 6.

The mean recovery of K^+ extracted from the reconstituted ethanol extract into butanol was 6.1% (s.d. = 2.2%), a sixteen-fold reduction in the K^+ content of the extract. The mean K^+ content of the butanol extract obtained from 5 ml. of normal human blood was 8.6 μ.Eq. (s.d. = 2.86). When the extract is re-constituted in 2 ml. de Jalon's solution, 0.1 ml. of the extract, the maximum usually added to the 2 ml. tissue bath, contains 0.43 μ.Eq. K^+, which is safely below the threshold range of 4 - 8 μ.Eq. per ml. of bath fluid.

Sample	K^+ Content (μ.Eq.) in Extract from 5 ml. blood		Recovery %
	Ethanol Extract	Butanol Extract	
1	140.0	4.50	3.2
2	135.0	12.50	9.3
3	135.0	9.00	6.6
4	135.0	8.00	5.9
5	155.0	9.00	5.8
Mean	140.0 (s.d. = 8.66)	8.6 (s.d. = 2.86)	6.1 (s.d. = 2.2)

Table 6

The Exclusion of Blood K^+ by Butanol Extraction

10. The exclusion of 5-HT by butanol extraction

Butanol extraction at alkaline pH (c. pH 10), is often used in the routine purification of 5-HT (Hanson 1966). 5-HT has a high pK_a (pK_a = 10.0) therefore at the acid pH of 1.5 used in the butanol extraction procedure, the 5-HT would be highly ionized (Appendix 5). It should thus remain in the aqueous phase, and be only very slightly extracted into the butanol. 5-HT contracts the isolated oestrus rat uterus, but the presence of the 5-HT antagonist, 2-bromolysergic acid diethylamide (BOL-148, Sandoz) (0.5 mg. per litre) in the bath solution, as described in Appendix 3, raises the threshold from 1 - 2 ng. 5-HT per ml. to c. 2 µg. per ml.

To test the efficiency with which 5-HT is excluded by the plasma kinin purification procedure, a series of recovery experiments was carried out. Because the volunteer blood donors were at a hospital at some distance from the laboratory, the following procedure was used. Venous blood samples were collected from five subjects, using standard disposable syringes and siliconized needles, and placed in heparinized polythene pots. The samples were rapidly frozen for transit to the laboratory.

At the laboratory, each sample was thawed, and 5-HT creatinine sulphate was added in a small volume of aqueous solution

(0.12 ml.), sufficient to give a concentration of 100 µg. per ml. of blood. 5 ml. of the blood was immediately taken through a siliconized needle, into a polystyrene syringe, and mixed with cold absolute ethanol, as described in Appendix 2 (a). From this point onwards, the sample was treated as described in the appendix for the extraction of free kinin. The resulting dried butanol extract was taken up in 0.3 M. borate buffer, which was saturated with butanol and sodium chloride, at pH 10, and extracted with an equal volume of butanol. A second extraction was then made from the butanol into an equal volume of 0.1 N HCl, and the 5-HT content was estimated spectrophotofluorometrically, as described by Ashcroft et al (1964).* The results are given in Table 7.

The control level of blood 5-HT for each subject, in the absence of added 5-HT, was not measured, since the normal level, 0.5 µg. per ml. of blood (Ashcroft et al, 1964; Stacey 1966), is negligible when compared to the quantity of 5-HT added. Any error arising from this, would in any case mean that the true recovery was in fact even lower than that measured.

The mean recovery of added 5-HT (100 µg. per ml. of blood) was found to be 3.2% (s.d. = 0.86%). The isolated rat uterus

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* Spectrophotofluorometric estimation of 5-HT was carried out by Dr. D. Eccleston.

Subject	5-HT Added per 5 ml. Blood (µ.gm.)	5-HT Recovered per 5 ml. Blood (µ.gm.)	Recovery %
1	500	17	3.4
2	500	10	2.0
3	500	16	3.2
4	500	22	4.4
5	500	15	3.0
Mean			3.2 (s.d. = 0.86)

Table 7

The Recovery of 5-HT from Blood following Ethanol and Butanol Extractions

in a 2 ml. bath, in the presence of BOL-148, (0.5 mg./l.) has a contraction threshold of 3 - 4 µg. of 5-HT added directly to the bath. For free kinin assay, the butanol extract is dissolved in 2 ml. de Jalon's solution: The maximum volume of this solution usually, added to the tissue bath is 0.1 ml. 3 µg. of 5-HT can be added before reaching the contraction threshold, and with the recovery factor of 3.2%, this is equivalent to an original 5-HT content of 375 µg. of 5-HT per ml. of blood. Concentrations of this order are unlikely to be reached, even in individuals suffering from carcinoid and other indole secreting tumours. When pathologically high levels of free plasma kinin are present, the reconstituted extract is diluted further before adding to the tissue bath and interference in the bio-assay from 5-HT is thus highly unlikely.

11. <u>The exclusion of histamine by butanol extraction</u>

The butanol extraction of kinins is carried out at pH 1.5. Histamine (pK_a 9.7) is therefore highly ionized (Appendix 5) and should not be extracted into the butanol. The extracted kinins are routinely assayed on the isolated oestrus rat uterus, which is insensitive to histamine. In fact, high doses of 0.5 - 1.0 µg. of histamine per ml. bath fluid tend to lower the muscle tone. The following experiment was carried out to determine the efficiency with which the butanol extraction procedure excludes histamine.

Two 5 ml. samples of venous blood were collected from each of four volunteers and inactivated in ethanol as described in Appendix 2 (a). To one sample in each pair, 50 µg. of histamine in 0.2 ml. de-ionized water was added as the blood was being inactivated. A fifth pair of ethanol tubes was prepared as described, except that no blood was added. All samples were then processed as described in Appendix 2 (a) and (b), to give dried butanol extracts.

All samples were reconstituted in 2 ml. of hot (60°C) Tyrode solution, and assayed against standard histamine on the isolated guinea pig ileum, using a simple close-bracketing assay. To determine whether the activity being assayed was due only to histamine, samples were re-assayed on the same tissue in the presence of the antihistamine, mepyramine maleate (10^{-3} g. per litre). In no case was there residual activity in the presence of the mepyramine. The results are given in Table 8.

The mean recovery of added histamine (10 µg. per ml. of blood) was found to be 0.4% (s.d. = 0.08%). Normal levels of blood histamine in man lie within the range 20 - 100 ng. per ml. (See Lindell and Westling, 1966), and, except in some patients with chronic leukaemia, pathologically raised blood levels are all less than 1 µg. per ml. In leukaemic patients, blood histamine

Subject	Histamine Content (ng.) in Butanol Extract		Recovery %
	5 ml. of Blood	5 ml. of Blood + 50 µg. Histamine	
1	<4	240	0.5
2	<4	150	0.3
3	<4	200	0.4
4	<4	200	0.4
Blank	<4	750	1.5
Mean (excluding blank)			0.4 (s.d.=0.08)

Table 8

The Recovery of Histamine Following Ethanol and Butanol Extractions

levels of up to 27 µg. per ml. have been recorded (Lindell and Westling, 1966). However, assuming that even at this high level, the histamine recovery is still only 0.4%, the butanol extract would contain a total of 540 ng. of histamine; or, when reconstituted in 2 ml. of de Jalon solution, 0.1 ml. added to the 2 ml. tissue bath would contain only 27 ng. Thus interference from histamine during the bio-assay of the kinin content of the butanol extracts can be ruled out.

EXPERIMENTS TO DEVISE A METHOD FOR THE ESTIMATION OF KININOGEN IN WHOLE-BLOOD SAMPLES

1. Rationale

Once the method of free kinin extraction using ethanol had been decided upon, it was necessary to determine whether it was practicable to estimate both free kinin and precursor in the same blood sample. As described in Appendix 2 (a), the sample is taken from a vein into a polystyrene syringe, and inactivated in absolute ethanol. The ethanolic supernatant is removed for free kinin assay, together with washings, leaving behind an insoluble coagulum containing cell debris and precipitated protein. Plasma kinin precursor is precipitated together with the other plasma proteins.

In their widely used method of plasma kininogen estimation, Diniz et al (1961, 1963), having first spun down blood cells, destroyed enzymic activity in plasma by boiling it with dilute acetic acid. The denatured plasma was then incubated with excess trypsin to liberate the potential kinin, which was assayed biologically. Lahiri (1962) has described a method more suitable for adaptation to the present work. The principle of Lahiri's method is similar to that of Diniz and his colleagues, but instead of the use of acetic acid as the denaturing agent, Lahiri denatured plasma proteins by boiling in ethanol.

Thus, to destroy permanently the latent enzymic activity contained in the whole blood precipitate produced by preliminary treatment of the sample, the precipitate was re-suspended in 80% (v/v) ethanol, and heated in a boiling water bath for ten minutes. This procedure produces leathery lumps of precipitate which cannot satisfactorily be broken by hand. In order to obtain small aliquots of precipitate containing manageable quantities of kininogen (from the point of view both of the trypsin incubation and of the bio-assay), the precipitate was washed with distilled water, and homogenized in concentrated saline (2.5 M). The sodium chloride increases the density of the solution, and this aids suspension of the homogenate. Aliquots (0.2 ml.) could then be incubated with trypsin (200 µg. per ml.) in phosphate buffer at pH 7.35.

In one further point the incubation procedure described differs from that of both Diniz and his colleagues, and Lahiri. Both of these workers added ethanol, prior to boiling, to inactivate the trypsin. Diniz et al then evaporated the resulting suspension to dryness, and reconstituted this to form a new suspension for bio-assay; while Lahiri removed the solids and evaporated the ethanolic supernatant to dryness for bio-assay. This use of ethanol seemed unnecessary in the present work. The volume of incubate (5 ml.) was small enough, and the wall of the

incubating vessel sufficiently thin for the temperature to rise outside the functional range of trypsin almost as soon as it was immersed in the boiling water. The resulting procedure for the estimation of kininogen in whole-blood samples is described in Appendix 2 (c).

2. Trypsin incubation of isolated blood cells

The main difference between the procedure described in Appendix 2 (c) for the estimation of plasma kininogen and that described by Lahiri, is the unavoidable presence of blood-cell debris in the trypsin incubate. Thus a preliminary experiment was carried out to determine whether the incubation of isolated blood cells with trypsin released oxytocic activity when tested on the isolated oestrus rat uterus.

Two 5 ml. venous blood samples were taken from each of five subjects, using polystyrene syringes fitted with siliconized needles. One sample was treated as described in Appendix 2 for the assay of kininogen content. The other sample was placed in a polythene centrifuge tube containing heparin (250 units in 0.1 ml. saline), and centrifuged at 4°C for fifteen minutes at 1500 'g'. The supernatant was discarded, and the cells were gently washed with 10 ml. Eagle's medium (Eagle, 1959), and the washings were discarded. The cells were re-suspended in Eagle's medium to a volume of 5 ml. The resulting suspension

was drawn into a disposable syringe, and inactivated by ejection into ethanol as described in Appendix 2 (a). From this point onwards, the samples were treated as for whole blood for the assay of kininogen.

In no case was rat uterus contracting activity detected in the isolated cell incubates. If a trace of activity was present, but below the threshold of detection of the procedure, it represented far less than 1% of the activity obtained from the whole blood incubates (See Table 9).

Subject	Oxytocic Activity Released on Incubation with Trypsin (ng. BK Eq./ml. Whole Blood)		Upper Limit (%) of Total Activity Contributed by Erythrocytes
	Erythrocytes	Whole Blood	
1	<13	6,900	<0.20
2	<13	4,400	<0.30
3	<13	5,000	<0.26
4	<13	3,800	<0.35
5	<13	3,800	<0.35

Table 9

The Kininogen Content of Blood Cells

3. Comparison of Plasma Kininogen Levels Calculated from Whole-Blood Estimations, with those Obtained by Direct Estimation of Plasma.

In order to compare the values obtained for plasma kininogen level as calculated from the whole-blood kininogen content (using the haematocrit value), with that measured directly in plasma, the following experiment was carried out.

From each of five subjects, two venous blood samples were taken into two syringes, but through the same needle. The first was a 6 ml. sample, which was treated for the assay of kininogen content exactly as described in Appendix 2. The second was a 10 ml. sample which was placed in a polythene centrifuge tube containing 10 units of heparin in 0.05 ml. of saline. The tube was centrifuged at 1500 'g' for fifteen minutes at $4^{\circ}C$, and 4 ml. of plasma were taken into a disposable syringe and inactivated by vigorous ejection into 15 ml. ethanol. The plasma protein precipitate was then treated identically to the whole-blood precipitate for the assay of kininogen content, as described in Appendix 2. The resultant procedure for the direct estimation of plasma kininogen was effectively that of Lahiri (1962), but with minor modifications.

The values obtained are given in Table 10. The mean value for plasma kininogen level calculated from the whole-blood level

Subject	Haemato-crit (%)	Kininogen Content (µg. BK Eq./ml. plasma)		A/B as %
		A	B	
		Value Calculated from Whole-Blood	Direct Estimation in Plasma	
1	55.5	5.74	6.39	89.8
2	60.0	5.75	5.75	100.0
3	53.4	4.57	5.33	85.7
4	51.0	5.04	5.66	89.0
5	52.9	4.48	5.66	79.1
Mean		5.12	5.76	88.7 (s.d. = 5.8%)

Table 10

Comparison of Plasma Kininogen Levels Assayed in Whole Blood with those Assayed in Plasma

was lower than that found by direct estimation, and the mean ratio of the two was 88.7% (s.d. = 5.8%). This result was surprising, since the preparation of plasma for the direct estimation of kininogen meant that the blood was subjected to a greater amount of handling before enzymic inactivation in ethanol. There was thus more opportunity for inadvertent kinin release and a consequent lowering of the estimated kininogen level. As shown in the following section, once the blood has been inactivated in boiling ethanol, it does not contain any significant enzymic activity, and so differences in residual enzymic activity can be ruled out as a cause of the discrepancy. It is possible that erythrocytes contain a trypsin inhibitor of some kind. It is equally possible that the difference is due to some error or assumption inherent in the use of the haematocrit value in the conversion of whole-blood kininogen levels to plasma kininogen levels. However, since the levels as measured by the two methods were so close together, and the discrepancy was so constant (the s.d. for the ratio of 88.7% was only 5.8%), the problem was not investigated any further. The values used for plasma kininogen level in the rest of this thesis are those calculated from whole-blood estimations, and no further correction has been made.

4. Estimation of Residual Enzymic Activity in Whole-Blood Homogenate Denatured in Boiling Ethanol.

Dilute acetic acid, as used by Diniz et al (1963) for the inactivation of plasma enzymes, boils at 90 - 96°C; 80% ethanol boils at about 80°C. Although in acetic acid solution, at temperatures above 90°C, it is very probable that complete denaturation of plasma enzymes occurs, it seemed possible that at the lower temperature of boiling ethanol enzymic inactivation might not be complete. The following experiments were carried out to detect possible residual kallikrein or kininase activity in whole blood homogenates which had been inactivated in boiling ethanol as described in Appendix 2 (c).

To detect possible residual kinin-releasing activity, venous blood samples taken from four subjects were treated for the assay of kininogen content as described in Appendix 2. After homogenization in saline, 0.2 ml. aliquots were taken as usual for thirty minutes trypsin incubation in 5 ml. phosphate buffer, but in the absence of trypsin. All samples were then processed identically and assayed on rat uterus as described. In only one out of four cases was any spontaneous kinin release detectable, and this was only 0.44% of the total kinin released by the trypsin (Table 11 (a)).

Residual kininase activity was estimated in venous blood samples obtained from two subjects. The samples were once again treated as for kininogen estimations, but in this case, incubates contained only 0.2 ml. homogenate, 50 ng. bradykinin, and buffer to 5 ml. As a control, 50 ng. of bradykinin was incubated for thirty minutes in 5 ml. phosphate buffer. In neither sample was kininase activity detected (Table 11 (b)), and in fact, in all incubates, surprisingly little kinin loss occurred during thirty minutes incubation. The apparent increase in kinin content of one of the controls lies within bio-assay limits.

It must be mentioned that an alternative interpretation of the above results is the possibility that both kinin-releasing and kinin-destroying activity were present in all the homogenates tested, but that the one exactly balanced the other. This seems reasonably unlikely, and it is thus concluded that following inactivation in boiling ethanol, whole blood homogenates contained no significant amounts of kinin forming or destroying activity.

Table (a)

Subject	Bradykinin Released (ng. BK Eq./ml. of incubate)		Spontaneous Release as % of Trypsin Release
	Trypsin Present	Trypsin Absent	
1	100.0	0.44	0.44
2	80.0	<0.44	<0.55
3	83.3	<0.44	<0.53
4	80.0	<0.44	<0.55

Table (b)

Subject	BK Content after Incubation (ng. BK Eq./5 ml. of incubate)		Bradykinin Destruction
	50 ng. BK + Buffer + Homogenate	50 ng. BK + Buffer	
1	50.0	52.6	0
2	48.0	48.0	0

Table 11

(a) Kinin-releasing Activity of Blood Homogenates Inactivated in Boiling Ethanol

(b) Kininase Activity of Blood Homogenates Inactivated in Boiling Ethanol

5. Preparation of a Stable 'Control' Substrate for the Production of Kinin by the Action of Trypsin.

Some plasma kininogen assays in the early part of this work were invalidated by the use of a moisture contaminated batch of trypsin which slowly lost its potency during storage. In order to check routinely the constancy of the incubation procedure, it was necessary to prepare a stable kinin releasing substrate for trypsin. As a very simple preparative procedure was required, the following original method was used.

10 ml. of human venous blood were taken through a siliconized needle into a disposable syringe, and placed in a polythene centrifuge tube containing heparin (500 units in 0.1 ml. saline). The blood was centrifuged at 1500 'g' for fifteen minutes at 4°C. 0.2 ml. aliquots of the plasma were taken into siliconized glass pipettes and squirted into 0.8 ml. redistilled ethanol contained in heavy glass ampoules. The ampoules were placed in a boiling water bath for three minutes, centrifuged, and the supernatant discarded. The samples were washed twice with distilled water and then dried over P_2O_5 in an evacuated dessicator for forty-eight hours. The ampoules were filled with nitrogen, sealed, and stored at -20°C.

The samples were included in routine trypsin incubations as standards. For incubation, 0.2 ml. of 2.5 M saline was added to each standard ampoule. The samples were then incubated with trypsin in phosphate buffer as described in Appendix (c). In order to check the constancy of the standard, five samples from the same batch, but incubated with trypsin on different dates over a period of five months, were stored at -20°C, and assayed together on the same isolated rat uterus (see Table 12).

Since the plasma precipitate had been subjected to the same procedure as the whole-blood precipitate, it was considered to be equally enzymically inactive. This was confirmed in a single experiment to test each of kinin releasing and destroying activity in the manner already described for the whole-blood precipitate. The mean amount of kinin released on incubation with trypsin was 88.7 ng. BK Eq. per ampoule. The standard deviation was only 2.8 ng. BK Eq. (i.e. 3.1%). The kinin precursor samples prepared in this way were thus acceptable standards for use in detecting variation in the conditions of the trypsin incubation used in the routine plasma kininogen estimations.

Sample	Date of Incubation	Kinin Released (ng. BK Eq. per Ampoule)
1	28. 6.64	90.0
2	14.10.64	85.5
3	21.10.64	90.1
4	4.11.64	85.5
5	12.11.64	92.6
Mean		88.7 (s.d. = 2.8)

Table 12

Stability of a Control Kinin-releasing Substrate for Trypsin.

(The batch was prepared on 28.6.64. Sample (1) was incubated and assayed on this date. The remaining samples were incubated on the dates stated and assayed on 27.11.64.)

NORMAL VALUES

1. Introduction

A survey of the literature shows great variation in the estimations of normal plasma levels of kinin and kininogen (Tables 2 and 3). Apparently identical or very similar methods can give very different estimates of the normal level of both kinin and kininogen in the hands of different groups of workers. Much depends on care in the manipulation of the blood before the enzymes have been inactivated, where this is not done immediately following sampling. Much also depends on a critical bio-assay technique, such as in the frequent checking of kinin standards (which decay spontaneously in very dilute solution), and in sufficiently close bracketing. Between these two stages in the various methods, the errors should be constant for each particular procedure, and may be accounted for by adequate recovery experiments.

Even if "normal" ranges found with a particular procedure do not accurately reflect the true normal values, findings of consistent and reproducible departures from those levels associated with a given set of conditions, must indicate the involvement of the kinin releasing system under examination. Before the procedure described in the previous sections could be applied to the assay of kinin and kininogen levels in pathological conditions,

it was necessary to establish the "normal" values for the method. It was in fact necessary to determine whether accurate estimates of normal values even exist, or whether there is great variation in the levels measured in normal subjects such as is described by Allwood and Lewis (1964), whose estimate of normal venous whole-blood free kinin ranged from 4 to 72 ng. per ml. (mean 32 ng./ml.; s.d. $\pm$ 23).

It was possible to use the procedure for the assay of plasma kininogen described in Appendix 2 (c) before the extraction procedure for the assay of free kinin (Appendix 2 (b)) had been satisfactorily developed. Thus separate series of control samples were assayed for free kinin and kininogen.

2. Plasma Kininogen

Kininogen estimations were carried out on venous blood samples from three groups of subjects, a group of eleven elderly women (61 - 85 years) and six men (49 - 61 years) convalescent in hospital, and thirteen active healthy young men (20 - 30 years). Samples were taken in all cases from an arm vein, and treated exactly as described in Appendix 2 for the assay of plasma kininogen. The results are given in Tables 13 (a), (b) and (c).

The mean kininogen level for the women was 7.1 µg. BK Eq. per ml. of plasma (s.d. = 1.1), and for the older men it was

Subject	Age (Yrs.)	Plasma Kininogen (µg. BK Eq./ml.)
1	61	6.7
2	61	7.3
3	65	7.9
4	68	6.2
5	72	8.3
6	74	8.7
7	75	7.2
8	77	6.9
9	77	7.6
10	85	8.2
11	Unknown	6.7
Mean		7.1 (s.d. = 1.1)

Table 13

Plasma Kininogen Levels

(a) Convalescent Elderly Women (61 - 85 years)

Subject	Age (Yrs.)	Plasma Kininogen (µg. BK Eq./ml.)
1	49	5.7
2	51	5.2
3	52	5.0
4	58	7.7
5	59	6.5
6	61	7.3
Mean		6.2 (s.d. = 1.1)

Table 13

Plasma Kininogen Levels

(b) Convalescent Men (49 - 61 years)

Subject	Age (Yrs.)	Plasma Kininogen (µg. BK Eq./ml.)
1	21	4.3
2	21	4.6
3	22	4.5
4	22	5.0
5	22	5.7
6	22	5.8
7	22	5.9
8	22	6.0
9	23	4.5
10	25	2.9
11	26	7.3
12	27	6.2
13	30	3.7
Mean		5.1 (s.d. = 1.2)

Table 13

Plasma Kininogen Levels

(c) Young Healthy Men (20 - 30 years)

6.2 µg. (s.d. = 1.1). For the young men, the kininogen level was 5.1 µg. BK Eq. per ml. of plasma (s.d. = 1.2). The mean value for the three groups considered together is 6.1 µg. BK Eq. per ml. (s.d. = 1.4). The kininogen values measured in the young men included one of 12.0 µg. BK Eq. per ml. of plasma. This value, obtained from an apparently normal subject, was much more than three standard deviations greater than the mean for that group, and has been excluded from the Table and associated calculations.

3. Free Plasma Kinin

Free plasma kinin estimations (Appendix 2) were carried out on venous blood samples taken from sixteen healthy male volunteers aged from 20 - 60 years. At the time of sampling, these subjects were showing no obvious abnormal symptoms. One subject who suffered vaso-vagal fainting during sampling was excluded from the "normal" group, and will be considered separately. The results are given in Table 14.

The mean free kinin level for this group was found to be 2.8 ng. BK Eq. per ml. plasma (s.d. = 1.7). This value is artificially raised, since where no free kinin was detectable, an arbitrary value determined by the assay threshold was used for the calculation. This approximation raises the mean normal level very little, the true mean for the method lying within the limits 2.2 ng. and 2.8 ng. BK Eq. per ml. of plasma.

Subject	Age (Yrs.)	Plasma Kinin (ng. BK Eq./ml.)
1	20	6.3
2	21	0.56
3	21	1.7
4	23	<0.30
5	23	3.7
6	25	<3.0
7	25	2.4
8	26	2.5
9	28	<3.5
10	39	1.7
11	46	<2.7
12	60	2.9
13	Not given	0.90
14	"	2.6
15	"	4.3
16	"	6.0
Mean		2.8 (s.d.=1.7)

Note: Subjects 13 - 16 were 4th year medical students (aged 21 - 25 years)

Table 14

Free Plasma Kinin Levels in Normal Healthy Males

DISCUSSION

1. The Method of Free Plasma Kinin Estimation

In the sections describing the derivation and testing of the procedures for the assay of free kinin and kininogen in blood samples, the reasoning behind each step and its ultimate effectiveness have already been fully discussed. The first part of this discussion will, in the main, merely summarize the conclusions arising from these sections.

Ethanolic precipitation of whole blood was chosen as the first stage in the treatment of clinical blood samples. This procedure inactivated blood enzymes, whilst at the same time separating the free kinin from its precursor and enabling both substances to be obtained in a form suitable for assay. Lahiri (1962) found that ethanolic extracts of whole blood from the guinea pig, rat and rabbit, when evaporated to dryness and suitably reconstituted, contained levels of potassium sufficient to stimulate the isolated rat uterus preparation. For his study this was of little significance, but the levels were high enough to interfere with the assay of the very low normal levels of free plasma kinin. In the present work, it has been found that extracts from human whole blood contained quantities of potassium even greater than those found by Lahiri. It was impossible to assay

satisfactorily whole blood concentrations lower than 10 ng. bradykinin per ml.

Moreover, it seemed highly probable that in pathological conditions of interest for kinin analysis (such as inflammation, allergic conditions, shock etc.) the biologically active amines, histamine, 5-HT and adrenaline were likely to cause serious interference in the kinin assay. The possibility of spurious findings as the result of such interference was demonstrated by Lewis and Nustad (1965). When bradykinin is infused intra-arterially in human subjects and ethanolic extracts are prepared from venous blood samples, their kinin content appears to rise at the outset of the infusion and to return to normal as the infusion continues, if assay is carried out on the isolated rat uterus without further purification (Allwood and Lewis, 1964). Lewis and Nustad (1965) found that infusion of bradykinin in the rabbit caused the release of adrenaline into the circulation, and the increasing level of adrenaline in the ethanolic extracts inhibited the rat uterus and caused the apparent fall in the plasma kinin level. When the animals were adrenalectomized, the plasma kinin level rose at the start of bradykinin infusion and did not subsequently fall. A secondary purification procedure for plasma kinin was thus sought.

In a preliminary series of experiments, separation of bradykinin from K^+ and small molecules was attempted using various small-column chromatographic procedures, including filtration on Sephadex G-25 eluted with water or 75% ethanol, and separation on "ion retardation" resin. Such procedures would have constituted a single step purification with a possibility of sub-fractionation of the kinin peptides, and would have provided a salt-free extract. However, no procedure gave a recovery above 40% nor was any really satisfactory separation obtained.

Of the other methods considered, the method first used by Gomes (1955) and subsequently modified by Gaddum and Horton (1959), for the purification of urinary kinin, seemed the most promising. These workers reported kinin recoveries of 80-100% varying with minor modifications. It also seemed likely, although not previously reported, that this procedure would exclude most of the potassium, which is very insoluble in butanol. Since the extraction takes place from an aqueous phase at low pH, it should also, in theory, exclude bases with high pK such as the biologically active amines previously mentioned. Preliminary recovery tests having shown that the butanol extraction procedure would give high recoveries of bradykinin, the method was investigated. As finally modified (Appendix 2 (b)), the procedure gave a bradykinin recovery of 81.5% (s.d. = 8.0%) in a concentration range of 10 - 50 ng. of

added bradykinin per ml. of blood. The interfering substances tested were also satisfactorily excluded by the procedure. There was at least 90% reduction in the K^+ content. The recovery of 5-HT added in high concentration to whole blood was 3.2% (s.d. = 0.86%), while that of histamine was only 0.4% (s.d. = 0.08%). Adrenaline recovery was not tested, but as its pK was intermediate between those of histamine and 5-HT, this was not considered necessary.

2. Method of Plasma Kininogen Estimation

The method for the estimation of plasma kininogen was predetermined by the choice of ethanol extraction for the preliminary purification of free kinin. The most suitable method was that of Lahiri (1962), who used boiling ethanol to destroy the enzymes and pro-enzymes in plasma prior to returning to aqueous solution for the activation of the kinin precursor with trypsin. The procedure, originally used with plasma, was adapted for the estimation of the kininogen content of whole blood (Appendix 2 (c)). This value was then corrected, using the haematocrit value (Appendix 4) to give the kininogen concentration in plasma.

It was found that isolated blood cells contained no detectable kininogen, and that the values for plasma kininogen levels calculated from whole blood were lower (88.7% s.d. = 5.8) than those determined directly using plasma. Since the denatured whole blood was found

to contain neither significant kinin forming nor destroying activity, this relatively constant and small discrepancy was disregarded in calculation. As a check on the constancy of incubation conditions, a standard kinin-releasing substrate was included as a control with each batch of samples. The control substrate was prepared from ethanol denatured plasma. It was dried and stored in standard quantities, in nitrogen filled ampoules.

3. Kinin Analysis in Clinical Blood Samples

The procedure fulfilled all the conditions necessary (P. 61) to make it suitable for use in the kinin analysis of clinical samples. Blood was taken in standard disposable equipment, and satisfactory kinin recovery was still obtained after storing the sample for twelve hours at 4°C. immediately following ethanolic inactivation. Thus the sample could be stored for a limited period by the clinician whilst awaiting processing. Both kinin and kininogen were obtained in high recovery, and thus both substances and the haematocrit could be estimated in a single 6 ml. blood sample. The purification procedure for the free plasma kinin excluded many interfering substances, giving the method a high specificity. Perhaps the most important drawback to the process from the point of view of routine analyses, was the fact that it was very time consuming.

4. Normal Values of Free Plasma Kinin and Kininogen

The procedure described was used to assay plasma kininogen levels in venous blood from three control groups of subjects, a group of 11 elderly convalescent women aged 68 - 86 years, a group of 6 convalescent men aged 49 - 61 years, and 13 active, healthy young men aged 20 - 30 years. The mean kininogen levels were 7.1 µg. (s.d. = 1.1), 6.2 µg. (s.d. = 1.1) and 5.1 µg. (s.d. = 1.2) bradykinin equivalents per ml. of plasma for the three groups respectively, while the mean value for all the subjects was 6.1 µg. bradykinin equivalents (s.d. = 1.2).

The values from which the mean levels for each group were calculated, were obtained at various times as suitable volunteers became available. When this is considered together with the fact that the values depended on a biological assay, the small size of the standard deviations is remarkable. This reflects, perhaps, the homogeneity of the groups and indicates that under normal conditions the plasma kininogen level is a fairly constant value from person to person in a homogeneous group.

The mean 'normal' free plasma kinin value of 2.8 ng. bradykinin equivalents per ml. of plasma (s.d. = 1.7) was obtained from a control group of 16 healthy males (20 - 60 years). As was found with the kininogen levels, under normal conditions the plasma free-kinin level varied very little, and as will be shown in the following sections, all subjects having a plasma kinin level greater

than an arbitrary value of 10 ng. bradykinin equivalents per ml. showed vasomotor symptoms (the 99% fiducial limit for the normal mean is 7.9 ng. per ml.).

When the normal values found in the present work are compared with the normal values found by other workers (Tables 2 and 3), it can be seen that both the free kinin and precursor values lie in the range of values found by the majority of workers. All the normal precursor values so far published are of a similar order of magnitude. If it is assumed that they are all estimates of the same mean value, and a very rough average is calculated from Table 2 (using mid-points where only ranges are given), a value of 5.4 µg. bradykinin equivalent per ml. of plasma is obtained. This value compares very favourably with the value of 5.1 µg. bradykinin equivalent per ml. of plasma obtained in the present work for the group of active males, and the value of 6.1 µg. bradykinin equivalent per ml. obtained when the two convalescent groups are included. The published normal free-kinin values are not all of the same order, two out of the six being several thousand per cent greater than the rest (Table 3). The value of 2.8 ng. of bradykinin equivalent per ml. of plasma (i.e. approximately 1.1 ng. per ml. of whole blood supports the conclusion that normally there is little or no free kinin in blood or plasma.

EXPERIMENTAL

II. APPLICATION OF METHOD

THE CARCINOID SYNDROME

1. Introduction

The carcinoid syndrome is the name applied to a complex consisting of some or all of the following symptoms; episodic or sometimes continuous flushing, abdominal colic, intestinal hypermotility, watery diarrhoea, attacks of dyspnoea due to bronchoconstriction, oedema, and ascites due to right heart failure. The patient often has a patchy cyanotic appearance, and pellagra-like skin pigmentation. The dramatic transient crimson flush may be provoked by such stimuli as ingestion of alcohol or food, emotion, intravenous adrenaline or noradrenaline, or compression of the tumour; and there is often a fall in blood pressure at the commencement of flush. Raised blood levels of 5-HT and other indoles, and a raised urinary excretion of 5-HIAA and other indoles are diagnostic of the condition. The carcinoid state has been fully discussed in a number of reviews, amongst which are those by Stacey (1966), Smith (1960-1; 1965), and three reviews given at a Symposium on the Clinical Chemistry of Monoamines by Campbell (1963), Gowenlock and Platt (1963) and Snow (1963).

The name Karzinoiden was first applied to certain tumours of the intestinal tract by Oberndorfer (1907). Carcinoid tumours may be found anywhere along the gastro-intestinal tract, or in

its appendages, the commonest sites for the primary tumour being in the appendix or ileum (Gowenlock and Platt, 1963; Sanders and Axtell, 1964). Cases have also been described in which the carcinoid syndrome has been associated with bronchial adenomata (Williams and Azzopardi, 1960; Gowenlock and Platt, 1963).

The origin of carcinoid tumour cells from the chromaffin cells of Kultschitzky (1897), found normally in the mucosa in the gastro-intestinal tract, was established by Gosset and Masson (1914), who first demonstrated the characteristic affinity of both types of cell for silver stains. These workers suggested that the tumour had an endocrine activity, a hypothesis which remained unconfirmed for some forty years, when the presence of a carcinoid tumour was first shown to be related to the complex of symptoms making up the "carcinoid syndrome".

In 1953, Isler and Hedinger reported three patients having carcinoid tumours, and right heart valvular lesions, which they described as a definite syndrome. In Sweden, Biörck, Axén and Thorson had earlier, in 1952, described a patient having a carcinoid tumour of the small intestine, and exhibiting the symptoms of flushing, cyanosis, oedema, asthmatic attacks, pellagra-like skin lesions, and cardiac lesions, without recognizing the syndrome. They subsequently reported a number

of cases showing these symptoms, and described the typical carcinoid syndrome, which they suggested might be due to 5-HT secreted by the tumour (Thorson, Biörck, Björkman and Waldenström, 1954). It had already been shown that tumour tissue contained high concentrations of 5-HT, and a second, uncharacterized smooth muscle contracting substance (Lembeck, 1953), and the Swedish workers went on to show that there was an abnormally high concentration of 5-HT in the blood of carcinoid patients (Pernow and Waldenström, 1954).

Many reports of raised levels of 5-HT, and its metabolite 5-HIAA, in both blood and urine have since appeared in the literature (Gowenlock and Platt, 1963), and this has become diagnostic of the carcinoid condition. This relationship, and the fact that when 5-HT is administered parenterally in man it can produce some of the symptoms of the carcinoid syndrome, including intestinal hypermotility (Hendrix, Atkinson, Clifton and Ingelfinger, 1957), and flushing (Page and McCubbin, 1953; Roddie, Shepherd and Whelan, 1955), resulted in the generally accepted assumption that 5-HT was the sole humoral agent responsible for the carcinoid flush. This assumption was challenged in 1962 by Robertson, Peart and Andrews, who showed that intravenous injections of adrenaline and noradrenaline provoked the carcinoid flush, doing so indirectly, by the

liberation of some secondary humoral factor. They further showed that the appearance of the flush was not always accompanied by an increase in circulating 5-HT. They found that intravenous infusion of 5-HT in patients in whom a flush could always be provoked by adrenaline was not always followed by a flush, even though doses high enough to cause other systemic effects were used. Even when a flush was produced by 5-HT, it differed qualitatively from both the spontaneous flush and that provoked by adrenaline, being more cyanotic. These workers concluded that 5-HT was probably not the humoral factor responsible for carcinoid flush. Conjecturing on the identity of this factor, they excluded for various "circumstantial" reasons such vaso-active substances as histamine, substance P, adenosine triphosphate, and nicotinic acid, and suggested bradykinin as potentially the most likely culprit.

An editorial comment on this hypothesis (Lancet, 1962) related earlier findings of raised levels of the proteolytic enzyme, pepsin, in the urine of some carcinoid patients (Smith, Nyhus, Dalgleish, Dutton, Lennox and Macfarlane, 1957), and the abnormal presence of a peptidase in carcinoid tissue (Pearse and Pepler, 1957) to the fact that bradykinin is released from its precursor by proteolytic enzymes. It was furthermore suggested that the provocation of carcinoid flush by catecholamines may be

related to the observations of Hilton and Lewis (1956) that catecholamines will release kinins in the perfused cat salivary gland. Similar considerations prompted the investigations of Oates, Melmon, Sjoerdsma, Gillespie and Mason (1964), who showed that raised levels of a kinin-like polypeptide appeared in the hepatic venous blood of eleven patients during carcinoid flushing. They also found that whilst hepatic tissue from non-carcinoid patients contained no demonstrable kallikrein activity, hepatic carcinoid metastases contained a kinin-releasing enzyme. They completed their investigation by showing that in carcinoid patients, intravenous injection of synthetic bradykinin produced a flush similar to the carcinoid flush. It was at this point, as a result of Oates' paper, that the present series of investigations into plasma kinin and kininogen levels in carcinoid patients was initiated. The only experiments characterizing the active principle purified from the blood of carcinoid patients reported by Oates and his colleagues, were the contraction of isolated rat uterus, the depression of rat blood pressure, and destruction with chymotrypsin. This prompted the suggestion (Lancet, 1964) that the activity was due to substance P, a peptide possessing the appropriate biological properties, and present normally in the gastro-intestinal tract. However, Oates and his colleagues have since reported that the chromatographic behaviour of the active substance, its actions on bio-assay

preparations, and its rates of enzymic destruction were indistinguishable from those of bradykinin (Oates and Melmon, 1966; Oates, Pettinger and Doctor, 1966).

Finally, although histamine has never had any wide acceptance as the major carcinoid humoral factor, it should be mentioned that the literature contains very numerous reports of raised urinary and blood histamine levels associated with the carcinoid condition. Raised histamine levels have frequently been found in the presence of both the "typical" carcinoid tumours (5-HT secreting, associated diagnostically with raised blood 5-HT, and raised urinary 5-HIAA) and the "atypical" carcinoid tumours (5-HTP, secreting, associated diagnostically with raised urinary 5-HT, 5-HTP, and 5-HIAA) (Pernow and Waldenström, 1954, 1957; Gowenlock and Platt, 1963). Histamine possesses many of the properties required of a carcinoid humoral factor, including the production of a crimson flush, and hypotension (Rocha e Silva, 1966), and gut stimulating activity (Parrot and Thouvenot, 1966). However, the subjective symptoms experienced on the intravenous infusion of histamine differ from those of the carcinoid syndrome (Robertson et al, 1962), and the actions of histamine can be inhibited by specific antihistamines, which is not usually the case with carcinoid symptoms. The presence of metastases in

the stomach, which normally contains histamine secreting cells, or the finding of Feldberg and Smith (1953) that tryptamine and 5-HT are histamine releasers are explanations often advanced for the presence of such abnormal histamine levels.

2. Clinical Material and Procedures

Samples have been investigated from four male patients having carcinoid tumours, and exhibiting the carcinoid syndrome with varying degrees of severity, and a female patient, who, at the time of investigation exhibited a carcinoid-like condition, with flushing and raised 5-HIAA excretion. Patient (1) (Kinloch, Webb, Eccleston and Zeitlin, 1965) and patient (2) (number (1) in the series of Smith, Preshaw and Sircus, 1965) have been described with clinical details elsewhere.

Patient (1) flushed continuously, and his whole-blood 5-HT level was 415 ng. per ml. when flushing was mild, and 820 ng. per ml. when intense (normal range 50 - 300 ng. per ml.). He excreted in his urine an average of 31 mg. 5-HIAA per twenty-four hours. He died two months after admission to hospital, and at autopsy, histological examination showed the presence of an oat-cell carcinoma of the bronchus, with metastases present in many tissues, including the liver, pancreas, both kidneys, and thyroid glands.

Patient (2) showed the typical transient flushing of the carcinoid syndrome. For experimental studies, flushing could be provoked by intravenous injection of adrenaline (10 µg.). Carcinoid tumour had been diagnosed four years earlier. At that time he had been admitted to hospital with a history of flushing and diarrhoea. Laparotomy had revealed a caecal carcinoid tumour containing argentaffin cells. He also had a raised urinary 5-HIAA excretion, and a raised whole-blood 5-HT level. At the time of the present investigation, his peripheral blood contained abnormally high concentrations of a number of indoles (Crawford, Ashcroft, Eccleston and Smith, 1965), his whole-blood 5-HT level being 2.0 µg. per ml. Urinary excretion of 5-HIAA was 70 mg. per twenty-four hours for this patient.

Patients (3) and (4) had histories of diarrhoea, abdominal discomfort, and repeated attacks of flushing. Patient (3) had a whole-blood 5-HT concentration of 0.37 µg. per ml., and excreted 255 mg., of urinary 5-HIAA in twenty-four hours. Necropsy disclosed a bronchial oat-cell carcinoma with anaplastic hepatic metastases. At the time of the present investigation, flushing could not be provoked in this patient.

In patient (4), laparotomy had shown the presence of carcinoid tissue in mesenteric lymph nodes and liver. The primary tumour was never identified, but was thought to be in the duodenum.

Transient flushing could be provoked in this patient by intravenous injection of adrenaline (10 µg.). Before, and during flush, his whole-blood 5-HT level was 0.35 µg. per ml., and his twenty-four hour urinary 5-HIAA excretion was 9.3 mg.

Patient (5), a 45 year old woman, had entered hospital some two years previously with a duodenal ulcer, hypersecretion, steatorrhoea, and a hepatic carcinoma. She had a history of flushing since hysterectomy nine years earlier. Her condition was diagnosed as the Zollinger-Ellison syndrome, which is usually associated with a non-insulin producing tumour of the pancreas. The patient accordingly underwent partial pancreatectomy, partial gastrectomy, and total vagotomy. No tumour tissue was found in the pancreas, and no argentaffin granules were seen in the hepatic metastases. She was discharged from hospital free from her earlier symptoms, including flushing. Two years later she was admitted to hospital in a diabetic coma, and a week after this, although she was exhibiting neither flushing nor other carcinoid symptoms, a blood sample was routinely assayed for free kinin. During the next two months her diabetes disappeared spontaneously, and two and a half months after the first kinin assay, she started to have carcinoid-like flushing. A second blood sample was obtained for kinin assay during flush. Her urinary 5-HIAA at this time was reported to be four times the normal value (medical case notes).

3. Results

a) Kinin and Kininogen Levels in Venous Plasma

Kininogen and free kinin levels were measured in the venous blood of carcinoid patients (1) - (4), who showed symptoms of varying severity (see Table 15). In only one of these patients (patient (3)) was it possible to obtain samples before, during and after the flush.

Patient (1), a terminal case, showed the most severe symptoms, and displayed a continuous lobster flush and general oedema. He also had the highest free kinin level, 53.7 ng. BK Eq. per ml. of plasma, or nearly twenty times the mean normal level of 2.8 ng. BK Eq. per ml.

Patients (2) and (3) showed the typical transient brick red flushing of the carcinoid syndrome. At the height of the flush provoked by 10 µg. intravenous adrenaline, patient (2) had a free kinin level of 26.3 ng. BK Eq. per ml. of venous plasma, or about nine times the normal level. Samples from patient (3) were assayed before, during, and after the flushing episode (see Fig. 4). The pre-flush control level for this patient, although a little high, was within 3 standard deviations of the mean normal level. At the height of flush provoked by 10 µg. intravenous adrenaline, the venous free kinin level for this patient was 24.3 ng. BK Eq. per ml. plasma, which is almost nine times the normal level.

PATIENT	CONDITION	SEVERITY of SYMPTOMS	BRADYKININ EQUIVALENTS OF:	
			KININOGEN (µg./ml. plasma)	FREE KININ (ng./ml. Plasma)
1	Carcinoid	Continuous lobster colour	16.6	53.7
2	"	Transient flush	4.9	26.3
3	"	Transient flush	7.0 (Control)	7.3 (Control)
			5.8 (Flushing)	24.3 (Flushing)
			3.5 (After Flush)	14.7 (After Flush)
4	"	Non-flushing	6.6	2.0
5	Zollinger-Ellison	Non-flushing Transient flush	not done 15.6	1.2 23.9

Table 15

Plasma Kinin and Kininogen Levels in Patients with the Carcinoid Syndrome

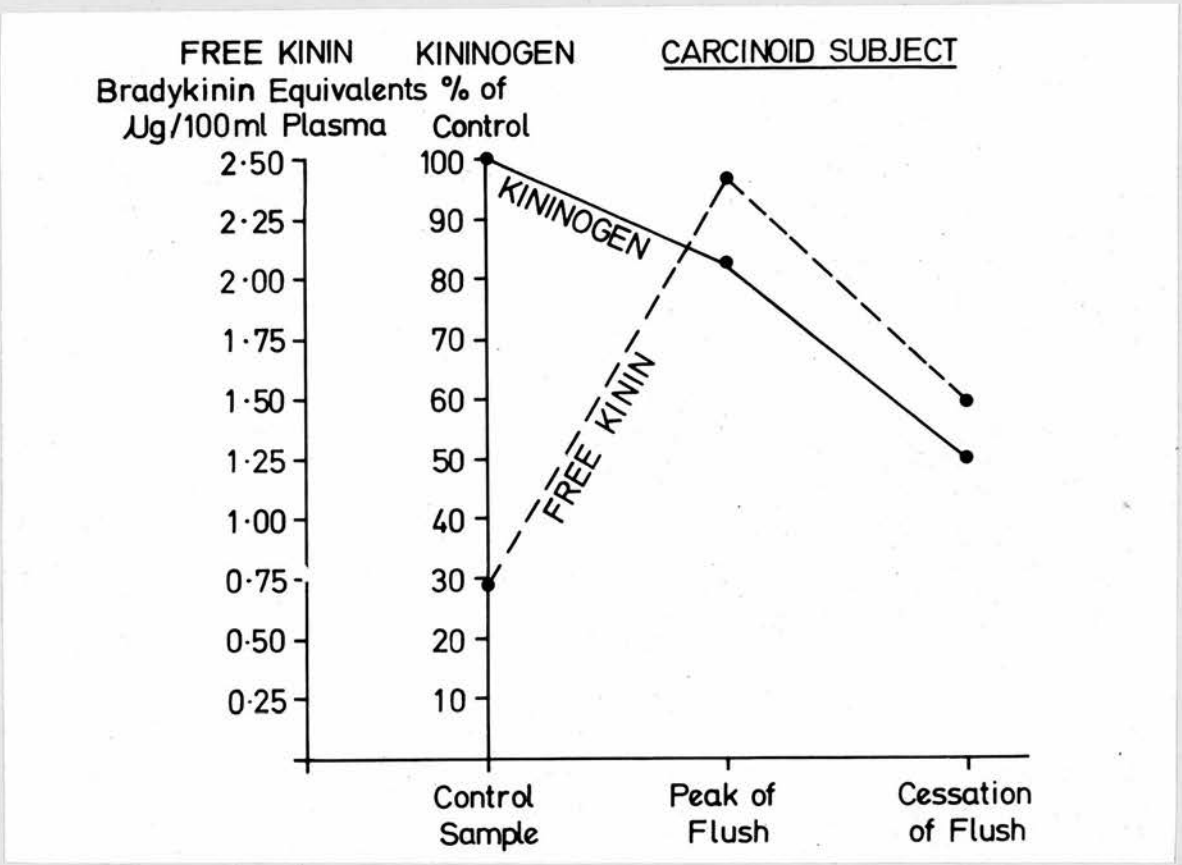

Fig. 4: Patient 3: Levels of free kinin and kininogen in venous plasma before, during, and after the flush provoked by 10 µg. intravenous adrenaline. (Note: 1 µg./100 ml. plasma = 10 ng./ml. plasma).

At the cessation of flush, the level approached normal again. The venous plasma kininogen level for this patient fell throughout the onset and cessation of symptoms (Fig. 4 and Table 15).

Patient (4), although showing other features of the carcinoid syndrome, could in no way be provoked to flush. His venous free kinin level, assayed on a number of occasions, lay at all times in the normal range. An exploratory operation was carried out on this patient. During the operation, because he had a high 5-HIAA excretion, blood samples were taken from the portal and hepatic veins and the hepatic artery for indole assay. Simultaneous samples were also taken for kinin and kininogen assay. Although metastases were present in the liver, there were no obvious indole concentration gradients (Eccleston, Personal Communication). The kinin and kininogen levels lay within the normal range (see Table 16) also, and showed no significant gradients. However, no real conclusion can be drawn from this finding, since the presence or absence of flush was not noted during the operation.

Samples from patient (5), investigated originally as a case of Zollinger-Ellison syndrome, were assayed on two occasions, separated by an interval of two and a half months. On the first occasion she showed no carcinoid symptoms, and was not flushing. Her plasma free kinin level was normal, 1.2 ng. BK Eq. per ml. Her symptoms subsequently became carcinoid-like, with typical

Sample	Bradykinin Equivalents of:	
	Kininogen (µg./ml. Plasma)	Free Kinin (ng./ml. Plasma)
Hepatic Artery	6.2	3.2
Portal Vein	5.6	3.0
Hepatic Vein	6.6	4.1

Table 16

Plasma Kinin and Kininogen Levels in Blood taken at Operation from the Hepatic Artery and the Hepatic and Portal Veins of a Carcinoid Patient (Patient 4).

transient flushing. A venous blood sample taken during "naturally" occurring flush, contained 23.9 ng. BK Eq. per ml. plasma.

When the free plasma kinin levels found in these patients are arranged according to the approximate severity of their vasomotor symptoms (Fig. 5), it can be seen that the kinin levels are highest during the most severe symptoms, and lowest in their absence.

Information gained from plasma kininogen estimation in these patients is less easy to evaluate. As already mentioned, serial kininogen estimations in patient (3) indicated the rapid release of kinin (Fig. 4). It is noteworthy that while all the kininogen levels measured in patients (2), (3), and (4) were in the normal range, those in patients (1), and (5), who incidentally showed the most severe general symptoms of their conditions, were very high, both being well over twice the mean normal level. No other plasma protein abnormalities were reported in these patients at the time of sampling. Although chronic severe depletion might cause compensatory over-production of kininogen, serial estimations during the course of the disease would be necessary before such a hypothesis could be made.

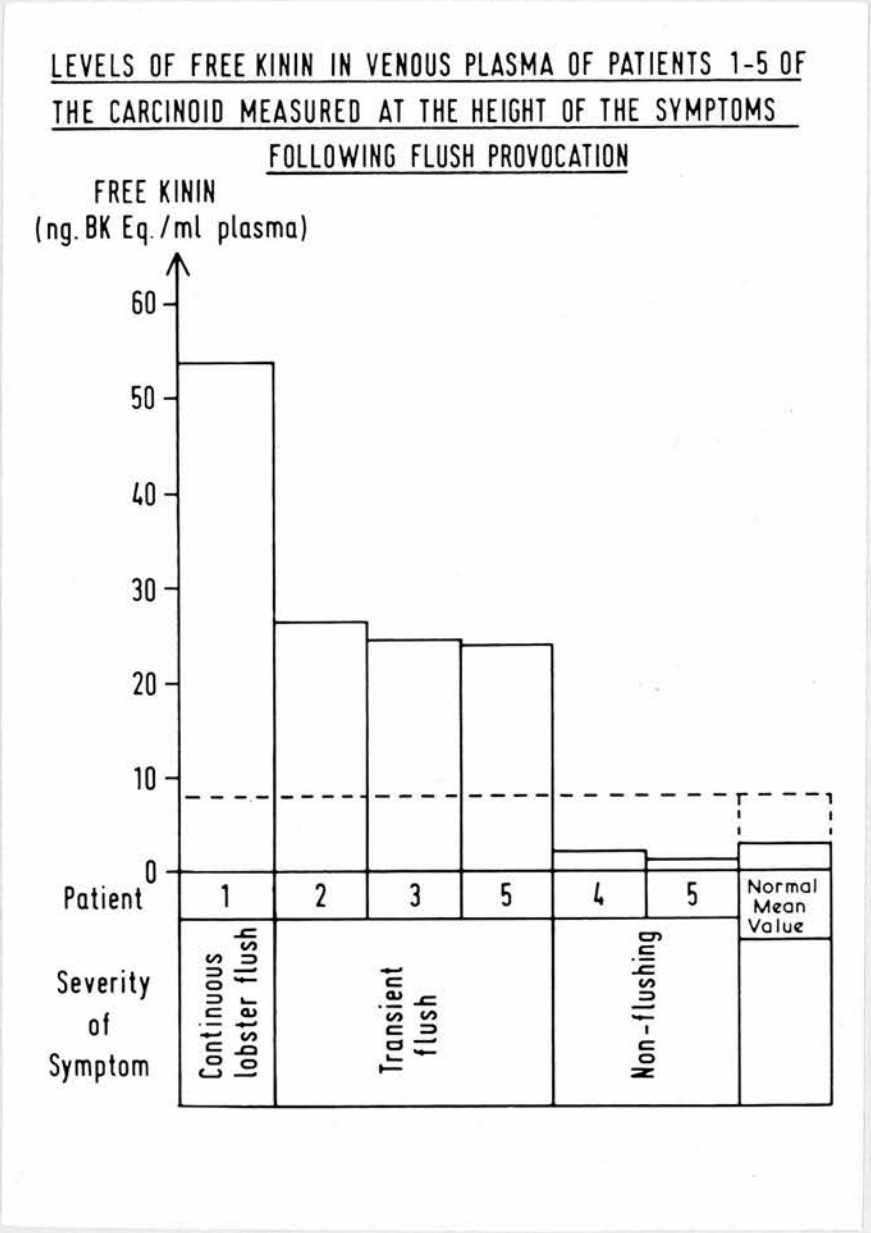

Fig. 5: Levels of free kinin in venous plasma of patients 1 - 5 of the carcinoid series measured at the height of the symptoms following flush provocation. The dotted line represents the 99% upper fiducial limit of the mean value found in symptomless healthy males. Therefore only 1 in 100 normal values will be greater than this.

b) Characterization of Bradykinin-like Activity

Although the plasma levels of kinin in the presence of flush in patients of the carcinoid series were very much greater than the levels in the absence of flush or in normal subjects, the total quantities of activity available were very small. As the amounts available were in the nanogram range, it was only possible to characterize the activity obtained from two of the carcinoid subjects by parallel qualitative bio-assay using the isolated rat duodenum (Fig. 6). This tissue relaxes in the presence of kinin, and contracts in the presence of most other smooth muscle stimulating substances. The activity so characterized contracted the isolated oestrus rat uterus in the presence of atropine and BOL. It was thus distinguished from catecholamines and histamine, which both inhibit the tissue; gastrin and SRS-A to which the tissue is insensitive; and from acetylcholine and 5-HT, which were antagonized with specific inhibitors. The active principle relaxed the rat duodenum, which further distinguished it from 5-HT, histamine, acetylcholine, gastrin, substance P, and angiotensin, all of which contract the tissue.

In addition, in patient (3), the appearance of free kinin-like activity occurred in parallel with a decrease in the plasma kininogen (Fig. 4). Thus the evidence indicates that the

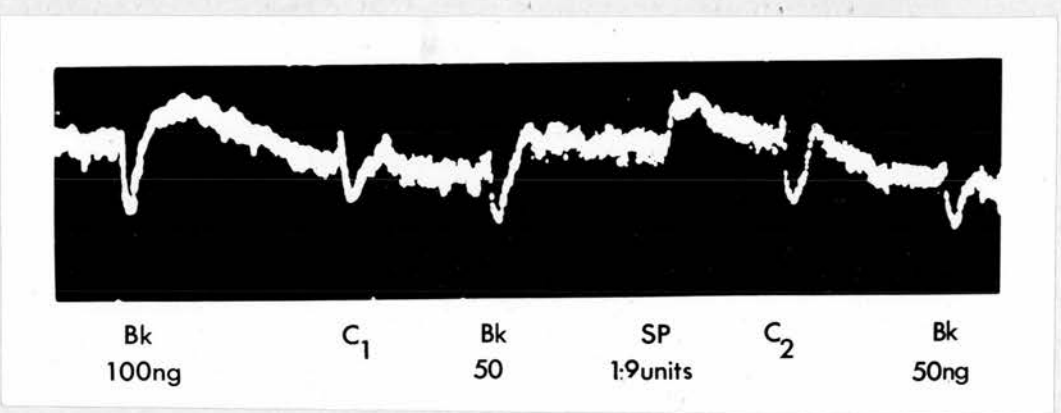

Fig. 6: Smoked-drum tracing showing relaxation of the isolated rat duodenum by bradykinin-like activity in extracts from the venous blood of flushing carcinoid patients. (Bk = Standard bradykinin; SP = Substance P; C_1 and C_2 = samples from two carcinoid patients).

increased activity assayed in these subjects in the presence of flushing was bradykinin-like, probably originating from a plasma kinin precursor.

4. Discussion

Of all the effects which bradykinin produces in man, the most striking is its ability to cause flushing. Intravenous bradykinin stimulates the blush areas of the human body in doses too low to produce other detectable physiological changes (Fox et al, 1961; Javett and Coffman, 1962). The argentaffin cells normally found in high concentration in the mucosa of the gastro-intestinal tract have long been known as the origin of carcinoid tumour (Gosset and Masson, 1914). As has already been discussed these cells are involved in indole metabolism, and abnormalities of indole metabolism are usually associated with the carcinoid condition. For this reason the vasomotor symptoms of the carcinoid syndrome have been generally considered to be mediated by the release into the circulation of raised levels of 5-HT.

5-HT infusion in man, however, causes cutaneous and venular vasoconstriction (Roddie et al, 1955; Fox et al, 1961) with eventual pooling of blood and development of a cyanotic flush similar to that seen in some carcinoid cases (Roddie et al, 1955). In carcinoid patients, Robertson et al (1962) found that

intravenous 5-HT did not produce the typical carcinoid flush and sometimes caused pallor, even in doses high enough to produce considerable systemic effects; while there was little correlation between free plasma 5-HT levels and flushing episodes. The intravenous injection of bradykinin in carcincid patients, unlike 5-HT, did produce symptoms closely resembling those of the syndrome (Oates et al, 1964).

Raised levels of free plasma kinin during carcinoid flush have been detected in the hepatic venous blood of 11 patients by Oates and his colleagues (Oates et al, 1964; Oates and Melmon, 1966), and in peripheral venous blood of a single patient by Webster (1966). The results reported in the present work support the findings of these workers. Plasma kinin release has been demonstrated in venous blood during flushing in 5 patients with carcinoid or carcinoid-like syndromes. Serial blood sampling indicated that raised plasma levels of free kinin occurred in a patient only during the appearance of flushing (Fig. 4). The level of free kinin measured in these patients seemed closely related to the severity of the flushing (Fig. 5), the patient with the most severe flushing having the highest level. The activity was shown to be kinin-like by parallel bio-assay on isolated oestrus rat uterus and rat duodenum, and the appearance of free kinin-like activity was associated with a fall in kininogen.

Whole blood 5-HT levels obtained after flush provocation and twenty-four hour urinary 5-HIAA levels are known for 4 of the patients (Table 17) and, although generally greater than normal, show no clear relationship to the severity of their symptoms. In these patients at least, although abnormal indole metabolism is obviously associated with their condition, the release of free kinin more reasonably accounts for the appearance of their vasomotor symptoms.

The origin of the kinin is indicated by the finding in carcinoid tissue of abnormal levels of proteolytic activity (Pearse and Pepler, 1957), later characterized chromatographically by Oates et al (1964) as a kinin-releasing enzyme different from that of blood. Oates and his colleagues found that during carcinoid flush, raised levels of this enzyme appeared in the circulation.

It should not be concluded, however, that plasma kinins are the only, or ultimate humoral carcinoid factors. A survey of the literature shows that the syndrome varies from patient to patient. Even the flush may range from the cyanotic colouration first described by Thorson et al (1954), possibly ascribable to circulating 5-HT, to the brick or lobster flushing seen in the present series of patients. It seems likely that 5-HT may be the major humoral factor in some patients, histamine in others,

Patient	Severity of Symptoms	Whole-blood 5-HT (µg./ml.)	Urinary 5-HIAA (mg./24 hr.)
1	Continuous lobster colour	0.82	31
2	Transient flushing	2.00	70
3	Transient flushing	0.35	9.3
4	Non-flushing	0.37	255
Normal Range		0.05 - 0.3	2 - 13

Table 17

Levels of Whole-blood 5-HT Measured during Symptoms, and Urinary Excretion of 5-HIAA in Carcinoid Patients

kinin in yet others. The inclusion in the present series of a patient (patient 5), whose formerly gastrin-secreting hepatic tumour changed over a period of years into a carcinoid-like tumour producing raised levels of indoles and plasma kinin, indicates the possibility of even more complex relationships.

THE DUMPING SYNDROME

1(a) Introduction

The dumping syndrome occurs in patients who have been subjected to gastric surgery. It consists of an intestinal component which may include hyperperistalsis, epigastric discomfort, bloating, fullness, cramps, nausea, vomiting and diarrhoea; and a vasomotor component which may include weakness, dizziness, pallor or flush, vertigo, desire to lie down, palpitations, sweating, tachycardia, and increased peripheral blood flow (Machella, 1949, 1950; Fisher and Cannon, 1955; Weidner, Scott, Bond and Shull, 1959; Silver, Anlyan, Postlethwaite, Morgan and Mengel, 1965). The dumping syndrome occurs during or immediately after eating, and particularly eating carbohydrates. The syndrome is usually provoked for experimental purposes by ingestion of hypertonic glucose solution (Machella, 1949; 1950; Fisher and Cannon, 1955; and others), although a variety of hypertonic solutions (including saline, xylose and protein hydrolysates) have been similarly used (Machella, 1949, 1950; Roberts, Randall, Farr, Kidwell, McNeer and Pack, 1954; Sessions, Reynolds, Ferguson and Scott, 1962; Geokas, Solymar and Beck, 1967; and others).

The very extensive experimental work on the subject has been fully reviewed by Miller and Peskin (1963), and the present review will be concerned in the main, with aspects most directly related to the work described in this thesis.

b) Development of a "Humoral" Theory of Dumping

Dénechau (1907) and Hertz (1913) were the first to describe functional disorders following gastric surgery for peptic ulcer. They noted that nausea, vomiting, epigastric fullness, and diarrhoea appeared in gastrectomized patients after meals. Using X-ray examination, Hertz found rapid emptying of the stomach remnant in these cases, and he ascribed the symptoms to the resultant distension of the jejunum. Mix, in 1922, introduced the term "dumping stomach" to describe the rapid emptying.

Direct distension of the jejunum by the bolus of food was, for many years, widely believed to be the primary cause of the dumping syndrome (Hertz, 1913; Zollinger and Hoerr, 1947; Irvine, 1948; Muir, 1949). In 1949, Machella (1949, 1950) made an important contribution to the study of the syndrome when he showed that the major factor in producing dumping was not the bulk, but the hypertonicity of the ingested substances. He noted that the symptoms occurred during the hyperglycaemic phase following ingestion of hypertonic glucose, and abated as the blood sugar fell. As earlier workers had suggested that dumping symptoms might be due to the hyperglycaemia (Glaessner, 1940; Zollinger and Hoerr, 1947), Machella showed that in dumping patients, hyperglycaemia alone, produced by intravenous glucose, caused no symptoms. He further showed that instillation of

virtually any kind of hyperosmotic solution (300 milliosmolar) via a tube into the proximal jejunum of both gastrectomized and normal subjects, could reproduce the typical dumping syndrome, while simultaneously causing a dramatic increase in the secretion of fluid into the intestinal lumen. Distension of the jejunum with a balloon produced some of the symptoms of dumping, and Machella thus postulated that the sudden outpouring of fluid into the small intestine to dilute its hypertonic contents, caused distension, and subsequent dumping symptoms. In the normal subject, however, the intact stomach acted as a reservoir for the gradual dilution of hypertonic contents, and only isotonic contents passed into the small intestine.

The findings of Machella were tested in 1953 by Roberts and her colleagues (Roberts et al, 1953, 1954). They confirmed that hypertonic solutions of a variety of solutes administered intrajejunally, would provoke dumping, but found that mechanical distension alone, of the jejunum, would not provoke dumping symptoms. During the course of these studies, Roberts measured blood volumes, and found a consistent decrease in plasma volume of 500 - 1000 ml. in patients during the acute phase of dumping. The finding of a decreased plasma volume has since been confirmed by many workers (Hinshaw, Joergensen, Davis and Stafford, 1957; Morris, Greenfield, Jordan, Peddie, Gordon and DeBakey, 1959; Scott, Weidner, Shull and Bond, 1959; Weidner et al, 1959;

LeQuesne, Hobsley and Hand, 1960; Sessions et al, 1962; and others). These observations formed the basis of what was, until recently, a very widely accepted concept of the dumping mechanism. The symptoms were thought to be provoked by the presence of hypertonic material in the jejunum. This causes a massive shift of fluid into the lumen to dilute its contents, producing distension, and consequent intestinal symptoms. The fluid shift also causes a reduction in plasma volume, which in turn triggers sympathetic vasoregulatory reflexes, and thus produces the vasomotor changes of dumping. However, this theory has been seriously challenged. The decrease in plasma volume has been found to correlate badly with the appearance of symptoms, while attempts to prevent the symptoms by intravenous saline infusions to maintain plasma volume have been unsuccessful (Webber, Bender and Moore, 1957; Duthie, Irvine and Kerr, 1959; Lawrence and Matthews, 1959; Butz, 1961).

In 1951, Capper and Butler published an observation which had been noted by most earlier workers, but had not been regarded as greatly significant. Gastrectomized patients who dumped when upright, showed no symptoms when supine. These workers inferred from this that the syndrome was produced by the abnormal weight of the over filled intestine dragging on the gastric remnant when the patient was in the erect position, while when the patient was

horizontal, the gut was adequately supported, and no symptoms were produced. Hinshaw et al, in 1957, showed that, contrary to general belief, there was an increase in cutaneous and peripheral blood flow in dumping patients with moderate to severe vasomotor symptoms (e.g. dizziness, weakness, sweating, tachycardia), the blood flow change being greater when symptoms were more severe. Patients who did not show these symptoms, but only the gastro-intestinal abnormalities during dumping, had a vasoconstrictor response, with decreased blood flow.

The picture became clearer when Cox and Allan (1961) measured the blood pressure and peripheral blood flow of dumping patients in both the upright and supine positions. They found that when recumbent, although patients showed considerable vasodilatation, their blood pressure and pulse rate rose slightly. Patients who stood during dumping, however, showed marked postural hypotension, with concomittant syncope-like symptoms. The findings of vasodilatation during dumping have since been confirmed in thorough studies by Castenfors (1961), and Christoffersson (1965). Hypotensive episodes were not seen consistently by these workers. However, this discrepancy may be explained by the fact that Cox and Allan (1961) made their measurements with patients standing, thus increasing any pressure fall resulting from failure of the homeostatic mechanisms, while Castenfors and Christoffersson made their observations with patients usually either sitting or lying.

c) Evidence for the release of a humoral factor

In 1961, too, Johnson and Jesseph initiated an important new line of investigation into the dumping mechanism. These workers found that portal venous blood collected from anaesthetized dogs which had received intrajejunal hypertonic glucose produced a reaction very like the dumping syndrome when transfused into conscious normal recipient dogs. Portal blood from normal donor animals produced no reaction when transfused into conscious dogs. This finding was the first real evidence for the release of a humoral "dumping factor" during the syndrome.

Although Johnson and Jesseph made no attempt to define this transfusible factor, much evidence was already in existence to give an apparent clue as to its possible identity. The tissues of the jejunum and duodenum were known to contain 5-HT (Erspamer, 1954; Resnick and Gray, 1961) and argentaffin cells (Erspamer, 1939) in high concentration. It had been demonstrated that both *in vitro* (Toh, 1954; Baker, 1958; Bülbring and Lin, 1958; Bülbring and Crema, 1959), and *in vivo* (O'Hara and Cole, 1959; O'Hara, Fox and Cole, 1959), 5-HT was liberated from the jejunal mucosa by distension and also by contact with hyper- and hypotonic sugar solutions. Serum 5-HT levels were known to be raised in man, following experimentally provoked increase

in bowel activity (Adams, 1960). Finally, it had been shown that intravenous 5-HT produced a marked suppression of pancreatic secretion (Drapanas, Pollack and Shim, 1961), and that external pancreatic secretion was diminished in animals following intraduodenal instillation of hypertonic glucose and saline (Lawrence, Khentigan, Hudock and Vanamee, 1961). It was thus suggested by Drapanas and Shim (1961) that the suppression of pancreatic function during experimental dumping in dogs might be mediated by 5-HT released from the intestine.

With this background of circumstantial evidence, plus the fact that intravenous 5-HT produces intestinal and vasomotor changes similar to those of the dumping syndrome (Hendrix, Atkinson, Clifton and Ingelfinger, 1957; Haverback and Davidson, 1958) it was not surprising that, in 1962, a number of groups should examine the release of 5-HT and its metabolites during dumping. Drapanas, McDonald and Stewart (1962), and Peskin and Miller (1962) found raised levels of 5-HT in venous blood draining the small intestine during experimental dumping in dogs. Walker, Turner and Hardy (1962) were unable to detect this increase in 5-HT without prior administration of an amine-oxidase inhibitor to prevent its destruction. Johnson and his associates (Sloop, Johnson and Jesseph, 1962) repeated their earlier transfusion experiments, while monitoring the transfused blood for 5-HT content. They found that blood causing dumping

in the recipient dog usually, but not always, contained a raised 5-HT level. These workers postulated the involvement of a second humoral factor to explain this result. In addition, Johnson's group (Johnson, Sloop, Jesseph and Harkins, 1962) and Peskin and Miller (1962, 1965) reported the suppression of dumping symptoms in both animals and man, using 5-HT antagonists.

In contrast to these findings, Sessions et al (1962) found no increase in peripheral blood 5-HT levels during dumping in three subjects; while Schmid, Meythaler, Schön and Henning (1962) found no increase in the human urinary excretion of 5-HIAA, the 5-HT metabolite, during dumping.

More recently, other workers have investigated 5-HT involvement in the dumping syndrome. Howe (1964) found no statistical correlation between 5-HIAA excretion, and the appearance or severity of symptoms in 13 dumping patients. Sullivan and Patton (1964) found that blood 5-HT levels in man do not vary in any particular way during dumping. Silver and his colleagues have examined several aspects of the problem. Working with dogs (McGregor and Silver, 1965; Silver, McGregor, Porter and Anlyan, 1966), they found that intrajejunal instillation of both hypertonic (50%), and nearly isotonic (5%) glucose caused an increase in portal blood 5-HT levels, together with an increase in the number of argentaffin staining sites in

jejunal mucosa obtained at biopsy. Increases in systemic blood 5-HT levels with dumping were not always found. Since the portal blood 5-HT level rose, following instillation of both 50% and 5% glucose solution, Silver then looked at the effect of intrajejunal pressure changes on 5-HT release (Silver et al, 1966). He found that increased intraluminal pressure was also accompanied by a rise in portal 5-HT. He completed his animal investigations by showing that a 5-HT antagonist (methysergide maleate) largely suppressed dumping symptoms in dogs.

These results, however, do not appear to parallel results obtained by this group from investigations into human dumping. They examined 88 dumping patients, and found no significant relationship between either peripheral venous plasma or platelet 5-HT levels or urinary 5-HIAA levels, and the presence or severity of symptoms. While, in a double blind controlled experiment with 14 dumping patients, Silver et al (1965) found that the 5-HT antagonist, methysergide maleate, produced no consistent or prolonged improvement of symptoms.

Most recently, evidence (albeit highly circumstantial) suggesting the existence of a humoral dumping factor in man, has been presented by Geokas and Beck (1966). These workers produced experimental dumping by intra-jejunal instillation of hypertonic glucose in a single patient suffering from cirrhosis of the liver. They did this before and after a portal-systemic

shunt. Before the operation, intrajejunal glucose provoked only mild vasomotor and intestinal changes. Post-operatively, dumping provocation produced a markedly exaggerated response, manifested by prolonged flushing and other vasomotor and gastro-intestinal symptoms. These authors pointed out that dog liver *in vivo* has been shown to remove up to 80% of infused 5-HT (Drapanas and MacDonald, 1963). Allowing the portal venous contents to by-pass the liver would permit any such humoral factor to pass directly into the systemic blood in unusually high concentrations. This, they postulated, might explain the increase in symptom severity.

d) **Some other theories**

The foregoing review has of necessity been highly selective, and has been presented with a view to giving the background directly leading up to the present work. In addition to the "humoral" postulate upon which this work is based, a number of other theories were referred to. Some further mechanisms which have been suggested as aetiological factors in the dumping syndrome, but which have not been mentioned in the main argument, include increased jejunal motility (Culver, 1949; Glazebrook and Welbourn, 1952), hypokalaemia (Smith, 1951), and adrenal stimulation (Pontes and Neves, 1953).

Many workers have reported hypermotility of the small intestine during dumping, amongst these are Glazebrook and Welbourn (1952), Jordan, Overton and DeBakey (1957) and Amdrup, Hjort, and Jörgensen (1958). Although this increased motility has been considered to be the main cause of the dumping syndrome (Culver, 1949; Glazebrook and Welbourn, 1952), a number of workers have more recently shown that hypermotility can be detected only when dumping consists of intestinal symptoms alone (Cox and Allan, 1960; Christoffersson, Kewenter and Kock, 1962; Wangel and Deller, 1965). These workers have found that, in patients exhibiting vasomotor dumping, the typical motility pattern consisted of a brief hypermotile burst, followed by an inhibition of motility during the period of vasomotor symptoms. The significance of this finding in respect of the present investigation will be considered in the discussion.

Hypokalaemia is not specific to dumping, but accompanies the deposition of glycogen which follows loading with glucose, glycogen deposition being associated with intracellular binding of potassium (Fenn, 1939). Hypokalaemia is not seen when dumping is provoked with hypertonic saline (Roberts et al, 1954). The fall in plasma potassium occurs long after the dumping symptoms have commenced (Roberts et al, 1954; Webber et al, 1957;

Duthie et al, 1959), and is seen in both gastrectomized and normal, dumping and non-dumping subjects following ingestion of glucose (Roberts et al, 1954; Webber et al, 1957). Smith (1951), who first suggested hypokalaemia as responsible for the dumping syndrome, was unable to prevent the symptoms in patients by intravenous infusion of potassium solutions.

Finally, the finding of eosinopaenia, cited by Pontes and Neves (1953) as one of the signs of increased adrenal stimulation during dumping in man, could not be confirmed by Roberts and her colleagues (1954). The other findings of Pontes and Neves, which include raised urinary uric acid, decreased urinary sodium chloride, increased endogenous steroid production, and other changes in the blood, which they claim to indicate increased activity of the adrenal cortex during dumping, have not been examined by other workers. However, it seems reasonable to expect an increase in adrenal activity as the result of the stress arising from the intestinal and vasomotor changes of dumping.

e) <u>The Present Investigation</u>

The dumping and carcinoid syndromes are alike in many respects. Both conditions have an intestinal and a vasomotor component. In both, the vasomotor phase is usually, but not invariably, dilator, and is often associated with facial flush and sweating.

In both conditions, 5-HT has been implicated as a possible humoral factor, but in neither does the release of 5-HT into the blood stream satisfactorily explain all the aspects of the condition. In the case of the carcinoid syndrome, the involvement of a second vasoactive substance, possibly kinin-like, was postulated (Robertson et al, 1962; Lancet, 1962), and the release of bradykinin during carcinoid flushing episodes was eventually demonstrated (Oates et al, 1964; Oates and Melmon, 1966). Many workers have suggested the release of another, unidentified principle during the dumping syndrome in addition to 5-HT, to explain anomalous results (e.g. Castenfors, 1961; Sloop et al, 1962; Howe, 1964). While Silver et al (1965), in a paper published after the start of this investigation, pointed out that kinin-like substances are capable of reproducing the symptoms of dumping in man, and could be involved in the production of the syndrome.

Prior to the present investigation, A. N. Smith (Zeitlin and Smith, 1965) had examined 5-HT metabolism during dumping in four partially gastrectomized male patients. Whole blood 5-HT levels were measured in their venous blood before and during vasomotor dumping provoked by oral ingestion of 250 ml. of 20% glucose. The mean 5-HT concentration, at the height of dumping symptoms was not significantly greater than the mean pre-dumping control. In these same patients, the urinary excretion of the

5-HT metabolite, 5-HIAA, was measured for a twenty-four hour period, during which repeated attacks, three or four times per day, had been provoked by normal food intake. The mean 5-HIAA excretion of these patients was more than 2.5 times that measured in seven healthy subjects, and this difference was highly significant. Since the urinary 5-HIAA estimate contained the accumulated excretion resulting from several dumping episodes for each individual, it was a more sensitive test for a release of 5-HT (and its consequent breakdown), than was the measure of blood 5-HT levels in a single dumping episode for each patient. Thus dumping patients were found to have an abnormal indole metabolism, possibly the result of a release of gut 5-HT into the bloodstream during the syndrome. However, at the height of the syndrome, the peripheral blood 5-HT levels, not significantly greater than normal, were certainly too low to explain the severity of the vasomotor changes seen in these subjects.

Because of the similarities between the carcinoid and dumping syndromes, and because a release of plasma kinin could reasonably account for most of the vasomotor changes of dumping, the present investigation was conducted.

2. Clinical Material and Procedures

Four patients with post-cibal dumping were investigated. They were males aged 30 - 55 years, and had all previously had a Polya partial gastrectomy. They were investigated at least six months after operation, and were selected because their vasomotor symptoms took the form of vasodilator, hypotensive episodes, with marked facial flushing.

For the purposes of the experiment, dumping was provoked by ingestion of 50 g. glucose in 250 ml. warm water, following overnight fasting. Blood samples were taken from an arm vein, before provocation, at the height of the vasomotor symptoms, and after each attack, and assayed for plasma kinin and kininogen as described in Appendix 2. The dumping features usually reached a peak between 10 - 20 minutes following ingestion of the glucose solution, and had generally subsided at 30 minutes. The blood pressure and pulse rate were examined while the patients were standing.

As a control experiment, a placebo of 250 ml. water, sweetened with saccharin comparable to the hypertonic glucose dumping stimulus, was administered to three of these patients. As a further control, 250 ml. of hypertonic glucose was administered to four healthy male volunteers. In both control experiments, blood samples were taken before and after the dumping stimulus, and assayed for kinin and kininogen content.

3. Results

Kininogen and free kinin levels were estimated in the venous blood of these patients before and during dumping. The results are shown in Table 18. With patient D, one dumping provocation was carried out; with each of patients A and C, dumping provocation was carried out twice on separate days. With patient B, one of the dumping experiments gave unusually high control and peak free kinin levels (excluded for the purpose of calculation of means etc.) and a third experiment was carried out with this patient. In every case, a striking increase in free kinin occurred during the syndrome (Table 18; Fig. 7). The peak levels showed wide scatter, but averaged 37 times the mean pre-dumping control level. Because of the wide scatter of the values found, the free kinin levels at the dumping peak were compared statistically with the pre-dumping control values. Such calculation is not a statistical analysis of the experiment, since, because of the scatter, this would require an impracticably large number of subjects to give a significant answer. It does, however, show whether for this set of results, the change in levels occurred too often to be explained by the scatter. The skewness of the values made them unsuitable for analysis using the usual parametric student 't' test, and the non-parametric Wilcoxon signed-ranks test (c.v. Documenta Geigy (1962), P. 191) was used (Appendix 6). The increase in

	Bradykinin Equivalents of:					
	Kinin precursor (µg./ml.)			Free Kinin (ng./ml.)		
	Pre-dump control	Dump peak	After Dump	Pre-dump control	Dump peak	After Dump
Dumping patients (provocation)*						
A	7.6	3.9	5.8	2.4	75.0	<1.0
	5.5	4.0	5.2	<2.7	140.6	<3.5
B	5.0	4.7	3.9	<3.5	43.7	3.7
	8.4	7.0	7.8	68.5	2615.0	1046.0
	6.9	5.6	6.5	1.5	38.0	8.5
C	12.8	12.6	13.1	6.3	62.9	<0.7
	4.9	1.8	2.0	0.6	70.5	139.9
D	8.1	7.2	7.2	<3.7	15.3	<3.7
Healthy volunteers (provocation)*						
E	3.7	3.5	3.6	1.7	2.7	2.7
F	4.5	4.6	4.5	3.7	2.4	2.6
G	2.9	2.8	2.5	<3.0	<3.0	4.7
H	2.1	2.0	2.1	<2.2	<2.2	<2.2
Dumping patients (placebo)+						
A	5.3	4.8		2.9	1.9	
B	4.9	5.0		2.5	8.8	
C	4.3	4.3		1.7	<1.7	

* Provocation by hypertonic glucose. + Saccharin.

Table 18

Plasma Levels of Kinin Precursor and Free Kinin in Dumping Patients and Healthy Volunteers

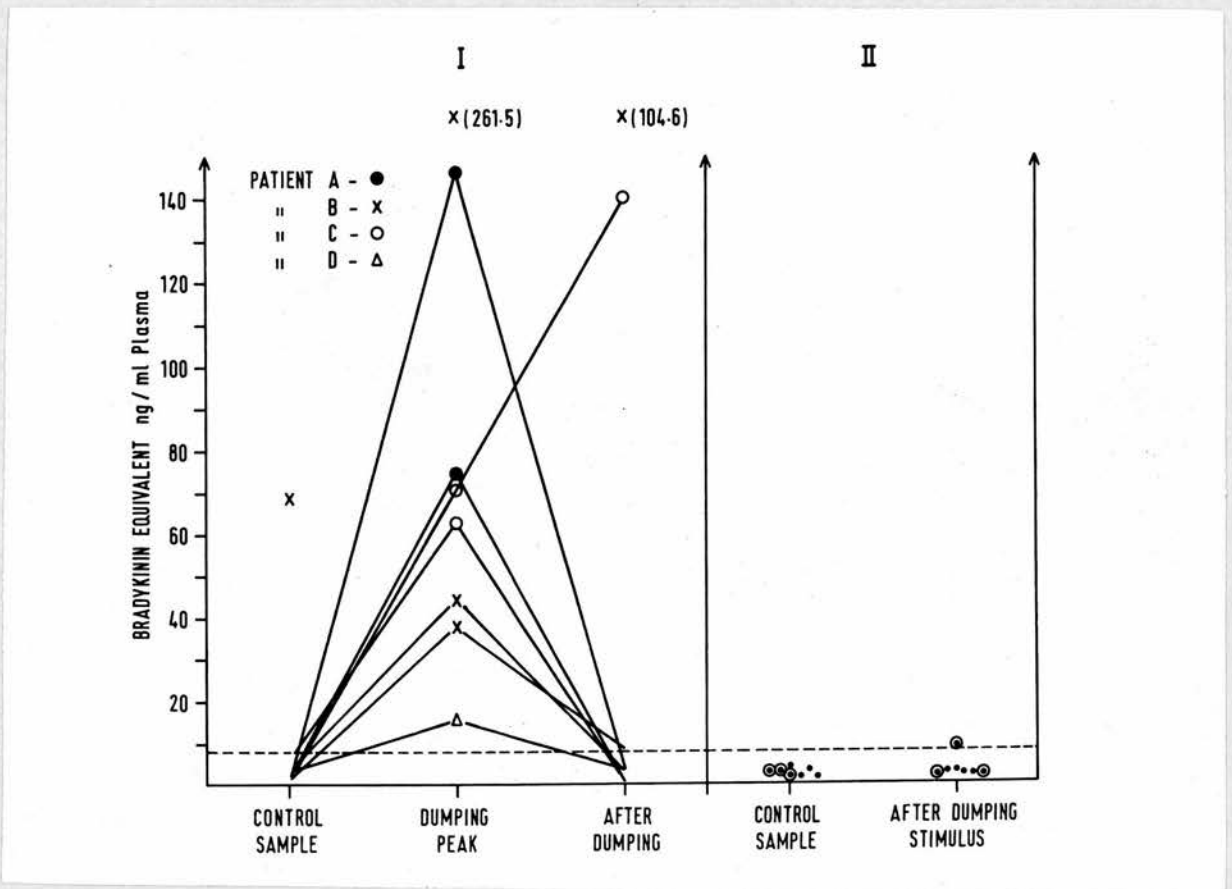

Fig. 7: Levels of venous free-plasma-kinin measured before, during, and after the vasomotor changes following dumping provocation. Interrupted line represents the 99% upper fiducial limit of the mean normal value.

I. Levels in dumping patients A - D; dumping provoked by ingestion of 250 ml. hypertonic glucose.

II. Levels in dumping patients A - C (⊙), after ingesting 250 ml. hypotonic saccharin placebo and in healthy controls E - H (•) after ingesting 250 ml. of hypertonic glucose.

free kinin level was found to be highly significant, with a $P \leq 0.01$. In every case, too, simultaneously with the increase in free kinin level, a fall in plasma kininogen was observed (Table 18; Fig. 8). The mean level of kininogen at the dumping peak was 75.8% of the pre-dump control value, and this change, too, was highly significant.

The increase in free kinin-like activity and fall in kininogen level for each patient was coincident with the appearance of facial flushing, and a transient hypotension (Fig. 9), which usually occurred 10 - 20 minutes after provocation.

To assess whether the kinin release was truly related to the dumping syndrome in these patients, and was not merely a non-specific response produced by ingesting 250 ml. of fluid, or perhaps by the emotional stress of sample taking, a sham experiment was carried out with three of the four patients. Provocation of the syndrome was attempted with a placebo consisting of 250 ml. of saccharin-flavoured water. In no patient did dumping symptoms occur, nor was there an increase in plasma kinin activity (Table 18; Fig. 7). The mean decrease in plasma kininogen for the dumping patients (1.6 µg. BK Eq. per ml. plasma) when provoked with glucose, was greater than the

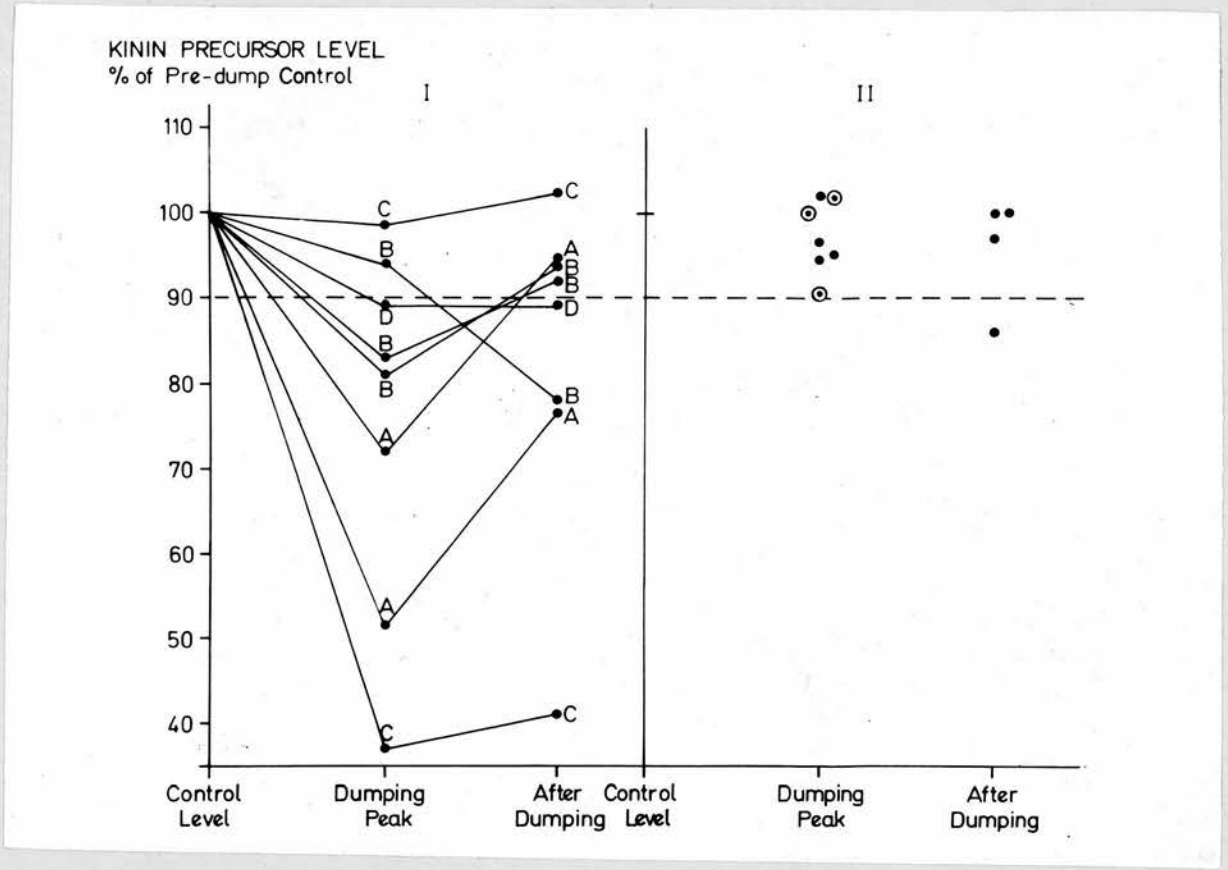

Fig. 8: Kinin precursor levels in venous plasma at the height of and the cessation of the vasomotor symptoms of the dumping syndrome.

I. Levels in dumping patients A - D; dumping provoked by ingestion of 250 ml. of hypertonic glucose.

II. Levels in dumping patients A - C (⊙), after ingesting 250 ml. of hypotonic saccharin placebo; and in healthy controls E - H (·), after ingesting 250 ml. hypertonic glucose.

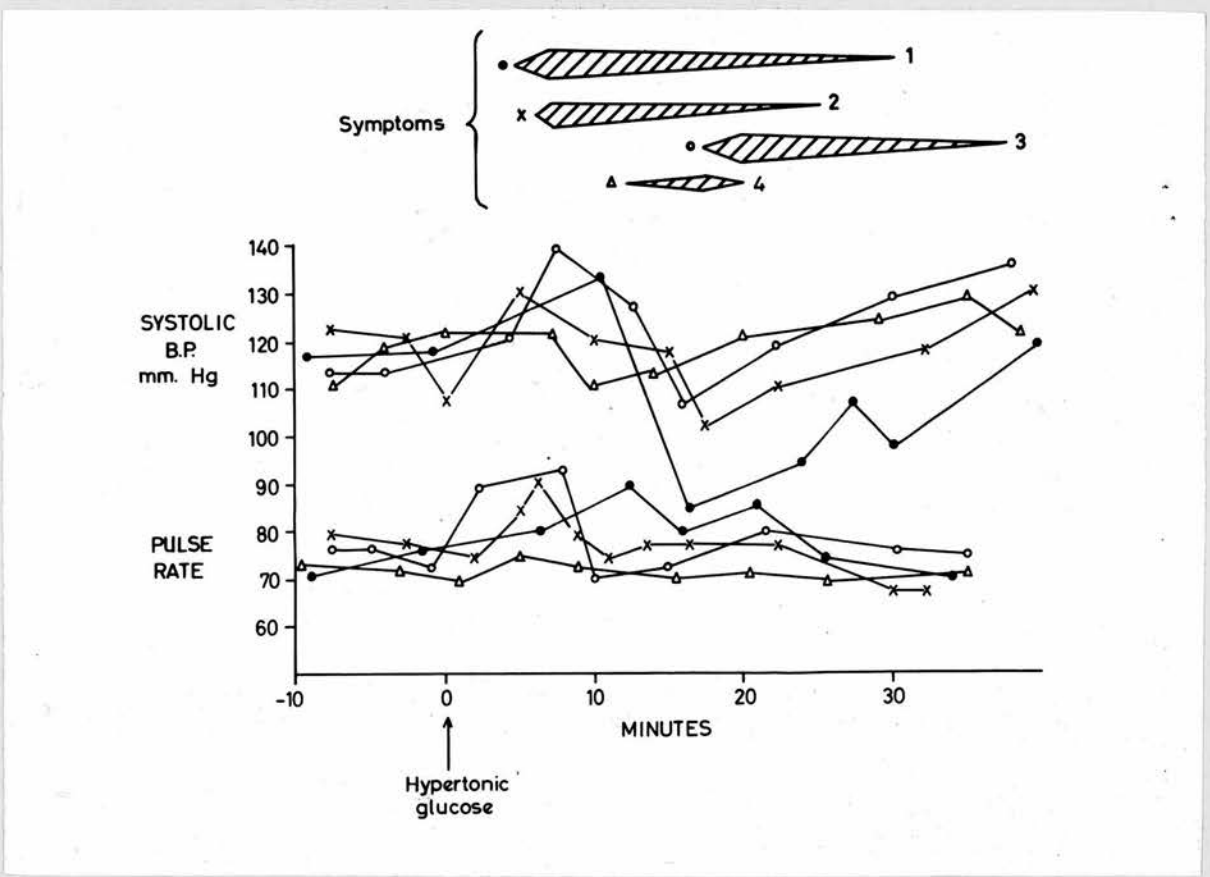

Fig. 9: Systolic blood pressures and pulse-rates measured in dumping patients A - D in the standing position, before, during, and after dumping provoked by ingestion of 250 ml. of hypertonic glucose. Hatched areas represent clinical assessment of the severity of symptoms.

change seen after saccharin placebo (0.2 µg. BK Eq. per ml.), and this difference was significant (P = 0.028).

Finally, to determine whether the kinin release found in the dumping patients was directly related to their dumping syndrome, and not merely an unrelated effect of ingesting hypertonic glucose, 250 ml. of hypertonic glucose was administered orally to four healthy volunteers. Again, in these individuals, no significant symptoms, nor change in free plasma kinin was detected, and the mean change in plasma kininogen level (0.125 µg. BK Eq. per ml. plasma) was significantly less than that seen in the dumping patients when provoked with hypertonic glucose (P = 0.014).

4. Characterization of Bradykinin-like Activity

The free kinin-like activity found in human venous blood samples in the presence of vasomotor dumping symptoms was shown to be bradykinin-like. The characterization tests have been necessarily limited owing to the small quantity of kinin-like activity available. However, it has been possible to carry the tests a stage further than those used on the carcinoid samples. As was found with the carcinoid samples, the active principle contracted the oestrus rat uterus in the presence of atropine and BOL, upon which it was routinely assayed, and relaxed the isolated rat duodenum (Fig. 10). As was also

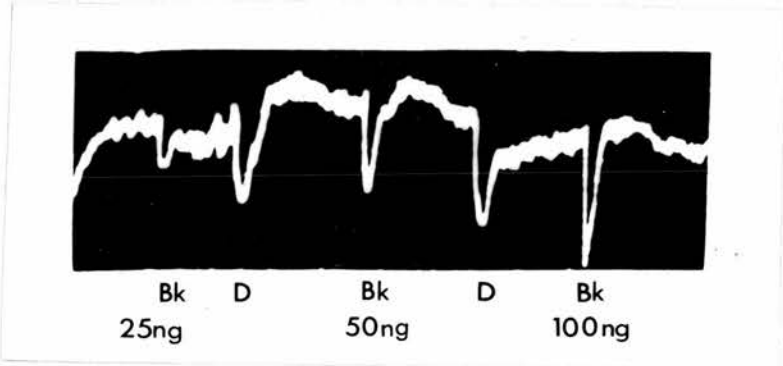

Fig. 10: Smoked drum tracing showing relaxation of the isolated rat duodenum by bradykinin-like activity in extracts from the venous blood of a dumping patient. (Bk = Standard bradykinin; D = sample from dumping patient).

described in the carcinoid section, by this means it was possible to distinguish the principle from catecholamines, histamine, gastrin, acetyl choline, 5-HT, substance P, angiotensin, and SRS-A.

In addition to this, activity from two dumping patients was destroyed when incubated with chymotrypsin (1 mg./ml.) in de Jalon's solution, at 37°C, for one hour (Table 19 (a)). One incubation experiment was assayed using the isolated rat uterus, and the chymotrypsin was found to have destroyed all but 2.5% of the activity measured in the control incubated in the absence of chymotrypsin. The other was assayed on rat duodenum, and the residual activity was found to be less than 5.0% of that measured in the control. The chymotryptic degradation of the active principle demonstrated its polypeptide nature.

The most conclusive evidence for the identification with bradykinin of the oxytocic activity released during dumping, was given by parallel bio-assay on three different tissues. Activity from one sample was compared with standard synthetic bradykinin using three different bio-assay tissues, the oestrus rat uterus, the guinea pig ileum, and the rat duodenum. When all three estimates were divided by the lowest estimate to give the indices of discrimination (Gaddum, 1955), they were found to be in the ratio of 1.1: 1.0: 1.0 for the rat uterus, guinea pig ileum, and rat duodenum respectively (Table 19 (b)). This indicates that

Table (a)

Sample	Assay Tissue	Kinin-like Activity (ng. BK Eq./ml.)		Residual Activity as % of Control
		Control	Control + Chymo-Trypsin	
D (1)	Rat Uterus	30	0.75	2.5
D (9)	Rat Duodenum	150	<7.5	<5.0

Table (b)

Assay Tissue	Sample D (2)		Index of Discrimination
	Total Kinin Content of Extract (ng. BK Eq.)		
	Brackets	Assay	
Rat Uterus	232.5-252.5	242.5	1.1
Guinea Pig Ileum	176.4-264.4	220.5	1.0
Rat Duodenum	142.9-285.7	214.3	1.0

Table 19

(a) Destruction of Free-Kinin Activity from Dumping Patients by Incubation with Chymotrypsin (1 mg./ml.) in de Jalon's solution at 37°C for one hour.

(b) Parallel bio-assay of Free-Kinin Activity from a Dumping Patient

each of the three tissues has almost the same sensitivities to both the active principle and bradykinin. This is unlikely to be the case if the two were not the same or very similar substances.

Finally, as has already been shown, the appearance of raised kinin-like activity was associated with a decrease in the plasma level of kininogen (Fig. 8). In fact, this fall in precursor level was directly related to the size of the increase in free kinin activity over the pre-dumping control level ($P < 0.05$) (See Appendix 6), the greater the kinin release, the greater the fall in precursor level.

Thus the evidence indicates that the activity found in human blood in the presence of dumping vasodilatation was a peptide, probably originating from a plasma kinin precursor protein. It was plasma kinin-like, and unlikely to be due to catecholamines, histamine, gastrin, acetyl choline, 5-HT, substance P, angiotensin, and SRS-A. Quantitative parallel bio-assay showed that the activity was closely identified with bradykinin.

5. Discussion

The similarity between the carcinoid and dumping syndromes in their haemodynamic pictures, their changes in intestinal motility and in their as yet unclear association with indole metabolism,

has been remarked upon by several authors (e.g. Sloop et al, 1962; Howe, 1964). This similarity has now been shown to extend to the release of plasma kinin during the vasodilator phase seen in many dumping patients.

The levels of plasma kinin and kininogen were examined during dumping in four male patients with partial gastrectomy. Their symptoms were characterized by syncope-like, hypotensive, vasodilator episodes. The symptoms were provoked by drinking a standard stimulus of 250 ml. of warm 20% glucose solution (isotonic $\simeq$ 5.34% glucose). In every case, with the onset of flush and hypotension 10 - 20 minutes after provocation, a dramatic rise was detected in free plasma kinin, characterized by parallel bio-assay and chymotrypsin degradation. This was associated with a fall in plasma kininogen. The changes in kinin and kininogen levels were directly related to the appearance of the patients' dumping symptoms. When the glucose stimulus was replaced by a saccharin flavoured placebo, neither the symptoms nor the kinin or kininogen changes were provoked. When the glucose stimulus was given to four healthy males possessing intact gastro-intestinal tracts, again neither symptoms nor changes in plasma kinin were seen.

The severity of the vasomotor symptoms were clearly related to the size of the kinin release, and there was a clear correlation between the maximum mean drop in systolic blood pressure

for each dumping patient and his mean peak free-plasma-kinin level ($P < 0.05$) (See Table 20). The release of plasma kinin as indicated by these results could thus reasonably account for the vasodilatation seen in the dumping syndrome.

Such a kinin release might provide one explanation for a puzzling aspect of the dumping syndrome. Hypermotility of the small intestine during dumping attacks was observed by, amongst others, Glazebrook and Welbourn (1952) and Jordan, Overton and DeBakey (1957), and this was generally believed to be the major intestinal symptom. However, not all patients with dumping syndrome showed this symptom. For instance, Jordan et al, (1957), found that 4/20 of their patients showed dumping symptoms and no increase in intestinal motility. More recently, improved techniques and a more critical analysis of the intestinal changes have shown that increased motility occurs as a major symptom only when there are no marked vasomotor symptoms. When vasomotor dumping occurs there is a brief hypermotile burst lasting from two to eight minutes (Christoffersson, Kewenter and Kock, 1962; Wangel and Deller, 1965), followed by a prolonged inhibition of motility which coincides with the vasomotor symptoms (Cox and Allan, 1960; Christoffersson, Kewenter and Kock, 1962; Wangel and Deller, 1965).

Patient	Mean Peak Free Kinin (ng. BK Eq./ml. plasma)	Mean Drop in Systolic Blood Pressure (mm. Hg.)
A	107.8	49
C	66.7	33
B	40.9	29
D	15.3	12

Table 20

Relationship between Free-Plasma-Kinin Level and Blood Pressure at the Height of Dumping Symptoms.

In vitro, in guinea pig ileum (Beleslin, Bogdanović and Radmanović, 1964) and in human jejunum, ileum and colon (Fishlock, 1966), it has been shown that while bradykinin contracts the longitudinal muscle, it relaxes the circular muscle and inhibits peristalsis. Intravenous infusion of bradykinin in the cat and rabbit produces inhibition of intestinal motility (Bauer, Gmeiner and Winkler, 1966). In man, while it is known that intravenous bradykinin inhibits colonic motility (Murrell and Deller, 1967) its effect on the small intestine is not yet known, however the in vitro results suggest that here too it would inhibit motility. It is possible that the vasomotor changes of dumping provoke the release of adrenaline into the circulation and consequent intestinal hypomotility. However, the coincidence of raised levels of plasma kinin with vasomotor dumping might also reasonably explain the inhibition of intestinal motility.

The many similarities between the carcinoid and dumping syndromes have already been discussed, but with respect at least to kinin release in the two syndromes, it seems likely that there is a fundamental difference. Raised levels of a kinin-releasing enzyme have been found in carcinoid tumour and in blood during the carcinoid syndrome (Oates et al, 1964); and this provides a likely explanation for the origin of the raised levels of kinin in this condition.

It seems unlikely that kinin released in the dumping syndrome can be attributed to a similar proliferation of kallikrein-containing cells, since it is a common observation that intrajejunal solutions can produce typical vasomotor dumping symptoms in perfectly healthy normal subjects (e.g. Machella, 1949, 1950, and others). The intestinal mucosa has been shown to contain kinin-releasing enzyme (Amundsen and Nustad, 1965). It is possible that gut hypermotility, contact with hypertonic solution, or distension of the intestinal wall may cause release of intestinal stores of kallikrein, similar to the release of 5-HT which is known to occur under such conditions. Another possibility, discussed in the following section, is that plasma-kinin release is secondary to the release of 5-HT or adrenaline.

KININ RELEASE AS A RESULT OF PHYSIOLOGICAL STRESS OR OF RAISED CIRCULATING LEVELS OF 5-HT OR ADRENALINE IN MAN AND DOG

1. Rationale

The experiments described in this section were prompted by three separate lines of thought. As has already been discussed, results presented in the literature indicate the release of 5-HT into the circulation as a factor in both the carcinoid and dumping syndromes. It has been shown that during carcinoid flush, raised levels of bradykinin may be found in the circulation. It has been further shown that plasma-kinin release can occur, not only during the carcinoid flush, but also during the vasodilatation often seen during dumping. Oates and his colleagues (1964) produced results indicating that the carcinoid tumour tissue itself contains abnormally raised levels of kinin releasing enzyme. These workers concluded that it is enzyme released from this source as a result of suitable provocation, rather than activated plasma kallikrein, which produces the vasomotor symptoms of carcinoid syndrome. For the reasons discussed in the previous section, a similar mechanism cannot be proposed for kinin release in dumping.

The plasma kinin release found in the clinical conditions may be secondary to the release of another humoral principle, such as 5-HT, from the gut or tumour tissue, or adrenaline from the

adrenal medulla. In both the dumping and carcinoid syndromes, not only has 5-HT release been implicated, but also both conditions are precipitated or exacerbated by emotional or other types of stress which may cause sympathetic stimulation and adrenaline release; while the intestinal symptoms of these conditions, in themselves, provide sufficient stress to stimulate further adrenaline release. The carcinoid syndrome, in particular, may be provoked by intravenous injection of small amounts of adrenaline (Robertson et al, 1962; Oates et al, 1964).

A possible relation between physiological stress, raised circulating adrenaline and kinin release, is indicated by two further sets of evidence. As long ago as 1917, Bainbridge and Trevan (Medical Research Council Report, 1917) had found that infusion of adrenaline in anaesthetized dogs produced only transient hypertension, followed by a fall in arterial pressure and circulatory failure. This finding was confirmed by other workers in both unconscious and conscious dog (Erlanger and Gasser, 1919; Freeman, 1933; Freeman, Freedman and Miller, 1940). Blacket, Pickering and Wilson (1950) found that infusion of both adrenaline and noradrenaline produced a similar result in rabbits, and postulated the release of a vasodilator substance into the bloodstream to explain it. It has been suggested that this vasodilatation is neurogenically mediated (e.g. Barcroft and Swan, 1953). The results of Lever, Mowbray and Peart, (1961), who used completely

denervated dog and rabbit muscle test preparations, indicated that during and following noradrenaline infusion in intact dog and rabbit, a vasodilator substance is released into the circulation, and that its presence coincides with the period of hypotension.

In man, facial flushing and hypotension following termination of adrenaline and noradrenaline infusions have been described (Green, Johnson, Lobb and Cusick, 1948; Lever et al, 1961), while flushing, excessive sweating, and vasomotor collapse may often be seen in patients with adrenaline secreting phaeochrome tumours (Barcroft and Swan, 1953). In order to explain the different actions of adrenaline when infused in man intra-arterially (transient vasodilatation) and intravenously (transient large dilatation followed by smaller, continuous dilatation), Whelan (1952) postulated the release of a secondary dilator substance into the circulation during intravenous infusion. It is now widely believed (Whelan and de la Lande, 1963) that most, if not all of the effects so far described can be explained purely in terms of Ahlquist's (1948) concept of α-(constrictor), and β-(dilator) receptors for the catecholamines. However, the demonstration of the release of an "isoprenaline-like" vasodilator principle in cats and rabbits following intravenous adrenaline (Eakins and Lockett, 1961; Roberts and Lockett, 1961) suggests that Ahlquist's theory may provide only part of the answer.

A third consideration is provided by examining the relationship between fibrinolysis and kinin release. Figure 11 presents, in simplified form, some associations between reactions which are definitely known to occur. Relationship (1) is a well known physiological observation and requires no further illustration, and relationship (2) has already been discussed in the preceding paragraphs.

One of the earliest recorded observations describing fibrinolysis was that of John Hunter (1794), who noted that in cases "of sudden death produced by many kinds of fits, by anger, electricity .... or by a blow in the stomach ....", the blood did not clot when removed from the body. It was later shown that clotting did occur, but that the clots dissolved spontaneously. Dastre (1893) observed this phenomenon in blood taken from dogs in severe haemorrhagic shock, and termed it "la fibrinolyse". The finding of increased fibrinolysis during shock and severe physiological stress is well substantiated (c.v. Macfarlane, 1964). Increased fibrinolysis has also been demonstrated in milder forms of stress (Fig. 11, Reaction 3), such as acute anxiety (Macfarlane and Biggs, 1946; Ogston, McDonald and Fullerton, 1962) and heat stress (Bedrak, Beer and Furman, 1963), and has been shown by many workers to occur during exercise (Biggs, Macfarlane and Pilling, 1947; Cash, 1966; Menon, Burke and Dewar, 1967; see last two papers for further references). The activation of fibrinolysis by adrenaline (Relationship 4) was first observed by Biggs et al (1947) and has since been confirmed many times (c.v. Macfarlane, 1964).

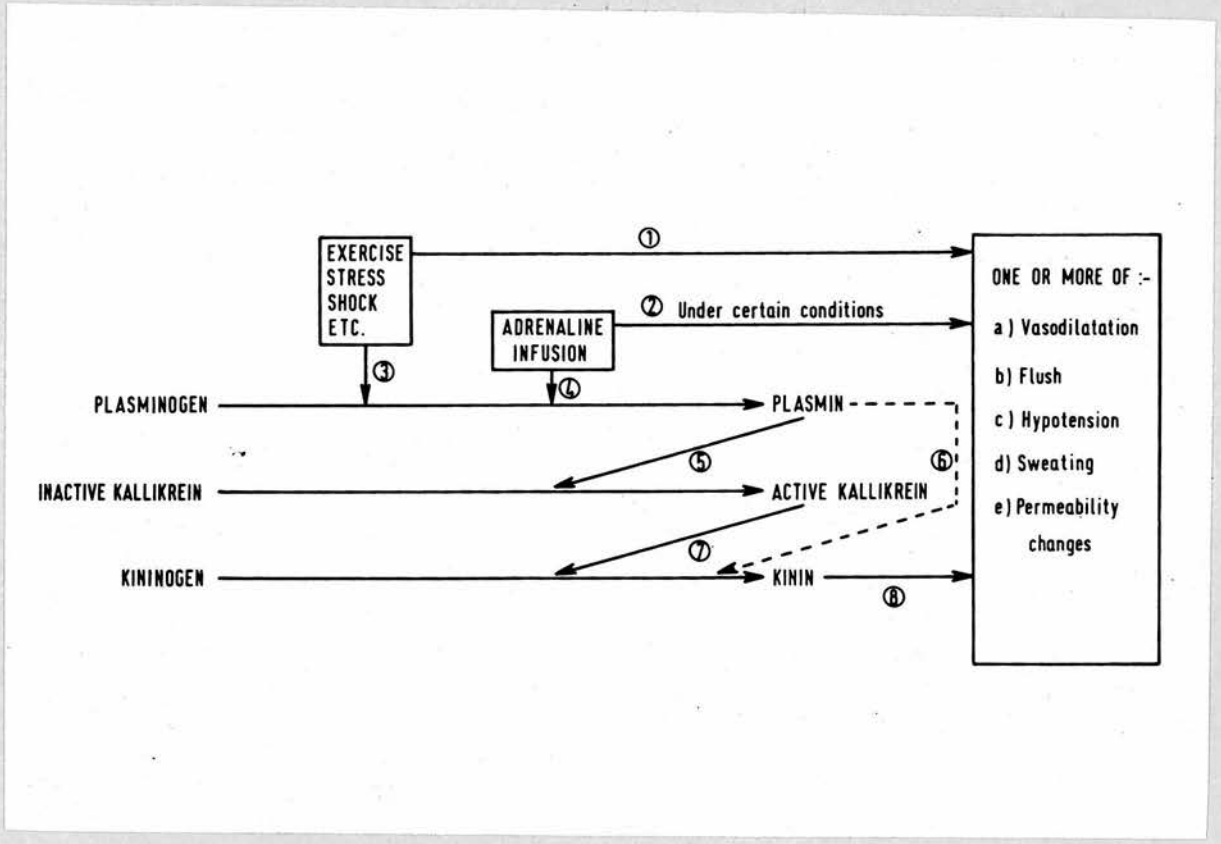

Fig. 11: Some relationships of the kinin-forming system.

The fact that plasmin, the fibrinolytic enzyme, can cause the release of plasma kinin (Fig. 11, Reactions 5 and 6) and has been well established, although the exact mechanism of this action is still under discussion (see General Introduction, P. 44). Reactions 7 and 8 in the figure, the release of plasma kinin by activated plasma kallikrein, and the pharmacological actions of plasma kinin, are now well known, and have been described in the General Introduction.

Thus through the consideration of a number of well established experimental relationships, it can be seen that symptoms produced by adrenaline infusion, exercise and physiological stress, may be reasonably attributed to the release of plasma kinins. As the result of the foregoing arguments, kinin release was investigated in man during certain types of stress, and in the dog during intravenous infusion of 5-HT and adrenaline.

2. Kinin Release in the Dog during Intravenous Infusion of 5-Hydroxytryptamine and Adrenaline.

a) Introduction

In this study, 5 dogs were given a thirty minute intravenous infusion of 5-HT in saline (20 - 80 µg./kg./min.), 5 dogs were given a thirty minute infusion of adrenaline (2 - 4 µg./kg./min.), and 2 control dogs were given a hundred minute infusion of physiological saline (0.9%). Arterial blood pressure and cutaneous temperature changes were monitored, and venous plasma kinin levels were estimated. Because of the lengthy nature of the purification and assay procedures, the number

of samples (six or more) obtained at each experiment, and the limited time available, it was not always practicable to measure simultaneous kininogen levels in this series of experiments. The dog was used, since it is the only animal, other than man, in which the "dumping syndrome" has been described, and has been widely used as an experimental animal for this purpose.

b) Method

Dogs of either sex and various breeds were used, with weights ranging from 7 - 20 kg. Anaesthesia was carried out using Nembutal (26 mg./kg.) administered via the cephalic (paw) vein. Following anaesthesia, an endotracheal tube was routinely inserted. Infusion of the test drugs was carried out by cutting down on to the femoral vein, and cannulating it using a suitable bore Portex nylon intravenous cannula. The cannula was fed into the vein to a depth of approximately 10 cm. On the occasions when deep cannulation was not carried out, particularly during 5-HT infusion, infusion flow was impeded, probably by thrombosis or venoconstriction.

Usually on the same side and through the same dissection, the femoral artery was similarly cannulated for measurement of arterial pressure changes. Pressure was measured using a Statham pressure transducer and Devices Multichannel Recorder. The arterial cannula was filled with heparinized saline (5 Units/ml.). Changes in cutaneous temperature were detected using a thermistor (a temperature sensitive resistor), which was set in one arm of a suitably balanced Wheatstone bridge, and the null point was detected using a sensitive voltmeter.

The thermistor was placed in close contact with shaved skin on the ear or belly, and changes in cutaneous temperature produced changes in its resistance. This caused an imbalance in the Wheatstone bridge, with a resultant deflection on the voltmeter. The size of this deflection gave a qualitative indication of the size of the temperature change. The true temperature at the thermistor depended not only upon the blood flow to the skin, but also upon the rate of heat loss from the skin, and this in turn depended upon the ambient temperature. It was thus not practicable to calibrate the device. Readings were allowed to reach a steady state during the control period. Venous blood sampling was carried out via a siliconized Rochester needle, which was inserted percutaneously into the external jugular vein. In some experiments, attempts were made to draw samples from the cephalic vein, however, not only was this arrangement prone to blockage by clotting, but during both 5-HT and adrenaline infusion, venoconstriction severely limited the rate of outflow of blood. The blood was drawn into a disposable syringe, and treated as described in Appendix 2 for the assay of plasma kinin or kininogen.

Infusion was carried out using either a Palmer infusion motor driving a 20 ml. plastic syringe or a peristaltic pump set to deliver 0.5 ml. per minute. Infusion of physiological saline

(0.9% NaCl) was continued for at least thirty minutes during which control blood samples were obtained. Following this, the animals were infused, for thirty to forty minutes at 0.5 ml./min., with saline solutions containing various concentrations of 5-HT or adrenaline. On two preliminary test dogs, different concentrations of 5-HT and adrenaline were tested, and the minimum concentrations to be used were arbitrarily selected as the lowest which still caused dramatic changes in blood pressure and respiration. The lowest effective infusion rate of 5-HT was 2 µg./kg./min., however, 20, 40, or 80 µg./kg./min. were used. The lowest infusion rate of adrenaline was 2 µg./kg./min., and, in the five dogs described in the adrenaline series, both 2 and 4 µg./kg./min. were used (see Table 21). The one dog in which 8 µg./kg./min. was infused immediately went into ventricular fibrillation, and could not be revived.

c) Results

(i) 5-Hydroxytryptamine Infusion

In each of the five dogs without exception, the cutaneous blood flow, which remained stable during the thirty minute control infusion of saline, was markedly increased during the intravenous infusion of 5-HT (20 - 80 µg./kg./min.) (Fig. 12). The arterial blood pressure, although unpredictable in its

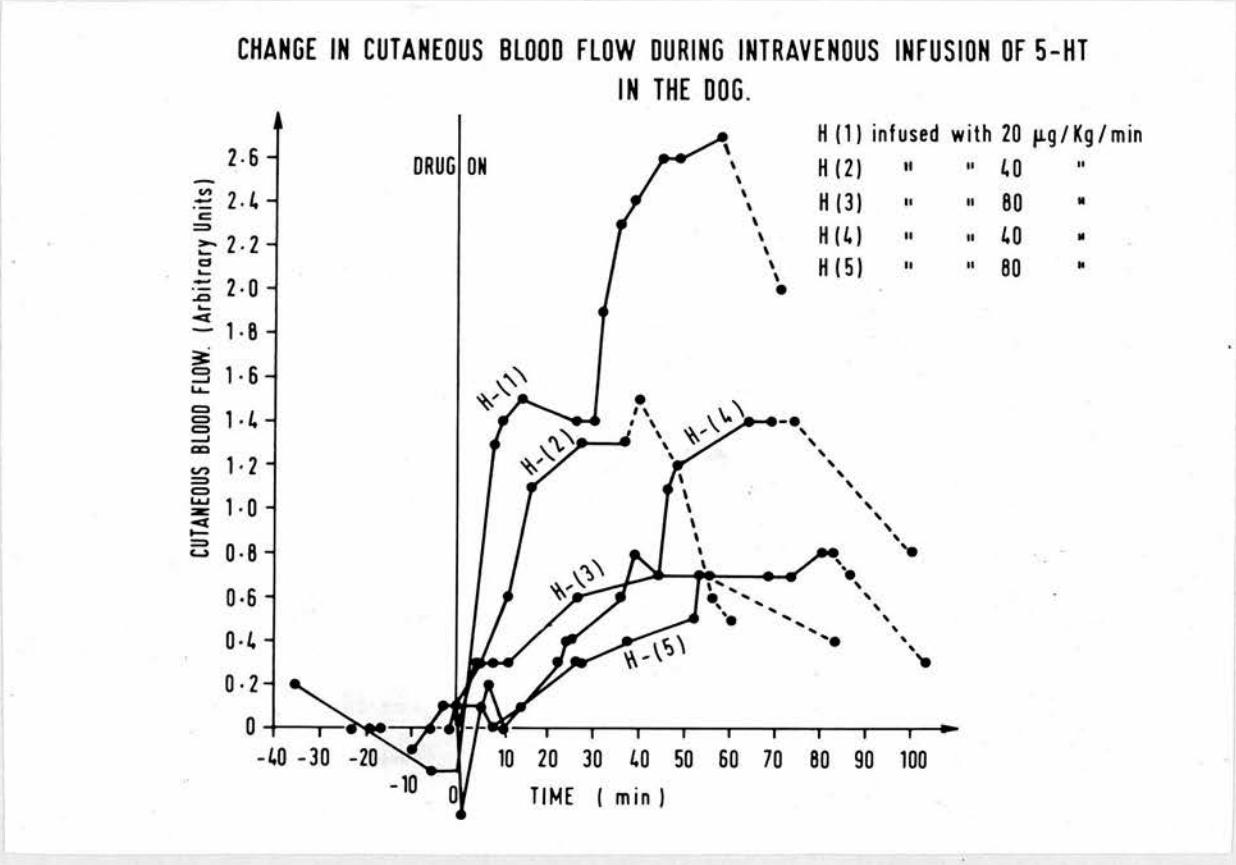

Fig. 12: Change in cutaneous blood flow during intravenous infusion of 5-HT in the dog. Dotted line indicates end of drug infusion. The axes are adjusted so that the mean control value is zero in each experiment.

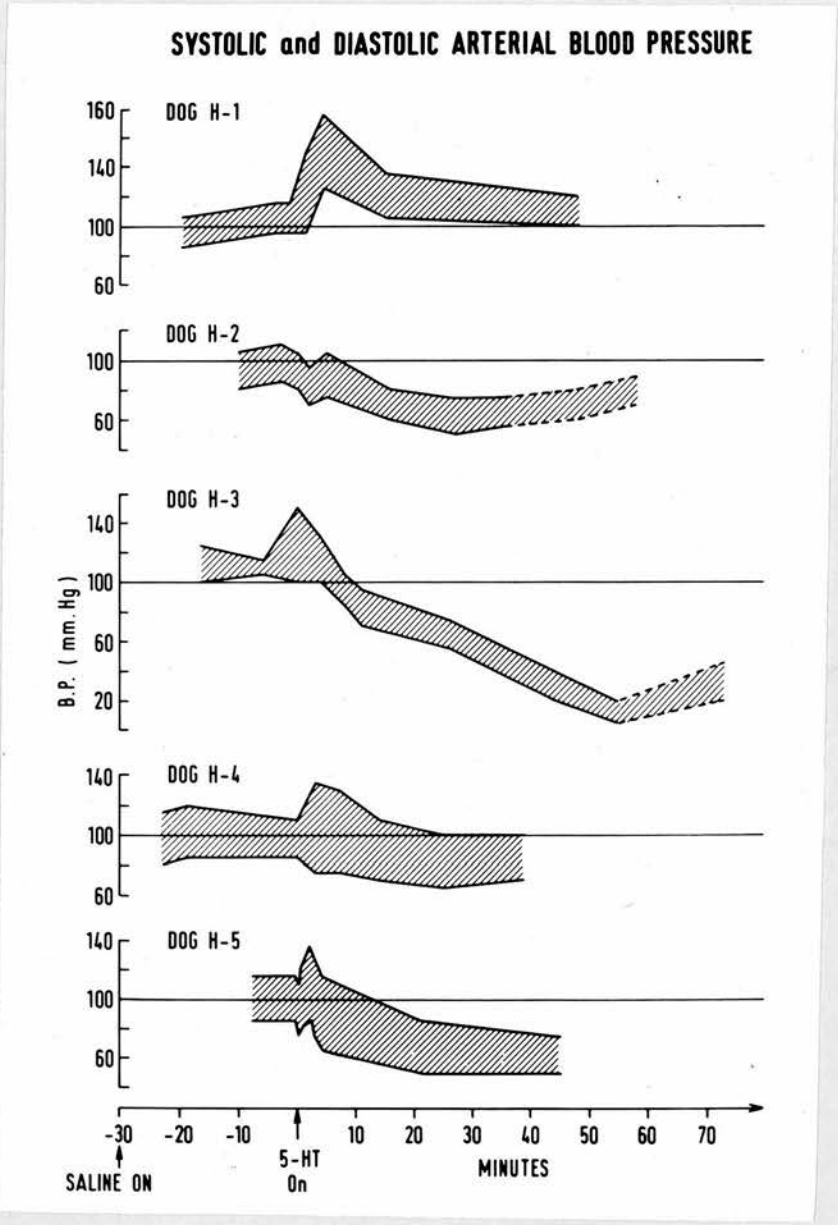

Fig. 13: Changes in arterial blood pressure during intravenous infusion of 5-HT in the dog. (Dotted lines indicate end of 5-HT infusion).

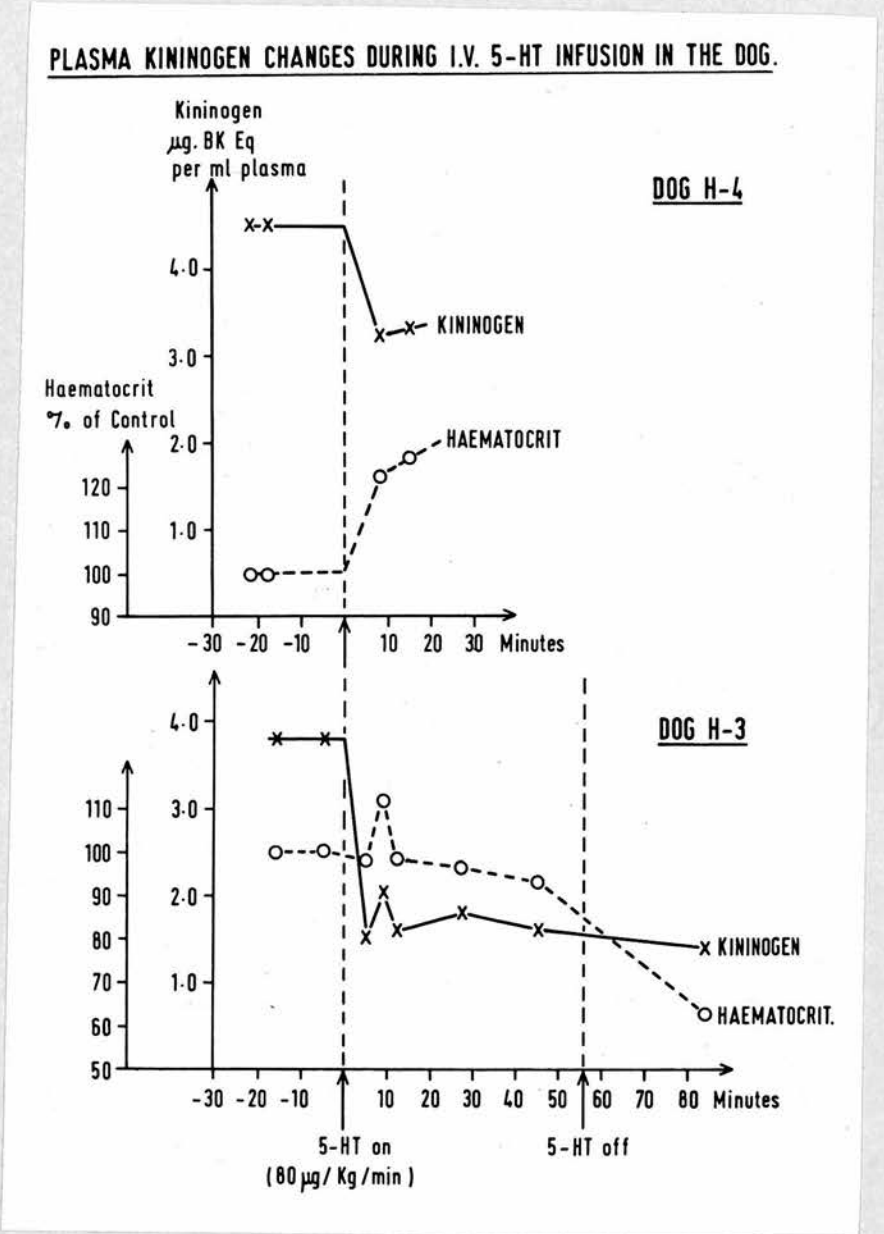

Fig. 14: Changes in plasma kininogen and haematocrit during intravenous infusion of 5-HT in the dog.

initial changes, slowly fell during the course of the infusion in four out of five animals (Fig. 13).

In only two blood samples from two different dogs (H-4 and 5) could any free kinin activity be detected (13 and 10 ng. BK Eq. per ml. plasma). These occurred during saline control infusion periods, and were considered too low to be of significance. In two of the five dogs, plasma kininogen levels were also measured (Fig. 14). In both animals, a marked fall in kininogen was observed following commencement of 5-HT infusion (80 µg./kg./min.).

(ii) Adrenaline Infusion.

Five dogs were given an intravenous infusion of adrenaline (2 or 4 µg./kg./min.). Of these five, two (A-1 and A-4) had detectable, but low, levels of free kinin present in their venous plasma during the control infusion of saline (Table 21; Fig. 15). In these two animals, however, when the saline control infusion was replaced by adrenaline, 2 µg./kg./min. in the one, and 4 µg./kg./min. in the other, a dramatic increase in free kinin was detected. In the first animal, the peak level reached was 60 ng. BK Eq. per ml. plasma, almost six times the control level; in the second animal, which had received the higher dose of adrenaline, the peak level reached was 328.2 ng. BK Eq. per ml., about 130 times the control level.

DOG	A - 1			A - 2			A - 3			A - 4			A - 5		
Weight (kg.)	7.5			10.5			13.0			17.2			7.0		
Infusion Rate (µg./kg./min.)	2			2			2			4			4		
	Time (Minutes)	Free Kinin (ng BK Eq/ml plasma)	Haematocrit (%)	Time (Minutes)	Free Kinin (ng BK Eq/ml plasma)	Haematocrit (%)	Time (Minutes)	Free Kinin (ng BK Eq/ml plasma)	Haematocrit (%)	Time (Minutes)	Free Kinin (ng BK Eq/ml plasma)	Haematocrit (%)	Time (Minutes)	Free Kinin (ng BK Eq/ml plasma)	Haematocrit (%)
SALINE				-10	1.0	44.1							-31	<3.3	39.0
INFUSION	-5	11.11	55.0	- 5	<1.1	44.1	-5	<3.5	50.5	-15	2.57	45.5	-25	<4.9	39.0
ADRENALINE INFUSION	0			0			0			0			0		
	7	20.3	63.0	7	<1.2	55.9	5	<3.5	60.3	6	165.9	55.0	50	<4.4	54.5
	20	16.3	54.1	21	<1.1	57.8	18	<3.5	61.0	15	82.96	55.0	60	<4.4	54.5
	37	63.2	56.5	34	1.1	57.5	35	<3.5	49.3	25	328.2	54.0	124	<4.4	54.5
	50	Adren off		35	Adren off		50	<3.5	57.7	37	4.76	58.0	127	Adren off	
	101	55.6	59.5	38	<1.1	58.0	52	Adren off		40	Adren off	52.0	145	<4.4	54.5
				41	1.2	56.0	55	<3.8	62.8	43	<2.0				

Table 21

Free Kinin Levels and Haematocrit Values during Intravenous Infusion of Adrenaline in the Dog.

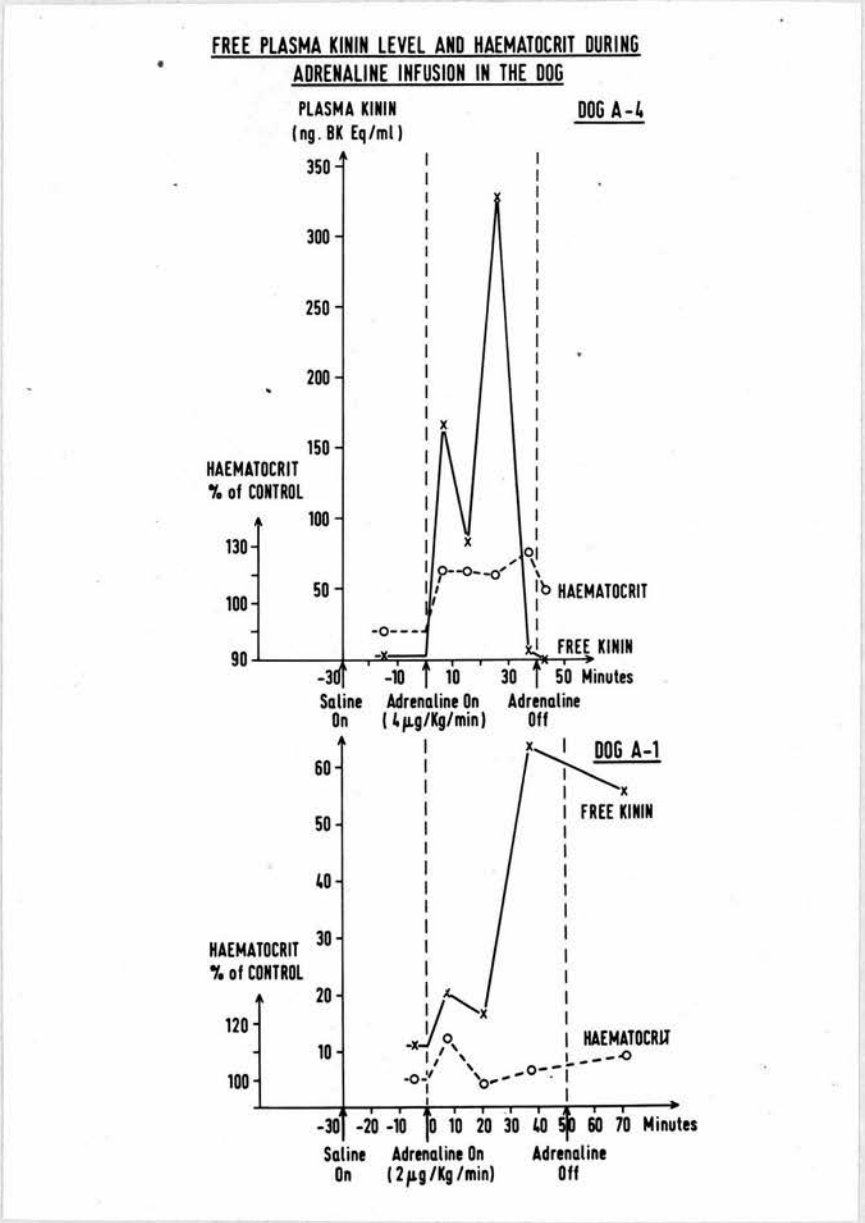

Fig. 15: Changes in free plasma-kinin and haematocrit during intravenous infusion of adrenaline in the dog.

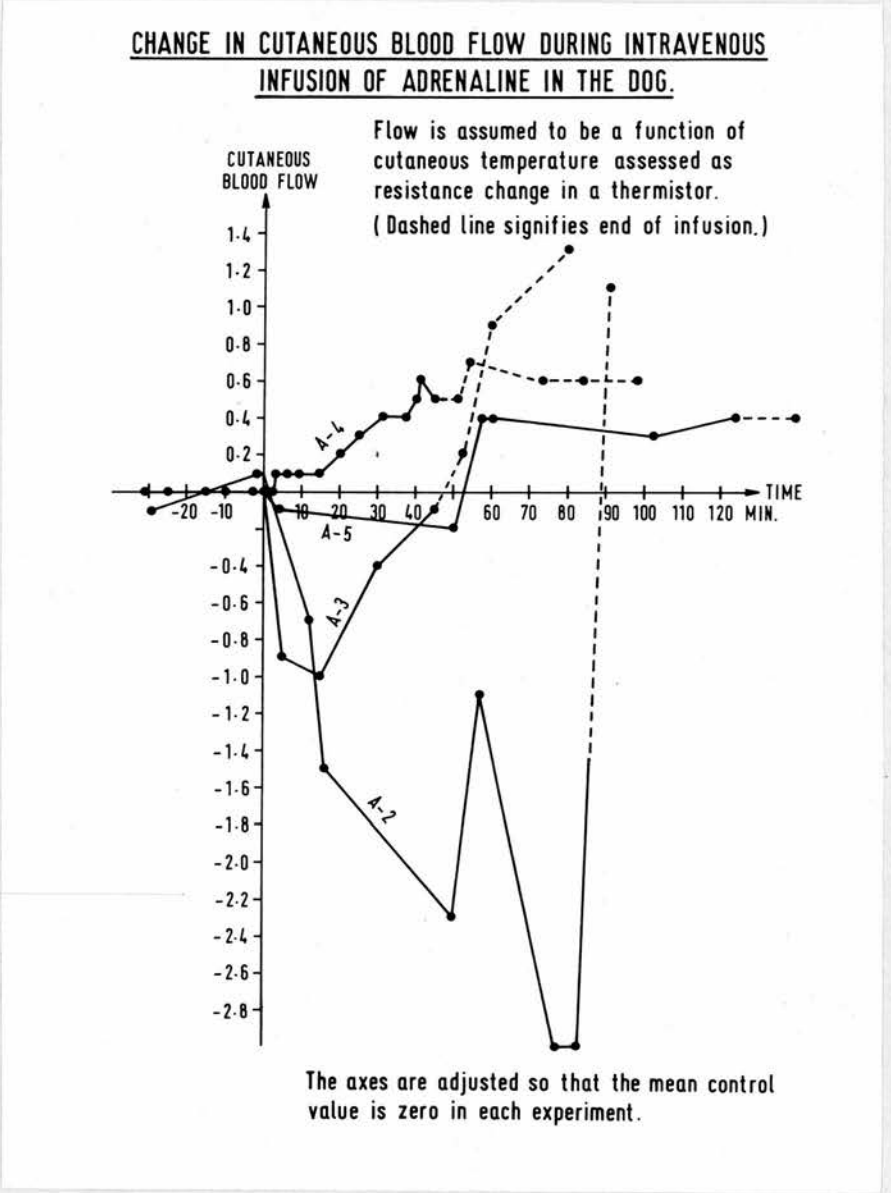

Fig. 16: Changes in cutaneous blood-flow during intravenous infusion of adrenaline in the dog.

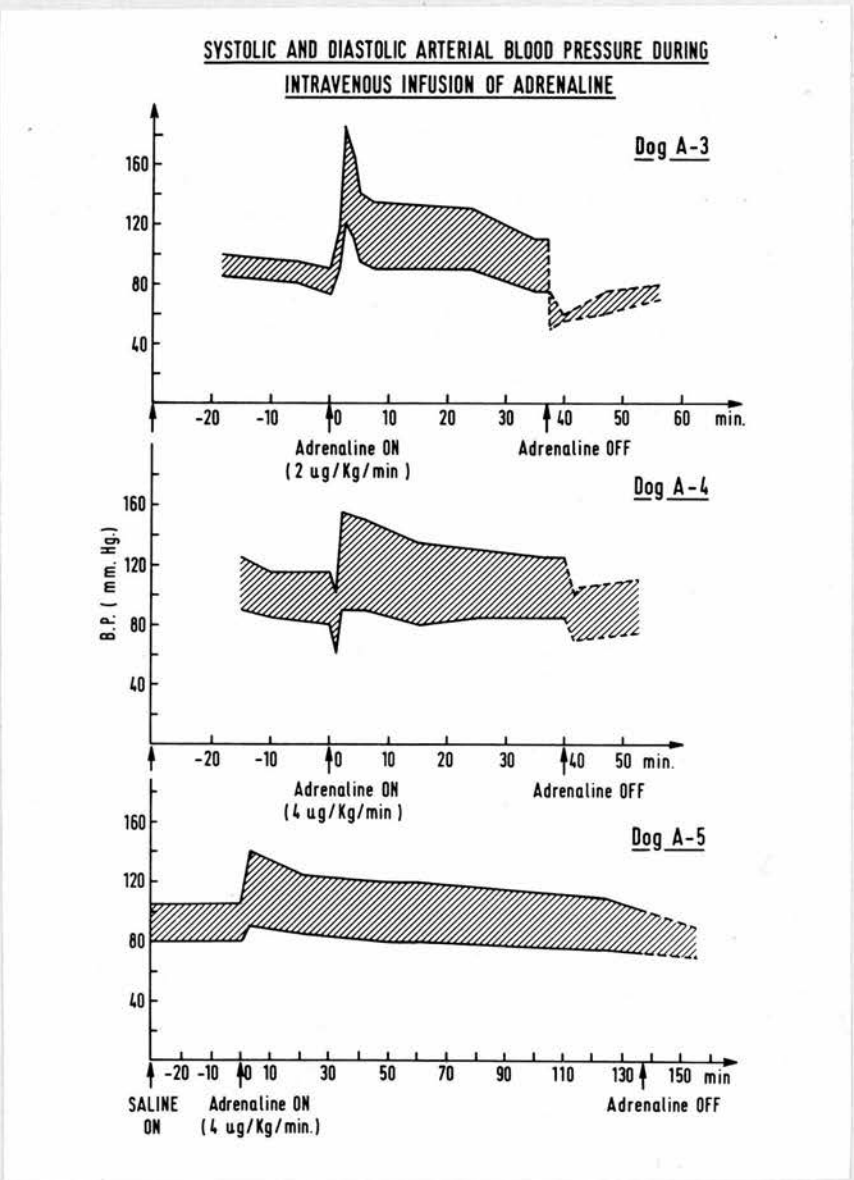

Fig. 17: Changes in arterial blood pressure during intravenous infusion of adrenaline in the dog. The dotted lines represent the end of adrenaline infusion.

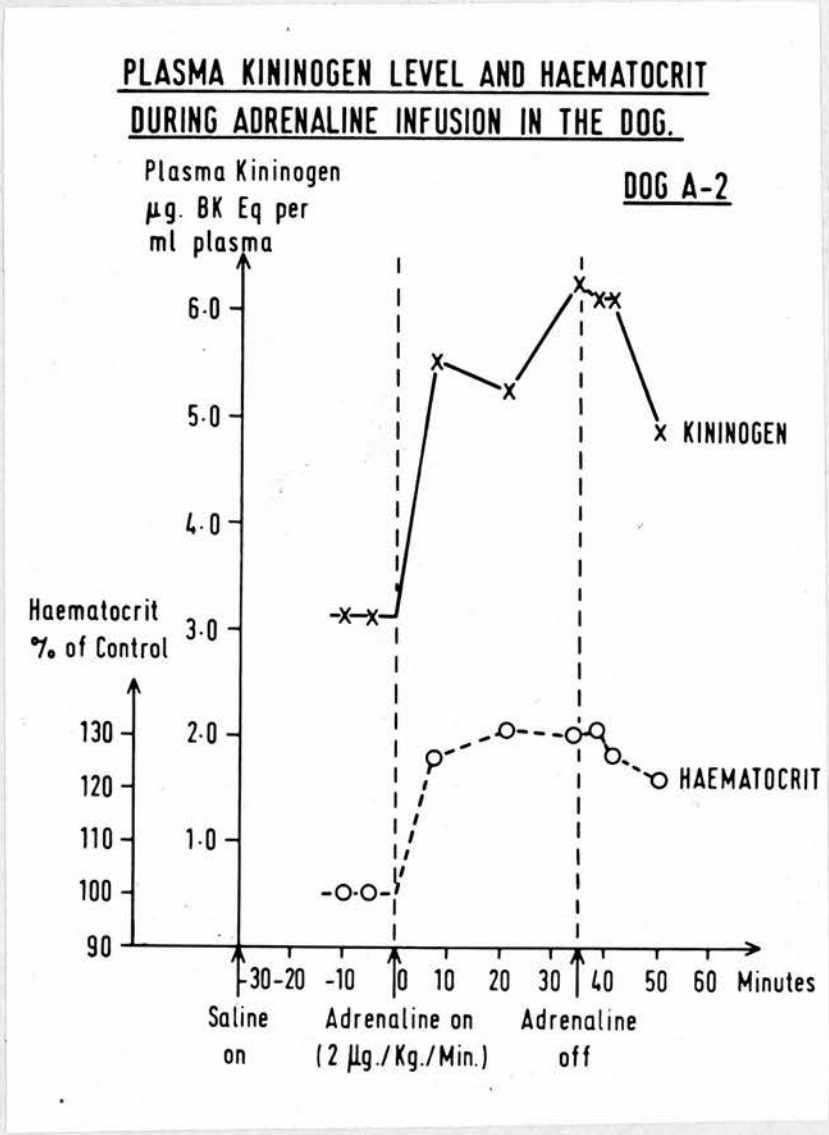

Fig. 18: Changes in plasma kininogen and haematocrit during intravenous infusion of adrenaline in dog A - 2.

Of the five dogs, cutaneous blood flow was monitored in four (A-2 to A-5) (Fig. 16), including one (A-4) of the two in which kinin release was detected. In all of the animals, the skin temperature was steady during the thirty minute control saline infusion period. Following commencement of adrenaline infusion, in three animals (A-2, 3, and 5), an initial fall in skin blood flow was detected. In two of these, flow increased spontaneously as the infusion continued, while in the third it continued to fall until the infusion ceased. In one animal (A-4) skin blood flow increased immediately following commencement of infusion, and no initial vasoconstriction was seen. This was also the animal in which kinin release was detected.

Arterial blood pressure was measured successfully in three of the five animals (A-3, 4, and 5), serious clotting invalidating the measurements from the remaining two. Although kinin release occurred in A-4, and not in A-3 or A-5, there was very little difference in the pattern of pressure change seen in these three animals (Fig. 17).

Plasma kininogen was measured in two dogs during adrenaline infusion, in A-2 and in C-1. The latter animal was one of two control dogs, infused with saline for a hundred minute period. At the end of this time, however, in the one animal, 2 µg./kg./min. adrenaline was infused. The kininogen level, which had been fairly constant during the hundred minute control infusion (Fig. 19)

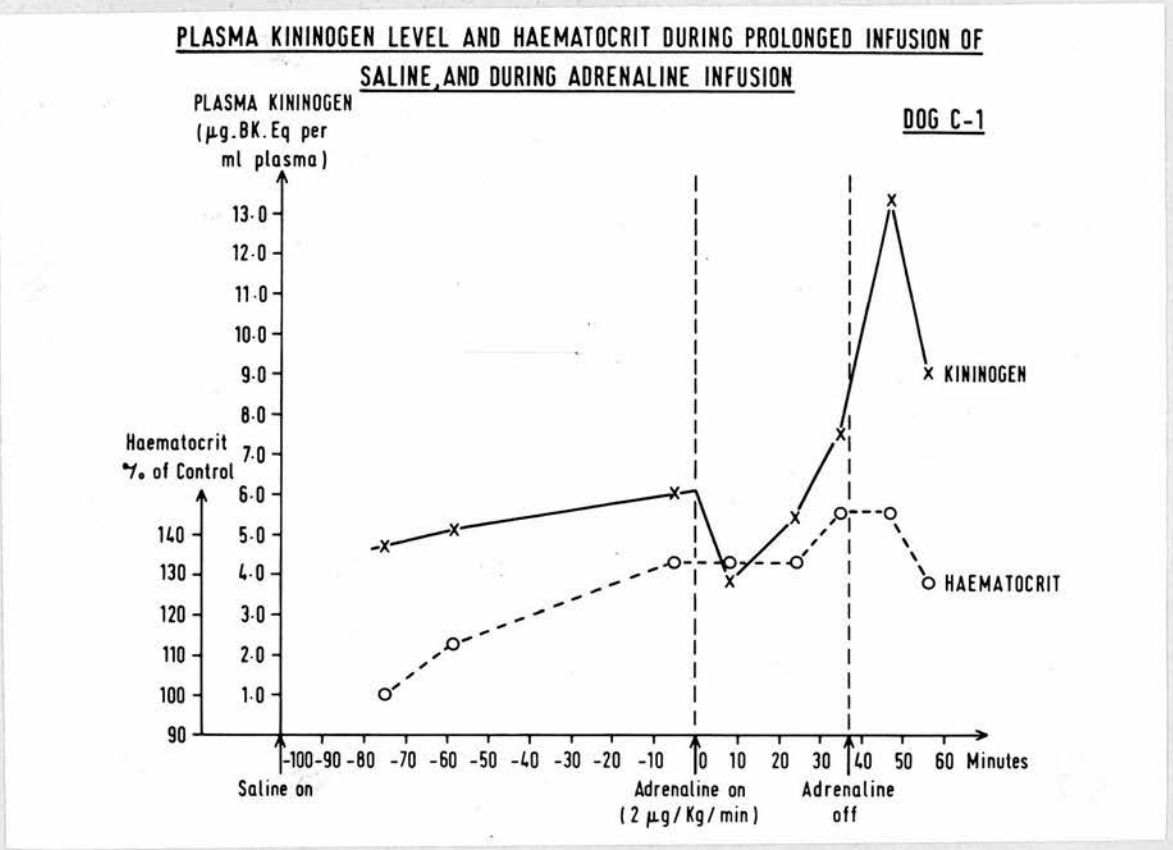

Fig. 19: Plasma kininogen and haematocrit levels in a dog during a 100 minute control saline infusion; and infusion with adrenaline.

fell a little at the start of the infusion, and then rose to more than twice the control value. A similar increase was also seen in the second dog (Fig. 18).

(iii) Control Infusions.

In addition to the thirty minute control saline infusion carried out on every animal prior to the infusion of the drugs, two dogs were infused with saline for one hundred minutes, being approximately the total time for which a dog in either test group was submitted to infusion. In no sample obtained from either animal was there any detectable free kinin. In the one animal in which kininogen was also estimated, the level increased slightly during the infusion (Fig. 19). Skin blood flow and blood pressure were unchanged throughout the infusion.

3. Kinin Release during Physiological Stress in Man.

a) Introduction.

In the present research, free plasma kinin and kininogen levels have been estimated in man during two types of stress situation. Free kinin levels have been estimated during vasovagal syncope, presumably the result of the emotional stress of sample taking, in two otherwise normal healthy young men. The subjects were controls in the normal free kinin, and bradykinin recovery series and as the result was not anticipated, simultaneous

plasma kininogen levels were not assayed. For obvious reasons, it has not been possible to repeat this experiment at will.

Plasma kininogen levels have been estimated in two healthy male subjects, while undergoing a standardized exercise procedure sufficient to produce flush and sweating. These two subjects formed part of a pilot group of six for whom free plasma kinin levels were being assayed during exercise. However, for reasons beyond the control of the author, experimental conditions were altered in an uncontrolled manner during the free kinin extraction, and the free kinin results are too unreliable to be quoted. The change observed in the plasma kininogen level in the two subjects in whom this was assayed, was so unexpected that these results will be described.

b) Methods.

(i) Vasovagal Syncope.

As already stated, the subjects were two apparently normal healthy males, aged 20 and 27 years. They were acting as controls, one in the Normal Free Kinin series, the other in a bradykinin recovery experiment. Sampling and assay were as described in Appendix 2. There was nothing unusual about the venous sampling procedure for these two subjects. The samples were taken from the two subjects on different weeks, and assayed on separate occasions. In each case, the subject was unusually apprehensive

prior to sampling, and collapsed while the blood was being withdrawn. In neither subject, following recovery, was there any unusual symptom, or subsequent history of illness. One of the two subjects volunteered again at a later date, and no abnormal symptoms were seen during sampling on this occasion.

(ii) Physical Exercise.

The subjects rested, lying down, for thirty minutes before the control blood samples were taken. Following the initial venepuncture, each subject walked at 3.4 m.p.h. and 120 strides per minute, on a treadmill at 5 degrees elevation for a period of 16 minutes. After the first 8 minutes, the subject left the treadmill for 60 to 80 seconds while a second blood sample was removed. At the completion of the exercise, a third and final sample was taken. The samples were assayed for kininogen content as described in Appendix 2. The standardized exercise used was that described by Cash (1966), as this was already known to activate proteolytic enzymes involved in fibrinolysis. The subjects were two healthy male volunteers aged 28 years (C) and 22 years (D).

(c) Results.

(i) Vasovagal Syncope.

The results are shown in Table 22 (a). In subject A, a 20 year old male, a free plasma kinin level of 31.1 ng. BK Eq. per ml. of plasma was measured during fainting. This was 11 times the mean normal level for the method, of 2.8 (s.d. = 1.7) ng. BK Eq. per ml. of plasma (measured in 16 males).

In subject B, a 27 year old male, a free kinin level of 30.1 ng. BK Eq. per ml. plasma was found. This was approximately 10 - 11 times the mean normal level. In this subject, a subsequent sample some months later, taken in the absence of symptoms, was found to contain less than 2.7 ng. BK Eq. per ml. plasma.

(ii) Physical Exercise.

The results are shown in Table 22 (b). As can be seen from Fig. 20, the level of plasma kininogen had markedly increased in both of the subjects examined after 8 minutes, in one case the level reaching nearly double the control level. By 16 minutes the level had fallen, but was still, in both cases, clearly above the control. During the exercise, it was found that the packed cell volume increased, with thus a concommittant decrease in plasma volume. Such a plasma volume decrease could produce an apparent increase in plasma kininogen level. However, as can be

Subject	Free Plasma Kinin (ng. BK Eq./ml. Plasma)	Packed Cell Volume %
Mean Normal Level (16 males)	2.8 (s.d.=1.7)	
Subject A, during syncope.	31.1	51.8
Subject B, during syncope.	30.1	50.2
Subject B, no symptoms.	< 2.7	48.5

Table 22 (a)

Free Kinin Levels during Fainting in Two Subjects

Subject	Time (Min.)	Plasma Kininogen (µg. BK Eq./ml. Plasma)	Plasma Kininogen as % of Control	Packed Cell Volume	
				(Percent)	% of Control
C	0	3.0	100.0	47.5	100.0
	8	5.9	196.6	52.0	109.5
	16	4.8	160.0	51.1	107.6
D	0	3.7	100.0	51.9	100.0
	8	4.9	132.4	56.9	109.5
	16	4.4	118.9	54.9	105.8

Table 22 (b)

Plasma Kininogen Levels during Exercise in Two Human Subjects

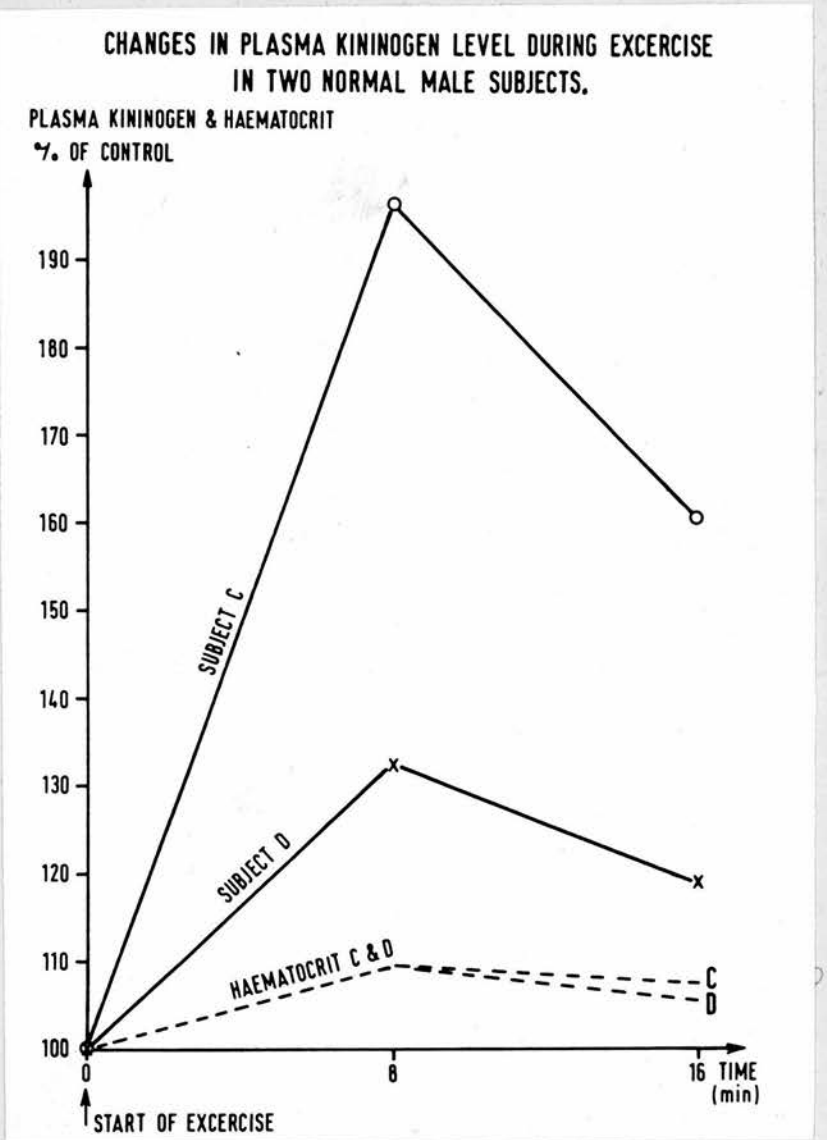

Fig. 20: Changes in plasma kininogen level and haematocrit in two human subjects during moderate exercise.

seen from Table 21 (b), the maximum changes in packed cell volume were less than 10% of control, and could not account for a kininogen change of more than 90% in the one case, and more than 30% in the other. In view of the earlier findings of kininogen fall during kinin release in the carcinoid and dumping syndromes, these results were unexpected.

Free plasma kinin estimations were carried out on the samples drawn from both these subjects and four similar subjects. However, controls included with these kinin extractions showed an added bradykinin (250 ng.) recovery of 20%. The extraction procedure used with these samples, the only ones not processed personally by the author, differed from the normal routine. They were allowed to stand for an unspecified, but lengthy period (one hour or more), at room temperature in acid saline contained in unsiliconized glassware. No free kinin was detected in any sample. Lack of time, and facilities did not permit a repetition of the experiment.

4. Discussion.

a) 5-Hydroxytryptamine Infusion in the Dog.

The work so far described indicates that plasma kinin release occurs in two pathological conditions in which there is also some evidence for the release into the circulation of 5-HT. To determine whether raised levels of circulating 5-HT would activate

plasma kinin releasing enzymes, varying concentrations of 5-HT (20, 40 and 80 µg./kg./min.) were infused intravenously in five dogs.

In all but one animal, after an initial unpredictable change the arterial blood pressure slowly fell during the infusion. With the onset of 5-HT infusion, in every animal, the cutaneous temperature rose indicating a marked increase in skin blood flow (Fig. 12). As the infusion continued, the blood flow eventually reached a plateau and started to fall as soon as the infusion was discontinued. This was in contrast to the finding of Fox, Goldsmith, Kidd and Lewis (1961) who measured not the temperature but the skin heat loss in man; these workers found that 5-HT produced a marked vasoconstriction.

A remarkable aspect of the skin flow measurements in the dog was the fact that the peak blood flow achieved appeared to decrease with increasing doses of 5-HT (Table 23 (a)). Three dose levels were used, one dog being infused with 20 µg./kg./min. of 5-HT, two dogs at 40 µg./kg./min. and two dogs at 80 µg./kg./min. Plotting the reciprocal of the mean peak blood flow at each dose, against the dose, shows that the peak flow is inversely proportional to the dose (Fig. 21). This finding is in contrast to the change in arterial blood pressure, since increasing the dose caused increased hypotension. After thirty minutes' infusion of 20 µg./kg./min. of 5-HT (Table 23 (b)), the mean blood pressure

Dog	Dose of 5-HT µg./kg./min.	Peak Blood Flow Reading (Arbitrary Units)	Mean Peak Blood Flow	1/(Peak Blood Flow)
H-1	20	2.7	2.7	0.37
H-2; H-4	40	1.3; 1.4	1.35	0.74
H-3; H-5	80	0.7; 0.7	0.7	1.43

Table 23 (a)

Relationship Between Peak Cutaneous Blood Flow and Dose of 5-HT Infused Intravenously in the Dog

Dog	Dose of 5-HT (µg./kg./min.)	Change in Mean Pressure at 30 min. (mm. Hg.)	Mean Change (mm. Hg.)
H-1	20	+ 13.4	+ 13.4
H-2; H-4	40	- 31.2; - 16.2	- 23.7
H-3; H-5	80	- 63.8; - 35.0	- 49.4

Table 23 (b)

The Change in Mean Blood Pressure from the Average Control Value after 30 Minutes Intravenous Infusion of 5-HT in the Dog

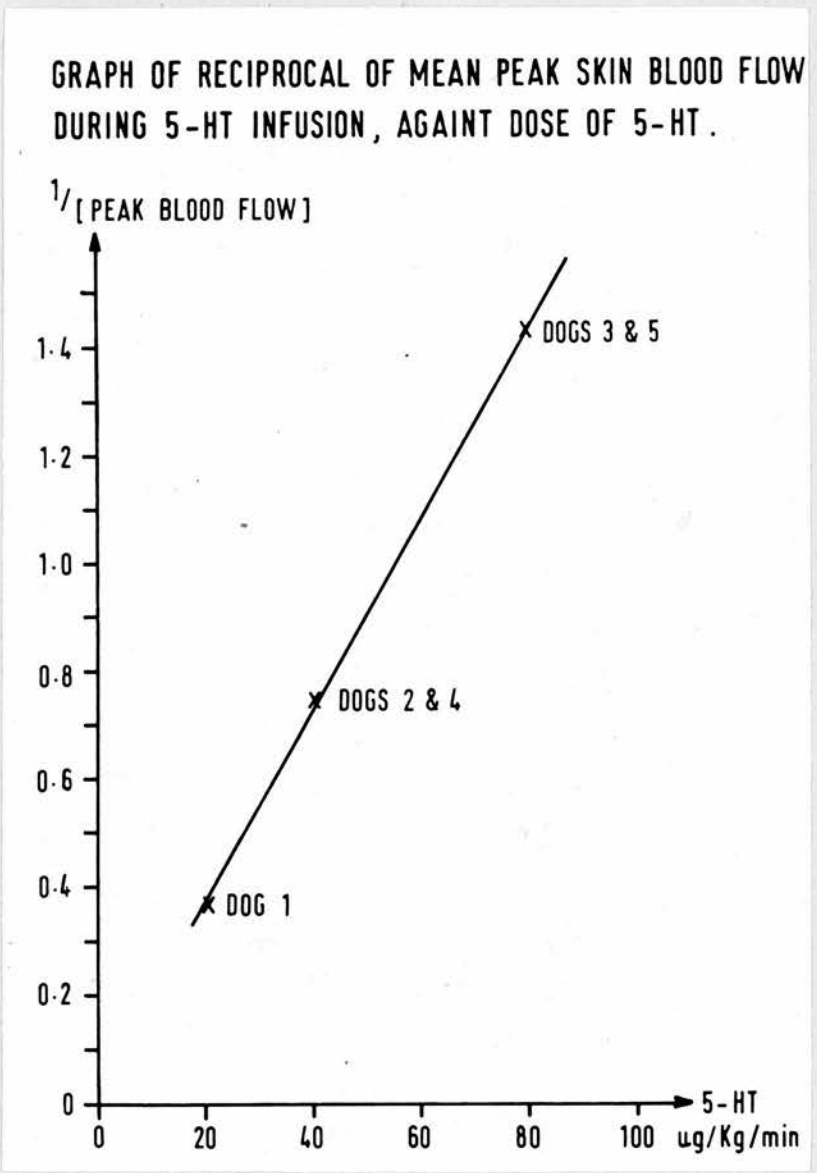

Fig. 21: Graph showing inverse relationship between the peak cutaneous blood-flow and dose of intravenous 5-HT in the dog.

had risen by 13.4 mm. Hg. above the average control value; however, doubling the dose produced a mean fall of 23.7 mm. Hg. whilst infusion of 80 µg./kg./min. caused a mean fall of 49.4 mm. Hg. This result is not easy to interpret. If, as found by Fox et al (1961), 5-HT is in fact constrictor to the skin vessels although dilator to other vascular beds, then possibly the infusion is causing the release of a dilator substance such as histamine (Feldberg and Smith, 1953) or plasma kinin. This secondary cutaneous dilatation would then be increasingly antagonized by the increased doses of 5-HT. Such a concept would be in accord with the increasing hypotension with higher dose levels.

In the two dogs in which plasma kininogen levels were measured, the levels fell by 60% and 30% respectively, indicating the release of plasma kinin. Neither in these, nor in any animal in which 5-HT was infused, were significant levels of free plasma kinin detected. These two results are hard to explain unless 5-HT can cause increases in plasma kininase activity masking an increase in kinin formation, or possibly the para-capillary circulation is slowed so that kinin formed there is destroyed before reaching the blood-stream. The kininogen might also be lost in some way other than kinin release, such as by leakage into the lymph.

b) Adrenaline Infusion in the Dog.

Of the dogs infused with 5-HT, two out of the five animals had detectable but low levels of free kinin in their blood during the control saline infusion. Two out of the five dogs (A-1 and A-4) infused with adrenaline also had low levels of free kinin (11.1 and 2.6 ng. BK Eq. per ml. plasma respectively) in their control blood samples. In these two animals only, when the adrenaline infusion was started (2 and 4 µg./kg./min. respectively) a dramatic increase in the free plasma kinin level occurred. The peak kinin level was greater in the animal (328 ng. BK Eq. per ml. plasma) receiving the higher concentration of adrenaline than in the animal (63.2 ng. BK Eq. per ml. plasma) receiving the lower concentration. In no sample from any other animal was there a detectable level of free kinin.

The adrenaline infusion produced a steep fall in skin temperature indicating vasoconstriction in those animals in which no kinin release occurred. The appearance of raised levels of bradykinin coincided with the onset of cutaneous vasodilatation. This vasodilatation was small when compared with the vasoconstrictor effect seen, and it seems certain that much of the dilator effect was masked by the constrictor action of adrenaline. The arterial blood pressure changes in this animal were not discernibly different from those in the dogs in which no kinin

release had occurred, however blood pressure is less sensitive to the action of bradykinin than is cutaneous vascular tone (Fox et al, 1961). Although the scale of this experiment was too small to permit any general conclusions to be drawn, it does indicate that raised circulating levels of adrenaline may, in some animals provoke the release of plasma kinins. Such a finding may explain the occurrence of anomalous vasodilatation. Simultaneous free kinin and kininogen levels were assayed in only two dogs during adrenaline infusion. While there were no detectable levels of free plasma kinin in either of these animals, the adrenaline infusion produced a quite surprising increase in the plasma kininogen levels. The kininogen level doubled in one animal, and in the other, reached nearly three times the control level. These results are of greater interest when related to the plasma kininogen changes seen in two human volunteers undergoing moderate exercise.

c) Kininogen Levels During Exercise in Man.

One of the features of the kininogen estimations in dumping patients was the rapid recovery of the plasma kininogen level following the initial fall during kinin release. In all but one dumping experiment, the level had markedly increased by the time symptoms had ceased some 10 - 15 minutes following the peak of the vasomotor effects. The origin of this additional kininogen was

not known. In the normal individual the plasma kininogen level is fairly constant, so if the increase in level is the direct result of synthesis, it must occur during either sub-normal destruction or super-normal synthesis of kininogen. There may be a kininogen pool independent of that in the circulating plasma, perhaps in the spleen or liver. Then, the rapid replacement of kininogen might merely be the result of equilibration between the two pools.

The phenomenon occurred again when changes in plasma kininogen level were examined in two human volunteers during mild physical exercise. These individuals were subjected to a standard moderate exercise procedure which had been shown by Cash (1966) to induce fibrinolysis in most subjects. In both individuals, the plasma kininogen had increased markedly after 8 minutes exercise, increasing by almost a third in the one case and nearly doubling in the other. After 16 minutes of exercise, the kininogen levels had fallen from this peak but were still well above the control levels. These findings are consistent with the existence of a kininogen pool in the body, apart from that in the general circulation. If transfer of kininogen into the circulation were to coincide with increased utilization, the plasma kininogen level would fall as soon as the second pool had been exhausted.

From the animal experiments already described, it appears that release of kininogen into the circulation from a secondary source can be stimulated in the dog by adrenaline infusion. In the dog, at least, it is possible that a pool of kininogen may be maintained, away from the sites of active kallikrein, in the blood stored in the spleen. Adrenergic stimulation causes this organ to contract and expel its store of blood into the general circulation. Barcroft and his colleagues (Barcroft and Stephens, 1927; Barcroft and Elliot, 1936) have shown that exercise, haemorrhage, anoxia and emotional conditions will cause such a reduction in the dog splenic volume. There is some doubt as to whether this response occurs in man (Grayson and Mendel, 1965). A similar increase in plasma kininogen has been noted by Diniz and Carvalho, (1963) during the first ten minutes following haemorrhage in the dog, but the kininogen level ultimately fell below normal. These workers considered that splenic contraction or induced synthesis were possible alternative sources.

Discussion of the findings concerning plasma kininogen poses more questions than it solves. It is clear that more extensive experimental work should be done concerning the distribution and production of the kinin precursor. Apart from confirming the preliminary results so far described, further

work might include adrenaline infusion of the splenectomized dog, and an examination of the rate of recovery of normal kininogen levels following depletion of plasma kininogen in the experimental animal.

d) Kinin Release During Emotional Fainting.

Free plasma kinin levels were assayed in the venous blood of two apprehensive but healthy subjects, who fainted during an otherwise uneventful venepuncture. Both subjects had free kinin levels of more than ten times the mean normal value of 2.8 ng. BK equivalents per ml. of plasma. In one of the subjects, plasma kinin levels assayed in the absence of symptoms were found to be normal.

When the return of blood to the right heart drops, fainting or syncope may occur. Such a decrease in venous return may be produced experimentally as the result of haemorrhage, or of 'bloodless haemorrhage' produced by tourniquet occlusion of the circulation in the lower limbs (Ebert and Stead, 1940). This was called the 'vaso-vagal syndrome' by Lewis (1932) who likened its effects to stimulation of the carotid sinus. Detailed analysis of the haemodynamic picture (Barcroft and Swan, 1953) showed that the explanation must be more complex.

As bleeding proceeds, prior to a faint, cardiac output and right atrial pressure steadily fall while arterial pressure declines more slowly. During this time, the heart rate and total peripheral resistance increase considerably. The vasoconstriction increases up to a point and then suddenly changes to vasodilatation, and there is a precipitous fall in blood pressure as the faint develops. This vasodilatation appears to be sympathetically mediated since its occurrence in a limb may be prevented by blocking the sympathetic supply. Barcroft and Swan (1953) have pointed out that this may not be the whole explanation; for instance, fainting can occur in totally sympathectomized subjects. The activation of kinin release by emotional stress, as suggested by the finding of raised levels of free kinin in fainting subjects, could well help to precipitate the faint.

C O N C L U S I O N

CONCLUSION

The work described in this thesis falls into two main categories, the derivation of suitable methods for the assay of plasma kinin and kininogen, and their application. In the methodology section, the aim was to devise a procedure for plasma kinin and kininogen assay which could not only be applied with the minimum of inconvenience in clinical situations, but also gave high standards of reproducibility and specificity. This aim has, to a large extent, been realised. The procedure described enables both free kinin and kininogen to be assayed in a single 6 ml. sample of blood. The small sample size enables investigations into kinin release to be carried out even in severely traumatic conditions without endangering the patient. Apart from the very simple inactivation procedure, the sample does not require immediate processing and does not cause the clinician undue inconvenience. The recovery of added bradykinin of 81.5% is better than any previously published for a similar method. The procedure, furthermore, largely excludes certain biologically active amines and potassium which would interfere with the biological assay of free plasma kinin.

For reasons already discussed and also because the amount of free kinin in the circulation needed to produce physiological changes (ng./ml.) is relatively minute when compared with the latent kinin carried as kininogen (µg./ml.), kininogen assay is an insensitive

method for the detection of kinin release. In the procedure described, the assay of both kinin and kininogen in a single blood sample enables changes in the concentrations of both substances to be monitored simultaneously. The estimation of the PCV of each sample not only enables the concentrations in plasma to be estimated, but permits a check to be kept on apparent changes in concentration as the result of fluid shift. The normal values of both free plasma kinin and kininogen in man, as determined by this method, are well within the range of those found by the majority of workers using a variety of methods.

The procedure has a probable source of error in the fact that the blood must pass into a syringe prior to inactivation, and it is likely that the concentrations assayed are not precisely those occurring *in vivo*. The need to use untampered-with standard sterile disposable equipment for routine clinical work, makes impossible the prior addition of enzyme inhibitors to the syringe. However, when sampling and assay conditions are kept constant, the reproducible appearance of an increase in free-kinin and a drop in precursor level must indicate an increase in kinin-releasing activity.

The assay procedure was used to investigate kinin release in the carcinoid and dumping syndromes. Raised levels of bradykinin-like activity were found in the blood of carcinoid patients during flushing, confirming the findings of Oates et al (1964).

Bradykinin-like activity was likewise found in the blood of patients during the hypotensive flushing episodes of the dumping syndrome. The findings indicated that irregularities in both indole metabolism and the kinin-releasing system were implicated amongst the many similarities between the two conditions. Nevertheless, from a consideration of the pathologies of the two conditions, it seemed unlikely that the precise mechanism of kinin release was the same in both cases. Since in both conditions there was much evidence for an increased formation of 5-HT, it was possible that 5-HT might activate plasma kinin release. Intravenous infusion of 5-HT, in the dog at least, did not cause the appearance of raised plasma kinin levels.

Both carcinoid and dumping syndromes may be provoked in varying degrees by similar types of stress. In both conditions, sympathetic stimulation with resultant adrenaline release seemed to be a likely factor. In the carcinoid syndrome, intravenous injections of minute amounts of adrenaline are used experimentally as a standard provocation of the symptoms. By inference, adrenaline is considered to activate carcinoid tumour kallikrein (Oates et al, 1964). When this was considered in the light of other evidence (see P. 177) it seemed possible that adrenaline might itself activate kinin release. In the present work, intravenous infusion of adrenaline was found to activate the release of large amounts of kinin in two dogs out of five. Emotional fainting in man is known to involve

extensive sympathetic stimulation (Barcroft and Swan, 1953) presumably with the resultant release of adrenaline. In two subjects undergoing emotional fainting, marked kinin release was detected. Adrenaline and various types of stress activate fibrinolysis (see P. 180) and the present findings lend tentative support to the existence of a similar action on plasma-kinin-releasing enzymes. Vasomotor dumping symptoms are often very similar to those seen in syncope. The stress produced by the intestinal and psychic components of dumping and the resulting sympathetic stimulation may prove to be an important factor in the provocation of the kinin release and a syncope-like reaction.

One final point of interest is the close association of 5-HT (Barter and Pearse, 1953; Lembeck, 1953) with kallikrein (Oates and Melmon, 1966) and kinin-like peptides (Pearse, 1968) in a single cellular store, the argentaffin cell. 5-HT tends to be vasoconstrictor and stimulates motility of the intestine, kinins are vasodilator and inhibit peristalsis. Vasodilatation and increased absorption are essential factors in the circulatory response to food (Grayson and Mendel, 1965). In the dog, feeding increases the blood flow of the small intestine (Grim and Lindseth, 1958); while distension of the isolated ileum has been shown to produce local vasodilatation (Lawson and Chumley, 1940). In man, there is a rapid increase in splanchnic blood flow following the ingestion of protein (Brandt, Castleman, Ruskin, Greenwald and Kelly, 1955)

and liquid carbohydrate (Castenfors, 1961). There is some evidence summarized by Castenfors (1961) that the circulatory response to dumping may be an intensified form of the normal circulatory response to meals. The opposing physiological actions of 5-HT and kinin make a suitable basis for a homeostatic mechanism. The local release of these two substances could well be a major factor controlling the normal vasomotor tone, permeability and motility of the intestine.

APPENDICES

APPENDIX 1

MATERIALS

1. Synthetic bradykinin (BRS 640; Sandoz Ltd., Basle).
2. Serotonin creatinine sulphate (Koch-Light Laboratories Ltd., Colnbrook, Bucks.).
3. Adrenaline hydrochloride (Parke, Davis and Co., Hounslow).
4. 2-bromo-lysergic acid diethylamide (BOL-148; Sandoz Ltd., Basle).
5. Atropine sulphate (British Drug Houses, Poole).
6. Salt-free, crystalline trypsin (Armour Pharmaceutical Ltd., Eastbourne).
7. Salt-free, crystalline chymotrypsin (bovine) (Armour Pharmaceutical Ltd., Eastbourne).
8. Heparin (Pularin; Evans Medical Ltd., Liverpool).
9. Pentobarbitone sodium (Nembutal; Abbott Laboratories Ltd., Queensborough, Kent).
10. 2% dimethyldichlorosilane in carbon tetrachloride (Silicone "repelcote"; Hopkins and Williams, Chadwell Heath, Essex).
11. Ethanol and butanol were re-distilled in glass and stored at 4°C until use).
12. Water was glass distilled, or de-ionized on an "Elgastat" where stated.
13. All reagents were Analar grade or equivalent.

APPENDIX 2

PROCEDURES FOR THE ESTIMATION OF PLASMA KININ AND KININOGEN IN SINGLE WHOLE-BLOOD SAMPLES

a) Preparation of the ethanol extract.

6 ml. of whole-blood were taken from an arm vein through a No. 19 sterile disposable siliconed needle into a 10 ml. disposable polystyrene syringe. 1 ml. was rapidly placed in a sequestrene tube for haematocrit estimations, and the remainder was immediately inactivated by forcibly ejecting it through the needle into 15 ml. of chilled ($4^{\circ}C$) glass-distilled ethanol in a stoppered glass centrifuge tube. The tube was then shaken to give good dispersion. Manipulations with whole-blood prior to inactivation were carried out as rapidly as possible, in order to minimize formation or destruction of free kinin as an artefact. The time between removing the blood from the vein, and its inactivation in ethanol was usually 60 - 80 seconds. At this point, but only when absolutely necessary, the sample was stored at $4^{\circ}C$ for up to eight hours.

Using a clean glass rod, any precipitate clinging to the stopper and walls of the centrifuge tube was washed into the ethanol. The sample was then centrifuged at 2000 'g' (3000 r.p.m.) for

thirty minutes. The supernatant containing the free kinin was placed in a 100 ml. stoppered flask, and the precipitate was washed with 5 ml. 80% (v/v) glass-distilled ethanol, and again centrifuged. The washings were added to the previous supernatant. The ethanolic extract was then placed in a boiling water bath for ten minutes to destroy any residual enzymic activity. The extract was finally evaporated to dryness under reduced pressure at the water pump.

b) Butanol extraction of kinin.

The dried ethanolic extract, prepared as described in Appendix 2 (a), was reconstituted in 8 ml. de-ionized water, and was warmed to 60°C on a water bath to get all the material into cloudy "solution". About 4 g. of sodium chloride was added to the warm solution, to give a saturated salt solution in contact with a little solid. Four drops (c. 0.2 ml.) of Analar concentrated hydrochloric acid were added with continuous shaking to lower the solution pH to c. 1.5 (measured with narrow range indicator paper). The resultant mixture (including undissolved NaCl) was transferred to a centrifuge tube, a further 3 ml. water being used to ensure complete transfer. The mixture was then centrifuged at 3000 r.p.m. (2000 'g') for fifteen minutes at 4°C, to give a fairly clear solution, with undissolved sodium chloride packed at the bottom of the tube,

and green insoluble lipid material floating on the surface. Using steady gently suction, all of the clear solution was removed, avoiding as far as possible, contamination with undissolved material, and transferred to a separating funnel. 10 ml. of re-distilled butanol was added, and the funnel was vigorously shaken for about thirty seconds to give complete mixing.

The resulting suspension was allowed to stand to permit separation of the phases. The lower (aqueous) layer was run into a clean tube, while the butanol layer was placed in a small flask. The aqueous layer (frequently containing freshly precipitated NaCl) was returned to the funnel, and re-extracted with a further 5 ml. of butanol, which was added to the earlier butanol extract. The butanol was then evaporated under reduced pressure in a rotary evaporator, starting cold (c. 15°C), and warming to 80°C. The last traces of butanol were removed by holding the extract at 80°C and 0.1 mm. Hg. for five minutes.

The dry extract was stored at -20°C until bio-assay. For bio-assay on oestrus rat uterus, the extract was routinely dissolved in 2 ml. warm (60°C) de Jalon's solution, and assayed against standard bradykinin, in the presence of atropine, and 2-bromolysergic acid diethylamide. The time during which the kinin was in solution, in particular that in the very acid solution, was kept to a minimum.

c) Estimation of plasma kininogen.

The precipitate obtained as described in Appendix 2 (a) was suspended in 10 ml. 80% ethanol, and placed in a boiling water bath for ten minutes to give complete denaturation. The resulting mixture was centrifuged at 2000 'g', and the supernatant was discarded. The precipitate was washed twice with 10 ml. distilled water, suspended in 15 ml. NaCl solution (2.5 M), and the suspension was homogenized in a ground glass homogenizer. 0.2 ml. aliquots of the suspension were placed in stoppered tubes in a thermostatically heated rocker-bath, and the temperature was allowed to equilibrate at 37°C for thirty minutes. To the tubes were added pure crystalline trypsin in 4.8 ml. of sodium phosphate buffer, to give 5 ml. of solution containing 200 µg. trypsin per ml. of 0.02 M. sodium phosphate at pH 7.35. The incubation was allowed to continue for thirty minutes, and then the trypsin was inactivated by heating in a boiling water-bath for ten minutes. The resulting incubates were immediately quick frozen (using solid CO_2 + Acetone), and stored at -20°C until assay. The isolated oestrus rat uterus bathed in oxygenated de Jalon's solution at 33°C in the presence of atropine (10^{-3} g. per litre) was used for the assay. A simple bracket assay was performed, using synthetic bradykinin as standard.

APPENDIX 3

ISOLATED TISSUE PREPARATIONS

Bio-assay Procedures.

The isolated uterus from the oestrus rat was routinely used for kinin assays in both the kininogen and free kinin estimations. The total amount of kininogen in blood was relatively large, while the changes were frequently less than 20%. The rat uterus easily detected changes of less than 10% in the applied dose of bradykinin (Fig. 22). Free kinin changes were generally much more than 100%; however, the total quantities were very small. The rat uterus is the most sensitive known routine assay tissue for kinin, and in addition is insensitive to histamine and the slow reacting substance of anaphylaxis.

Contraction of the isolated guinea pig ileum and relaxation of the isolated rat duodenum were used for parallel bio-assay when the characterization of bradykinin-like activity was required.

Rat Uterus.

Virgin albino rats (150 - 200 g.) were brought into artificial oestrus by a subcutaneous injection of stilboestrol (20 µg./100 g. of body weight) in 30% aqueous ethanol. Eighteen to twenty-two hours following the injection, they were killed and the uterine horns excised.

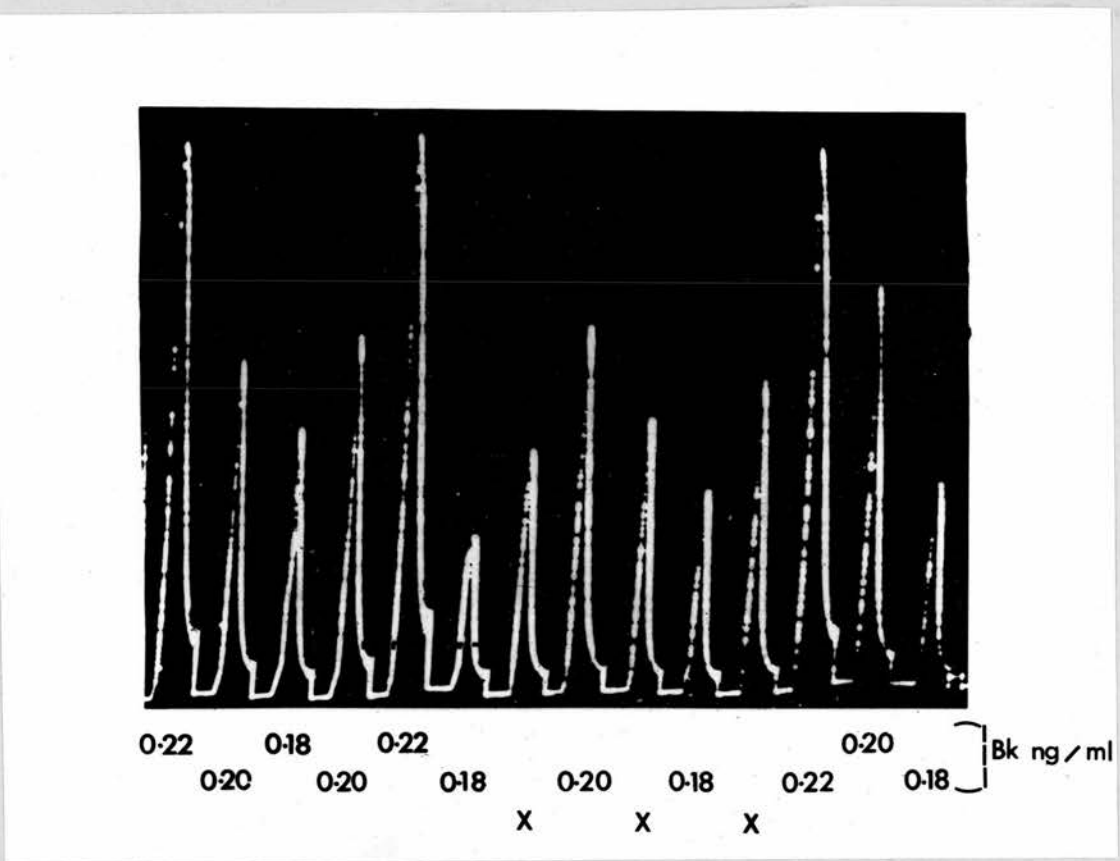

Fig. 22: Typical bracketing assay and dose-response curve from isolated oestrus rat uterus. (Bk = Standard bradykinin; X = unknown sample).

A length of 1 - 2 cm. was suspended in a 2 ml. bath for free kinin estimations, or 4 ml. bath for kininogen estimations. The bath contained oxygenated de Jalon solution with atropine sulphate (1 µg./ml.) at 33°C. For the free kinin assay, when 5-HT was to be excluded, freshly made 2-bromo-lysergic acid diethylamide (0.5 µg./ml.) was used. A frontal writing lever with a six-fold magnification exerted a tension of 0.5 g. on the tissue. The sample was allowed to remain in contact with the tissue for up to two minutes. A cycle of five minutes was usually employed.

In view of the comparatively long time cycle and the nature of the experiments, a simple bracketing assay was used. Such an assay is shown in Fig. 22.

Guinea Pig Ileum.

A piece of terminal guinea pig ileum about 2 cm. long was suspended in a 2 ml. bath at 34 - 35°C. The tissue was bathed in oxygenated Tyrode solution. The writing lever used was that described for the previous preparation. A drug contact of 30 - 45 seconds in a cycle of four minutes was generally used. Mepyramine maleate and atropine sulphate (1 µg./ml.) were used to increase specificity.

Rat Duodenum.

The proximal 2 - 3 cm. of duodenum from albino rats of either sex was suspended in 5 ml. of oxygenated de Jalon solution at 30°C. The tissue had been stored at 4° for two or three hours previously. The lever used, exerted a tension of 0.5 g. on the tissue and magnified the response about ten times. The tissue was kept under constant wash by slow overflow which was stopped twenty seconds before drug was added to the bath.

APPENDIX 4

CALCULATION OF RESULTS

a) **Free plasma kinin**

A butanolic extract, E, obtained as described in Appendix 2 (b), is dissolved in 2 ml. of de Jalon solution, and is subsequently diluted to give a final concentration of [E] per y ml.

On assay it is found that:-

x ml. of solution [E] /y $\equiv$ z ng. of synthetic bradykinin (BK)

Then 1 ml. of solution [E] $\equiv$ z.y/x ng. BK

But E contains the kinin content of 5 ml. of whole blood.

Therefore 5 ml. of whole blood $\equiv$ z.y/x ng. BK

And 1 ml. of whole blood $\equiv \frac{1}{5} \times \frac{z.y}{x}$ ng. BK

If the haematocrit is H%, then the plasma volume is (100-H)%.

Then 1 ml. of plasma contains $\frac{1}{5} \times \frac{z.y}{x} \times \frac{100}{(100-H)}$

$= 20.z.y/x(100-H)$

Thus if an extract from 5 ml. of whole blood, of haematocrit H%, is reconstituted in y ml. of de Jalon's solution, and x ml. of this solution on bio-assay is equivalent to z ng. BK, then the plasma kinin concentration is 20.z.y/x(100-H) ng. BK equivalents per ml. of plasma.

b) Plasma kininogen

If x ml. of kininogen incubate I, diluted 1/y is found on assay to be equivalent to z ng. of synthetic BK.

Then x ml. of solution [I]/y $\equiv$ z ng. BK

And 1 ml. of solution [I] $\equiv$ z.y/x ng. BK

The total volume of the incubate is 5 ml.

And 5 ml. of solution [I] $\equiv$ 5.z.y/x ng. BK

5 ml. of incubate contains 0.2 ml. of whole blood homogenate.

Therefore 0.2 ml. of homogenate $\equiv$ 5.z.y/x ng. BK

15 ml. of homogenate $\equiv 75 \times 5 \times \frac{z.y}{x}$ ng. BK

15 ml. of homogenate contains 5 ml. of whole blood.

Therefore 5 ml. of whole blood $\equiv 75 \times 5 \times \frac{z.y}{x}$ ng. BK

And 1 ml. of whole blood $\equiv 75 \times \frac{z.y}{x}$ ng. BK

If the haematocrit is H%, then the plasma volume is (100-H)%.

Thus 1 ml. of plasma contains $75 \times \frac{z.y}{x} \times \frac{100}{(100-H)}$ ng. BK Eq.

Or more conveniently = $75 \times \frac{z.y}{x} \times \frac{100}{(100-H)} \times \frac{1}{1000}$ µg. BK Eq.

= 7.5 (z.y/x(100-H))µg. BK Eq.

Thus if an incubate, obtained as described in Appendix 2 (c) from whole blood of haematocrit H%, is diluted 1/y, and x ml. is equivalent to z ng. of synthetic BK, then the concentration of kininogen is 7.5 (z.y/x(100-H))µg. BK equivalents per ml. of plasma.

APPENDIX 5

pK_a AND DEGREE OF DISSOCIATION OF BASE AT ACID pH DURING BUTANOL EXTRACTION

The dissociation equilibrium for a base is:

$$B + H^+ \rightleftharpoons BH^+$$
$$\alpha \quad \alpha \quad 1-\alpha$$

Where B is the un-ionized base, α is the degree of dissociation of ionized base.

Then equilibrium constant, $K_a = \frac{[B][H^+]}{[BH^+]}$

Therefore $pK_a = pH + \text{Log}([BH^+] / [B])$

Therefore $pH = pK_a + \text{Log}([B] / [BH^+])$

$= pK_a + \text{Log}(\alpha/(1-\alpha))$

The pH of the aqueous phase during the butanol extraction is pH 1.5. Assuming that only un-ionized base passes into the butanol, it is of importance to know at what pK_a the proportion of un-ionized base ceases to be negligible.

Take $\alpha = 10^{-5}$ as negligible.

Then $pH = pK_a + \text{Log}(\alpha/(1-\alpha))$

Therefore $1.5 = pK_a + \text{Log}(10^{-5}/(1-10^{-5}))$

$\simeq pK_a + \text{Log}\, 10^{-5}$

Therefore $pK_a = 1.5 + 5$

$= 6.5$

Then at pH 1.5, only 0.001% of a base, pK_a = 6.5, is in the un-ionized form.

The bases to be excluded from the butanol are all of $pK_a > 9$. e.g:

Base	pK_a	Reference
Histamine	9.7	Levy, M. (1935)
Adrenaline	9.90	Barlow, R. B. (1964)
5-HT	10.0	Vane, J. R. (1959)

Thus when pH = 1.5, and $pK_a = 9$

Then $pH = pK_a + \text{Log}(\alpha/(1-\alpha))$

That is $1.5 = 9 + \text{Log}(\alpha/(1-\alpha))$

Then $\text{Log}(\alpha/(1-\alpha)) = -7.5$

And $\alpha/(1-\alpha) = 10^{-7.5}$

Thus $= 10^{-7.5} /(1 + 10^{-7.5})$

$\simeq 10^{-7.5} = 10^{-8}\ 10$

Thus at pH 1.5, only $10^{-6}\sqrt{10}$% of a base of $pK_a 9$ is un-ionized. The bases under consideration are thus very highly ionized at this pH, and are unlikely to be extracted into the butanol.

APPENDIX 6

STATISTICAL ANALYSIS OF CHANGES IN FREE PLASMA KININ AND KININOGEN DURING THE DUMPING SYNDROME

Plasma Kinin Levels

The levels of plasma kinin found in the dumping patients showed a wide scatter. A simple statistical analysis was carried out to show that a positive increase in free kinin levels during dumping symptoms had occurred more often than may be expected by chance. These increases were in all but one case more than 100%, while in the control experiments, changes were negligible and in either direction. In such circumstances, the experimental conclusions are so clear that there is nothing to be gained by a statistical comparison of the results obtained from the dumping experiments, and those obtained from the control experiments.

Kininogen Levels

The changes in kininogen levels during the dumping syndrome were fairly small, being of the order of 20% of the pre-dumping control levels. Although difference between the changes measured during the dumping experiments and those seen during the control experiments were fairly marked, statistical analysis was carried out to decide whether or not this difference could be due to experimental variability. These analyses differed from that

carried out on the free kinin results in that they were analyses of the experiment and not merely of the array of figures. Thus, when non-paired groups were being compared, where the experiment had been repeated more than once for each patient, mean values were calculated, so that no patient was considered more than once (See Table 24).

Statistical Tests

Non-parametric tests were used, since the results did not always satisfy the requirements of the parametric tests, e.g:

(i) Observations must be independent.

(ii) Populations must be normally distributed.

(iii) Populations must have the same variance.

(iv) The variables must have been measured in at least an interval scale, so that normal arithmetic operations may be used on the scores.

(v) Effects of columns and/or rows must be additive.

The non-parametric tests, as used, on the other hand, are derived from variations and combinations of the 'Sign' test, and ranking or 'order' tests. These tests do not depend on the distribution of the population, and need require no unjustifiable assumptions.

Null Hypothesis

When two groups of results are being compared, the 'null hypothesis' will be made that there is no difference between them. The null hypothesis will be rejected at a probability level of $P < 0.05$.

a) In the presence of dumping symptoms, does an increase in the free plasma kinin level occur more often than would be expected from random scatter?

Test Used: Wilcoxon test for pair differences (c.v. Documenta Geigy (1962) P. 191).

Rationale

(a) Let d_i = Difference for any pair of values.

(b) Rank all the d_i's without regard to sign (i.e: -1 has lower rank than -2 or +2)

(c) Then affix sign to each rank.

(d) Sum the ranks having (+) sign $(T_{(+)})$ and ranks having (-) sign $(T_{(-)})$

(e) If the free kinin level does not increase during dumping, and the different values are only influenced by random scatter, then $T_{(+)} \simeq T_{(-)}$.

(f) The test gives the probability for a particular value of $T_{(+)}$ or $T_{(-)}$ occurring.

Null Hypothesis

(a) The null hypothesis is that the mean free kinin level at the dump peak $(\bar{x}_D)$ is not greater than that during the control period $(\bar{x}_C)$.

(b) The null hypothesis is rejected if $T_{(-)}$ is too small (or if $T_{(+)}$ is too large) to have occurred by chance.

Results

From Table 24 (b), $T_{(-)} = 0$.

Then from the Wilcoxon probability table (Documenta Geigy (1962) P. 128),

When number of pairs n = 7, the probability that $T_{(-)} = 0$ is $P \leq 0.01$.

Conclusion

The null hypothesis is rejected. The levels of free plasma kinin at dump peak tended to be greater than those during the control period. The probability that this result could occur by chance is $P \leq 0.01$.

Reaction	Free Kinin (ng. BK Eq. per ml. Plasma)		
	Pre-dump Control (x_{Ci})	Dump Peak (x_{Di})	Difference (d_i)
A(i)	2.4	75.0	72.6
A(ii)	<2.7	140.6	137.9
B(i)	<3.5	43.7	40.2
B(ii)	1.5	38.0	36.5
C(i)	6.3	62.9	56.6
C(ii)	0.6	70.5	69.9
D(i)	<3.7	15.3	11.6

Note: Where no activity was measurable, the value is taken as the threshold level for the purposes of calculation.

Table 24 (a) (From Table 18)

Rank	d_i
1	+ 11.6
2	+ 36.5
3	+ 40.2
4	+ 56.6
5	+ 69.9
6	+ 72.6
7	+137.9

Table 24 (b) (From above Table)

b) Statistical Analysis of Changes in Plasma Kininogen Level in Dumping and Control Experiments.

(i) Is the Fall in Plasma Kininogen greater in Dumping Patients than in Normals, following provocation with Hypertonic Glucose?

Test Used: Mann-Whitney 'U'-test. (Siegel, 1956).

Rationale

(a) Samples from two populations, A and B.

(b) Null hypothesis is that A and B have the same distribution. Alternative hypothesis is that A is stochastically larger than B.

(c) Alternative hypothesis may be accepted, if the probability of a score from A being larger than a score from B, is greater than $\frac{1}{2}$. That is, if 'a' is an observation from A, and 'b' is an observation from B, then $P(a>b) > \frac{1}{2}$.

Method

(a) n_1 = Number of cases in smaller of two independent groups, N_1.

n_2 = Number of cases in larger group, N_2.

(b) Combine both sets of results, and rank them in ascending order.

(c) Then, for N_1 say, let U_i be the number of times a score in N_2 precedes a score in N_1 in the ranking.

(d) Then $U_{N_1} = \sum U_i$

(e) The sampling distribution of U for the null hypothesis is known. Thus the probability for the occurrence, under the null hypothesis of any U as great as an observed value of U may be determined.

(f) $U_{N_1} = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1$ And, $U_{N_1} = n_1 n_2 - U_{N_2}$.

$U_{N_2} = n_1 n_2 + \frac{n_2 (n_2 + 1)}{2} - R_2$

Where R_1 and R_2 = Sum of ranks for N_1 and N_2 respectively.

(g) The smaller value of U is used.

Null Hypothesis

The fall in plasma kininogen in dumpers is no greater than in normals, following provocation with hypertonic glucose.

Results

From Table 26:

$$U_{N_1} = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1 = 4 \times 4 + \frac{4 (4 + 1)}{2} - 10$$

$$= 16$$

$$U_{N_2} = n_1 n_2 - U_{N_1} = 4 \times 4 - 16$$

$$= 0$$

The smaller value of U is used.

From tables (Siegel (1956), P. 271), the probability for the occurrence of this value of U for $n_1 = n_2 = 4$ is P = 0.014.

Conclusion

The null hypothesis is rejected. At the previously selected level of significance (P = 0.05), the fall in plasma kininogen in dumping patients is greater than that in normal subjects, following provocation with hypertonic glucose.

Experiment	Patient	Plasma Kininogen (µg. BK Eq. per ml. Plasma)	
		Fall in Kininogen (Control level - Peak level)	Means
Dumpers + Hypertonic glucose	A(i)	3.7	2.6
	A(ii)	1.5	
	B(i)	0.3	1.0
	B(ii)	1.4	
	B(iii)	1.3	
	C(i)	0.2	1.7
	C(ii)	3.1	
	D(i)	0.9	0.9
Normal Subjects + Hypertonic glucose	E	0.2	
	F	-0.1	
	G	0.1	
	H	0.1	
Dumpers + Saccharin	A	0.5	
	B	-0.1	
	C	0.0	

Table 25 (From Table 18)

Ranked Values of Fall in Kininogen (μg. BK Eq./ml. Plasma)	Patient	Rank
-0.1	N	1
0.1, 0.1	N, N	2.5, 2.5
0.2	N	4
0.9	D	5
1.0	D	6
1.7	D	7
2.6	D	8

D represents a dumping patient provoked with hypertonic glucose.
N represents a normal subject provoked with hypertonic glucose.

Table 26 (From Table 25)

(ii) Is the Fall in Plasma Kininogen greater in Dumping Patients following provocation with Hypertonic Glucose, than in Dumping Patients following provocation with Saccharin Placebo?

Test Used: Mann-Whitney 'U'-test.

Null Hypothesis

The fall in plasma kininogen in dumping subjects following provocation with hypertonic glucose, is no greater than that in dumping subjects following provocation with saccharin placebo.

Results

From Table 27:

$$n_1 = 3, \quad n_2 = 4$$

$$R_1 = 6, \quad R_2 = 22$$

$$U_{N_1} = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1 = 3 \times 4 + \frac{3 (3 + 1)}{2} - 6$$

$$= 12$$

$$U_{N_2} = n_1 n_2 - U_{N_1} = 3 \times 4 - 12$$

$$= 0$$

The smaller value of U is used.

From tables (Siegel (1956), P. 271), the probability for the occurrence of this value of U for $n_1 = 3$, $n_2 = 4$ is P = 0.028.

Conclusion

The null hypothesis is rejected. At the level of significance, $P = 0.05$, the fall in plasma kininogen in dumping patients following provocation with hypertonic glucose is greater than that in dumping patients following provocation with saccharin placebo.

Ranked Values of Fall in Kininogen (µg. BK Eq./ml. Plasma)	Patient	Rank
-0.1	S	1
0.0	S	2
0.5	S	3
0.9	D	4
1.0	D	5
1.7	D	6
2.6	D	7

D represents a dumping subject provoked with hypertonic glucose.
S represents a dumping subject provoked with saccharin placebo.

Table 27 (From Table 25)

(iii) Is the fall in Plasma Kininogen in Dumping Patients following provocation with Saccharin Placebo greater or smaller than that in Normal Subjects following provocation with Hypertonic Glucose?

Test Used: Mann-Whitney 'U'-test.

Null Hypothesis

The fall in plasma kininogen in dumping subjects following provocation with saccharin placebo is neither greater nor smaller than that in normal subjects following provocation with hypertonic glucose.

Results

From Table 28:

$n_1 = 3, \quad n_2 = 4$

$R_1 = 11.5, \quad R_2 = 16.5$

Note that $R_2 > R_1$, thus the kininogen change in the normals is apparently greater than in the placebo provoked dumping patients.

What is the probability for the chance occurrence of this inequality under the null hypothesis?

$$U_{N_1} = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R_1 = 3 \times 4 + \frac{3 (3 + 1)}{2} - 11.5$$

$$= 6.5$$

$U_{N_2} = n_1 n_2 - U_{N_1} = 3 \times 4 - 6.5$

$= 5.5$

The smaller value of U is used.

From tables (Siegel (1956), P. 217), the probability for the occurrence of this value for $n_1 = 3$, $n_2 = 4$ is $0.429 < P < 0.571$.

Conclusion

The data do not give evidence justifying the rejection of the null hypothesis at the level of significance, P=0.05. Thus there is no difference between the fall in plasma kininogen in dumping patients following provocation with saccharin placebo, and that in normal subjects following provocation with hypertonic glucose.

Ranked Values of Fall in Kininogen (µg. BK Eq./ml. Plasma)	Patient	Rank
-0.1, -0.1	N, S	1.5, 1.5
0.0	S	3
0.1, 0.1	N, N	4.5, 4.5
0.2	N	6
0.5	S	7

N represents a normal subject provoked with hypertonic glucose.
S represents a dumping subject provoked with saccharin placebo.

Table 28 (From Table 25)

(iv) Is there a Correlation between the Percentage Fall in Plasma Kininogen, and the Dumping Peak Level of Free Plasma Kinin expressed as a Multiple of the Pre-dump Control Level, in Dumping Subjects following Provocation with Hypertonic Glucose?

Test Used: Significance of the Spearman Ranking Coefficient. (Siegel, 1956).

Rationale

(a) Let N individuals be ranked according to two variables, denoting ranking according to one variable as X_1, X_2, X_3 ..... X_N, and according to a second variable as Y_1, Y_2, Y_3 ..... Y_N. Then a measure of the rank correlation may be used to determine the relation between the X's and the Y's.

(b) Perfect correlation would exist if $X_i = Y_i$ for all values of i. The differences $d_i = X_i - Y_i$ would give a measure of the disparity between the two sets of ranking, the larger the d_i's, the less perfect the association between the two variables.

(c) The total magnitude of the discrepancy between the two sets of ranking may be determined by calculating Spearman's rank coefficient, $r_s = f\ (\sum d_i^2)$.

(d) Then the sampling distribution of the statistic, r_s, is known for the condition of the null hypothesis that the two variables under study are <u>not</u> associated in the population, and the observed value of r_s differs from zero only by chance.

(e) Spearman's rank coefficient,

$$r_s = 1 - \frac{6 \sum_{i=1}^{N} d_i^2}{N^3 - N}$$

<u>Null Hypothesis</u>

There is a purely random relationship between the percentage of the pre-dumping control kininogen lost, and the number of times the free kinin at the dumping peak is greater than the control level.

<u>Result</u>

From Table 29 (b):

$$N = 7 \quad \sum d_i^2 = 8$$

Then Spearman's rank coefficient,

$$r_s = 1 - \frac{6 \sum_{i=1}^{N} d_i^2}{N^3 - N} = 1 - \frac{6 \times 8}{343 - 7}$$

$$= 0.8571$$

Then from tables (Siegel, (1956), p. 284),

The probability that for N = 7, the Spearman rank coefficient $r_s = 0.8571$ is, $0.05 > P > 0.01$.

Conclusion

The null hypothesis is rejected. At the selected level of significance ($P_{.} = 0.05$), the Spearman rank coefficient is too large to have occurred by chance. There is thus a significant correlation between the amount of plasma kininogen lost and the height reached by the free plasma kinin level.

Estimated at Dumping Peak				
Fall in Plasma Kininogen		RANK	Peak Free Plasma Kinin	
Patient	% of Pre-dump Control Level		Patient	Multiple of Pre-dump Control
C(i)	1.6	1	D(i)	1.27
B(i)	6.0	2	C(i)	9.98
D(i)	11.2	3	B(i)	12.49
B(iii)	18.9	4	B(iii)	25.33
A(ii)	27.3	5	A(i)	31.42
A(i)	48.7	6	A(ii)	52.07
C(ii)	63.3	7	C(ii)	125.89

Table 29 (a) (From Table 18)

Patient	Rank of Kininogen Change	Rank of Free Kinin Change	d_i	d_i^2
C(i)	1	2	-1	1
B(i)	2	3	-1	1
D(i)	3	1	2	4
B(iii)	4	4	0	0
A(ii)	5	6	-1	1
A(i)	6	5	1	1
C(ii)	7	7	0	0
			$\sum d_i^2 = 8$	

Table 29 (b) (From Table 29 (a))

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ACKNOWLEDGMENTS

ACKNOWLEDGMENTS

I am grateful to Professor W. L. M. Perry for his encouragement and support of this work, and to Dr. W. E. Brocklehurst for his interest and valuable advice. I wish to thank Mr. A. N. Smith of the Department of Clinical Surgery for his kind advice and co-operation during the investigations into the carcinoid and dumping syndromes.

I am indebted to Dr. D. Eccleston for performing the spectro-fluorimetric estimations of 5-HT, to Dr. H. Holgate of Sandoz Ltd., London, for gifts of bradykinin and bromolysergic acid diethylamide, and to Dr. I. Laszlo for a sample of substance P.

I would like to thank Miss I. Scott for her patience and care in typing the manuscript.

The award of a Graduate Research Scholarship by the University of Edinburgh during the period of the investigation is gratefully acknowledged.