STUDIES ON BLOOD BRADYKININ IN MAN

A Thesis

submitted for the degree of

Doctor of Medicine

of

The University of Adelaide, South Australia

by

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March, 1967

PREFACE

Although the plasma kinins (e.g. bradykinin) occur widely in body fluids, their function and significance as physiological or pathological mediators remain to be established. The pharmacological actions and potency of these endogenously occurring polypeptides , particularly their vasodilator actions are such as to indicate a possible role for them in both physiological and pathological processes.

This thesis describes investigations designed to throw further light on the role of bradykinin in man by study of its occurrence and metabolism in blood and its participation in a number of vasodilator phenomena.

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DECLARATION and ACKNOWLEDGEMENTS

I declare that this thesis is of my own composition and that it is a record of original work conducted during the years 1964, 1965 and 1966 in the Department of Human Physiology and Pharmacology, University of Adelaide. The work described herein has not been submitted for any other degree, award or diploma.

I wish to thank my supervisor Dr. M.L. Mashford for guidance, invaluable discussion and active participation in many of the experiments.

I would also like to record my gratitude to Professor R.F. Whelan and Dr. J.A. Walsh, with whom I had the privilege of working during some stages of these investigations and to Mr. F. Ledwith of the Psychology Department for helpful advice on statistical problems.

I am grateful to Dr. I.S. de la Lande for constructive criticism of the drafts of this thesis and to the technical staff of this department for their general assistance,

I am grateful to those colleagues, students and patients who volunteered for the investigations and to Dr. I. Collins of Sandoz Australia, Pty. Ltd., for the generous supplies of synthetic bradykinin (Sandoz, BRS 640).

GENERAL SUMMARY

PART 1 presents a historical survey of the advances in the elucidation of the plasma kinin system, including the isolation and synthesis of the important members of this group of polypeptides. A review is presented of the accumulating evidence for the possible participation of these substances in normal and disease processes.

PART 2 describes a method for the estimation of peripheral blood bradykinin levels, using a method of extraction and biological assay. With this it is possible to measure levels in the blood of all normal subjects so far examined. The average bradykinin level in brachial arterial blood is found to be 0.25 ng/ml ($\pm$ 0.23 ng/ml). These levels are lower than others reported but the average recovery of internal standard of 97% suggests that these figures are not low due to losses in the procedure.

In PART 3 attempts are made to relate blood bradykinin levels to peripheral circulatory changes. Direct heating of the hand causes the expected increase in blood flow and results in an increase in bradykinin levels in the venous blood draining that

part by up to 53%. In reactive hyperaemia and cold vasodilatation a fall in the venous bradykinin level is observed. It is shown that there is an arterio-venous difference in bradykinin levels across an extremity such as the hand,with arterial levels usually higher than the venous. The occasional observation of higher venous levels and the results of venous bradykinin assays during intra brachial artery bradykinin infusions, suggests that peripheral sites are able to produce bradykinin. It is also noted in this section that a generalised sympathetic discharge causes a lowering of endogenous arterial bradykinin levels, whereas sympathetic blockade produces an increase. The falling blood bradykinin levels observed in cold vasodilatation and reactive hyperaemia could be related to the painful nature of the experiments and the resultant sympathetic nervous system overactivity.

In PART 4 it is shown that infusions of adrenaline result in falls of blood bradykinin levels and this seems to be achieved by an acceleration of the destruction of the peptide by kininase. Since the adrenergic blocking agent phenoxybenzamine can block this enzyme activation, the possibility is discussed that catecholamines act on a circulating " receptor " which can be blocked by small concentrations (10^{-7} M) of a specific adrenergic blocking agent. Conclusions are drawn that catecholamines are powerful modulators of kininase activity and may be responsible for settingthe half-life of bradykinin in blood (and in interstitial

fluid) within the large range encountered in normal blood viz. 3 sec $\geqslant t_{\frac{1}{2}} <$ 40 sec , and thus regulating the activity of bradykinin in regions where it is possibly acting as a physiological (or pathological) agent.

In PART 5 the flushing and other cardiovascular changes in the carcinoid syndrome, which have been related to the appearance of large amounts of bradykinin in the hepatic vein blood by other workers, are here related more directly with bradykinin changes in the peripheral circulation. The carcinoid blood bradykinin levels are evaluated in terms of levels found in normal people and a comparison is drawn between the effects of intravenous adrenaline infusions in normal and carcinoid syndrome subjects.

A small group of patients with cirrhosis and another of menopausal women, suffering from severe flushing attacks , have been studied to determine whether bradykinin was involved in their abnormal vascular manifestations. Using this present method , no direct evidence of this could be obtained, but interesting facets of these conditions , and the experimental findings are discussed that suggest that a more refined or differently directed approach may yet implicate the plasma kinins.

Part 1 INTRODUCTION

a. THE PLACE OF ACTIVE POLYPEPTIDES IN BIOLOGY

b. THE PLASMA KININS

c. PLASMA KININS IN PHYSIOLOGY AND PATHOLOGY

d. THE SCOPE FOR STUDY

a. PLACE OF ACTIVE POLYPEPTIDES IN BIOLOGY

Many polypeptides with a wide spectrum of biological activity occur in nature. Some have well defined roles in the animal body as endocrine hormones e.g. A.C.T.H., M.S.H., A.D.H.,oxytocin and the less well understood fat-mobilising polypeptide (Fraction H) also from the pituitary gland.

Another group of polypeptides has a more local area of activity -- the gastrointestinal hormones, viz. gastrin,secretin,pancreozymin and cholecystokinin,which regulate the motility and secretory activity of the glands and organs of the upper gastrointestinal tract.

A group of polypeptides produced by soil bacteria have antibiotic properties and the polymixins,colistin,the bacitracins and tyrothricin have a valuable place in antimicrobial therapy.

Finally,a heterogenous group of polypeptides of diverse origins, having in common a small molecular weight and sharing a common property of marked action on smooth muscle cells,includes many substances of less well understood significance,structure and physiological role. An important polypeptide in this final group is angiotensin,an octapeptide which is liberated from an alpha$_2$ globulin in the plasma by the action of renin. The intense vasoconstrictor

and pressor properties of angiotensin have played a key role in its historical association with hypertensive disease,and the synthetic octapeptide has been introduced as a therapeutic agent. Angiotensin also has a stimulating effect on some extravascular smooth muscles,and it has a powerful stimulating effect on the secretion of aldosterone -- its property on extravascular smooth muscle is probably of minor importance. In contradistinction to angiotensin the other polypeptides in this last group do not have vasoconstriction as their chief property,in fact they are vasodilator on most vascular beds. Some,like angiotensin,are liberated by the action of enzymes on a plasma substrate -- again an $alpha_2$ globulin, and are called plasma kinins. The plasma kinins are a group of vasodilator polypeptides and possess an extraordinarily high degree of pharmacological activity. In low doses they cause vasodilatation,increase capillary permeability and produce oedema; evoke pain by some action on nerve endings and contract or relax a variety of extravascular smooth muscle. Three plasma kinins, bradykinin, kallidin (lysyl-bradykinin) and methionyl-lysyl bradykinin (Fig. 1), have now been identified and their amino acid sequence elucidated; all three have been synthesized. All three have very similar properties and differ in their actions only quantitatively. Other plasma kinins can also probably be liberated from the same substrate, their structure is uncertain, but they resemble the identified kinins in their pharmacological actions and

their general structure is probably similar.

Other kinins are found in animal tissues. They resemble the plasma kinins in some of their pharmacological properties,but because of incomplete characterization are identified rather by their site of occurrence. These include wasp kinin,substance P, colostrum kinin and neurokinin. Another very active polypeptide eledoisin,an endecapeptide,isolated from the posterior salivary glands of Eledone, a mollusc belonging to octopod cephalopods,has been studied and in some preparations has been found to be about fifty times a more active dilator than the plasma kinins. Physalaemin, isolated from the skin of Physalaemus Fuscumaculatus also possesses a powerful vasodilator action.

The kinin peptides are the least well understood of all biologically active polypeptides, but because of their widespread natural occurrence and because of their marked properties, have stimulated much study in an attempt to elucidate their complex metabolism and their precise role in the organism in health and disease.

b. THE PLASMA KININS

The pharmecological actions of these substances in the mammalian body were noticed long before their nature was appreciated. Soon after the demonstration of the pressor effects of suprarenal extracts by Oliver and Schafer in 1895 and before the establishment of the natural occurrence of histamine in the body by Best, Dale, Dudley and Thorpe in 1927, French workers Abelous and Bardier in 1909 described a substance extracted from urine of normal humans which had marked hypotensive properties. When injected intravenously into dogs it caused a marked fall in the carotid artery blood pressure, they called this substance in the urine l'urohypotensine. They suggested that this substance was a protein and had protease properties. They showed that in the presence of serum this substance was able to cause a contraction of smooth muscle and that the hypotension produced on intra-venous injection was peripheral in origin due to a vasodilator action on the peripheral blood vessels and not due to the weakening of the heart. Large intra-venous injections of this substance into animals caused torpor and somnolence and often death, which was ascribed to the compression of the brain which occurred due to the intense vasodilatation. It was suggested that this substance which is normally excreted by the kidneys may play a part in producing some of

the cerebral symptoms associated with uraemia.

The study of the hypotensive material in the urine was taken up again by German workers Frey and Kraut in 1928. Seemingly unaware of the findings of Abelous and Bardier, they 're-discovered' a hypotensive material excreted in the urine. The called it a "circulatory hormone" because it also occurred in the blood in the form of a reversibly inactive complex with an inactivator. The active substance was excreted in the urine after the inactivator had been split off by the kidney. They were able to distinguish this substance from histamine and acetylcholine and also suggested that it was an enzyme. Frey, Kraut and Schultz in 1930, in search of organs and tissues in the body that were producing this substance and passing it on to the blood stream, observed that the fluid in a pancreatic cyst found at operation produced the same cardiovascular effects on intra-venous injection as their urinary substance. Failing to find the substance in any other body fluids, they assumed the pancreas to be the major source of this material and called it kallikrein (Kallikreas = pancreas).

Werle, Götze and Keppler, in 1937 found that kallikrein had an indirect action, and, behaving as an enzyme, split off a pharmacologically active substance from some inactive precursor present in the serum. This substance which contracted isolated smooth muscle

they called DK (darmkontrahierende substanz) to indicate its pharmacological property.

In 1948 Werle and Berek re-named this active substance 'kallidin' and its precursor, a plasma protein,'kallidinogen'.

About this time, another line of study by Brazilian workers was being carried on,which initially had no bearing on plasma kinins, but which,as was subsequently seen,was the starting point of work that gave much of the early information about these substances. In 1948, Rocha e Silva and his group were investigating whether the venom of the snake Bothrops jararaca liberated histamine from dogs liver,as trypsin had been shown to do,(Rocha e Silva,Beraldo & Rosenfeld, 1949; Rocha e Silva,1955). The liver was perfused with defibrinated blood and the perfusate collected. Since dog's blood contains practically no histamine nor any other substance which stimulates the isolated guinea-pig gut,the appearance of a strong stimulating material in the effluent blood when the venom was introduced in the perfusing blood created much interest. The factor which immediately distinguished it from histamine was the nature of the contraction it produced. Furthermore,its action was not antagonised by histamine nor atropine.When the venom was put directly into the blood samples, it resulted in the production of a substance which had the same effect on the gut. It was obvious that the precursor of this substance was in the blood itself and not in the liver.

These workers also showed that the precursor was in the globulin fraction of the plasma. Two terms were now coined, "bradykinin" for the new pharmacologically active substance ('brady' -- because of its slow onset of action on the smooth muscle preparation), and "bradykininogen" was the term given to the globulin fraction of plasma from which bradykinin was released. Trypsin was shown to behave in the same way as the venom in liberating bradykinin from serum.

A degree of purification was achieved by precipitating out the nitrogenous contaminants with 80 - 90% alcohol from the 'crude bradykinin' and when the 'purified' substance was injected intravenously into rabbits and cats a sharp fall in arterial blood pressure followed. This early work also showed that bradykinin was probably a polypeptide or at least it had a peptide link,the integrity of which is necessary for its pharmacological activity. Bradykinin was found to be thermostable and dialysable. On the guinea-pig gut it had an effect many times more potent than histamine or acetylcholine.

Because of the different methods of preparation of the kinin peptides (bradykinin) by Rocha e Silva and (kallidin) by Werle, the pharmacologically active peptide prepared by treatment of serum globulin fractions with trypsin or snake venoms continued to be called bradykinin whereas that prepared by the action of kallikrein was called kallidin. The realisation of their marked

similarity grew, Holdstock, Mathias and Schachter in 1957 failed to distinguish between kallidin and bradykinin on pharmacological and chemical basis,whereas wasp kinin,which had been identified as a polypeptide with similar pharmacological properties by Schachter and Thain in 1954, was easily distinguishable. In spite of this,reservations about their identity were fostered because trypsin and kallikrein were often shown to release different amounts of the active material from the same substrate preparation (Holdstock et al. 1957), and kallikrein sometimes failed to release kallidin under conditions in which trypsin readily releases bradykinin (Schachter 1960). This discrepancy has now been accounted for by the realisation of the greater specificity of kallikrein for the substrate, e.g. cat salivary kallikrein will not release kallidin from horse serum (Bhoola, Morley, Schachter & Smaje, 1965). Also denaturation of the kininogen molecule by boiling renders it less susceptible to attack by kallikrein whereas tryptic activity may be favoured by the unfolding of the protein molecule (Werle & Trautschold, 1963).

Elucidation of the relationship between bradykinin and kallidin had to wait until their further purification. Although progress was made in the purification of bradykinin (Andrade, Diniz & Rocha e Silva, 1953; Andrade & Rocha e Silva,1956), it was not until 1960 that Elliott,Lewis and Horton finally purified brady-

kinin prepared by treating ox serum with trypsin,and were able to elucidate its structure. Simultaneously peptide chemists in Basle,Switzerland were attempting to synthesize the polypeptide according to the structure put forward by Elliott and his group. The result of this was that bradykinin,a nonapeptide, was synthesized by Boissonas, Guttmann and Jaquenoud in 1960. In 1960 the bradykinin released by the action of snake venom was isolated by Zuber and Jaques; and in 1961 purified "snake venom bradykinin", "trypsin bradykinin" and the synthetic nonapeptide were shown to be identical (Hamberg, Bumpus & Page, 1961).

Pierce and Webster in 1961 using human plasma as substrate and incubating it with human urinary kallikrein isolated two kallidins. One of these was identical with bradykinin and the second was a decapeptide containing the same sequence of amino acids as bradykinin but with an additional residue of lysine joined to the N-terminal residue of arginine (Fig. 1). The nonapeptide from the mixture was the first to be eluted from the carboxymethyl cellulose column in the purification, and was called kallidin 1. The decapeptide was then named kallidin II.

With the knowledge of the structure of kallidin II,Nicolaides, DeWald and M^{C}Carthy at Ann Arbor Michigan were able to synthesize the decapeptide in 1961. The synthetic decapeptide did not,however,exhibit the full biological activity of natural kallidin II,

PLASMA KININS

BRADYKININ

H–ARG - PRO - PRO-GLY - PHE - SER-PRO-PHE-ARG–OH

Lysyl — BRADYKININ (kallidin-10)

H–LYS - ARG - PRO - PRO-GLY - PHE - SER-PRO-PHE-ARG–OH

Methionyl — lysyl — BRADYKININ

H–MET–LYS - ARG - PRO - PRO-GLY - PHE - SER-PRO-PHE-ARG–OH

Fig. 1 (a). The amino acid sequence of the three major plasma kinins. Bradykinin (nonapeptide);lysyl-bradykinin (kallidin-10,decapeptide) and methionyl-lysyl-bradykinin (endecapeptide).

..LEU (?) –MET–LYS–**BRADYKININ**– SER-VAL-GLU NH_2-VAL-MET(?)...ALA-LEU

Fig. 1 (b). The kinin-yielding sequence of the kininogen molecule of bovine serum origin (Habermann & Helbig,1966). Pierce and Webster (1966) postulate also another kininogen molecule where the kinin moiety is at the C terminus.

and this was believed to be due to the extensive racemisation occurring during the coupling reaction of the dipeptide with the octapeptide in the synthetic procedure.However, when the synthetic decapeptide was treated by carboxymethyl cellulose chromatography, a small fraction appeared which had the full biological activity of kallidin II (Nicolaides et al. 1961). Using this synthetic - chromatographed decapeptide , Webster and Pierce (1963) compared its biological activity with synthetic bradykinin and found that the decapeptide was twice as active as a vasodilator in the dog as the nonapeptide. Differences in the cardiovascular effects of these two substances have been found and have been studied (Parratt,1964 ; Kjellmer & Odelram, 1965; Chou, Frohlich & Texter, 1965; Lochner & Parratt, 1966). On many isolated smooth muscles the synthetic decapeptide acts like bradykinin, but is less potent (Webster & Pierce, 1963). It seems that plasma can release other kinins apart from bradykinin and kallidin (Armstrong & Mills, 1963). Elliott, Lewis and Smyth (1963) have isolated an eleven amino acid peptide from acidified, dialysed and incubated bovine plasma fraction and have characterised it as methionyllysylbradykinin (Fig 1). The pharmacological actions of the endecapeptide are qualitatively similar to bradykinin and ithas now also been synthesized (Schroder, 1964).

The previous methods of peptide synthesis have now been improv-

ed and a new method introduced by Merrifield (1964). The Merrifield method is termed solid phase peptide synthesis and is based on the idea of attaching the protected C-terminal amino acid by a co-valent bond to an insoluble,solid,supporting particle and the peptide chain can then be constructed and lengthened in a stepwise process. The peptide chain can then be liberated in the form of a free peptide after the desired sequence has been assembled. With the availability of easier and more sophisticated methods of peptide synthesis,interest has been extended to the study of structure-activity relationships of peptides and in particular to the kinin analogues. Bodanszky, Ondetti, Sheehan and Lande (1963) pointed out the importance of the terminal arginine moieties,the length of the peptide chain and the direction of the amino acid sequence for biological activity. Preparation by Nicolaides, M^{c}Carthy and Potter (1965) of a bradykinin analogue containing arginines in the D-configuration which had negligible vasodilator activity indicated that the two terminal arginines of bradykinin must be of the L-configuration, in order for the molecule to exhibit its characteristic biological effects. The negligible activity of the D-arginine analogues demonstrates the stereospecificity of the N- and C- terminal amino acids of the bradykinin molecule and that the correct spatial configuration of the terminal ends of the peptide chain is an indispensible feature of the molecule.

Using the Merrifield method of peptide synthesis, Stewart and Woolley (1965) have investigated the changes in biological actions of bradykinin derivatives resulting from the addition of amino acids to the amino and carboxyl ends of the molecule. They showed that the lengthening of the molecule at the amino end has only a small effect on potency, at least if the lengthening is not greater than one or two residues. In contrast to this is the marked effect of lengthening from the carboxyl end; when just a single amino acid residue is added to the carboxyl end of the bradykinin, the potency is markedly reduced. This importance of the carboxyl end in biological activity extends to other biologically active peptides e.g. angiotensin, gastrin, eledoisin and oxytocin, and some biologically active proteins e.g. pancreatic ribonuclease and insulin.

c. PLASMA KININS IN PHYSIOLOGY AND PATHOLOGY

Speculations on the involvement of the kallikrein-kininogen-kinin system (Fig. 2) in physiological and pathological phenomena began from the time that Frey and Kraut in 1928 postulated that kallikrein was a circulatory hormone with potential vasodilator and vasodepressor properties. They were able to show that kallikrein was liberated from its complex with the inactivator at a low pH, and would then be able to exhibit its vascular effects. On the basis of the fall in pH which occurs with ischaemia, they postulated that kallikrein may be involved in producing the circulatory changes of reactive hyperaemia.

Because both the substrate and the releasing enzyme are endogenous substances and the kinin that is released has been shown to have such powerful pharmacological properties in in-vitro experiments on isolated smooth muscle preparations and on the cardiovascular system in laboratory animals, it has been a challenging riddle for investigators to implicate this system in physiological and pathological situations. This interest intensified as more and more information emerged about the nature and distribution of the kallikrein in the body. Kallikrein has now been demonstrated in the pancreas, in the salivary glands, in

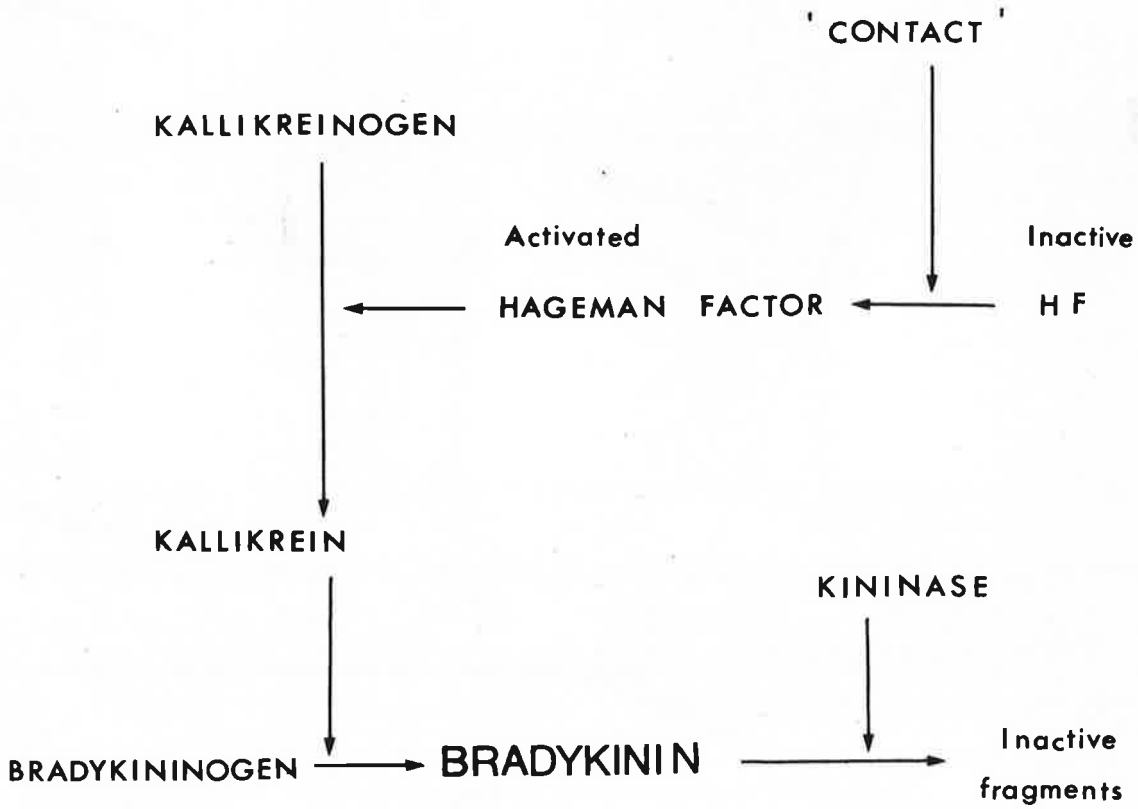


Fig.2 A simplified representation of the kallikrein-kininogen-kinin-kininase system in blood. Bradykinin is the representative kinin and its generation involves the action of the activated Hageman Factor. Its rapid degradation to inactive fragments is achieved by carboxypeptidase N (kininase).

glandular elements of the tongue, throughout the intestinal tract and in urine,saliva, sweat, faeces, in lachrymal secretions and probably in the cerebrospinal fluid. Kallikrein is found in the free active form in urine, sweat, saliva and faeces, however, in glandular tissues and in the plasma it exists as inactive precursor (Lewis, 1960; Webster 1966).

Of the many inferences that have been made about the participation of kinins in physiological and pathological changes,most have been made from observations of changes in one or more of the reactants in the kallikrein - kininogen - kinin system. Only in very few instances has it been possible to establish a convincing correlation between changes in local or general concentrations of plasma kinin and the expected functional change. The evidence for the activation of the kallikrein - kallidin - kinin system has come from measurements of increases in kallikrein or kallikrein activator, decrease in kallikrein inhibitor levels, diminished kininogen levels, increase in bradykinin or kallidin, increase or decrease in kininase or the reproduction of the symptoms or signs with kinins or kallikreins.

The presence of kallikrein in the salivary gland promised an explanation of the vasodilatation that occurs when the chorda tympani is stimulated. This has been difficult to account as being due to vasodilator nerve fibres in the chorda, because if

they are cholinergic, they could not be effectively blocked by atropine, whereas the secretory fibres could (Heidenheim,1872). Hilton and Lewis (1955 a, b; 1956) took up the study of the cause of functional hyperaemia in the cat's submaxillary gland. The lingual artery of the cat was cannulated so that the gland could still be perfused by natural circulation or artificially with oxygenated Locke's solution. When the gland was stimulated through the chorda tympani a substance appeared in the Locke's solution which when incubated with plasma produced a smooth muscle stimulating substance,and injected intra-arterially into the lingual artery produced a vasodilatation. The saliva also had this property and they concluded that the agents present in saliva and in the perfusate collected from the salivary gland on chorda stimulation are one and the same, and that bradykinin, or some closely related polypeptide is formed whenever they come in contact with plasma protein. They extended the possibility of this mechanism to other organs and tissues to account for the means of adjustment of local blood flow to local needs.

In 1956, Hilton and Lewis overcame the objection that could be raised to their use of plasma as a source of substrate for the glandular kallikrein --since plasma itself contains kallikreinogen which would be activated by dilution (Schachter, 1956). This time they used dog pseudoglobulin as substrate and the isolated

rat uterus as assay tissue, to achieve greater sensitivity. They were able to show an increased output of bradykinin forming enzyme on chorda stimulation, infusion of acetylcholine and during the post dilatation which occurred with injections of adrenaline or noradrenaline. They re-emphasised their previous conclusions by stating that no matter how the gland is activated, by chorda or sympathetic stimulation or by infusions of acetylcholine or sympathomimetic amines, a bradykinin-forming enzyme is released from the gland cells, and that the vasodilatation accompanying the glandular activity is produced by the bradykinin which is formed.

The observation of Bhoola et al. (1965), however, did not substantiate Hilton and Lewis' conclusions about bradykinin being the cause of the functional vasodilator in the cat's submaxillary salivary gland. They observed that the vasodilatation produced by the intra-arterial injection of cat salivary kallikrein did not mimic the decrease in resistance caused by stimulation of the chorda tympani. The vasodilatation produced by the injection of dialysed cat saliva was slower in onset, not as great, generally more prolonged, and became smaller with successive injections of saliva. Stimulation of the nerve still produced vasodilatation when the gland was perfused with horse serum (for three hours) -- a substrate from which cat kallikrein does not release kallidin.

Desensitisation of the gland to bradykinin by close arterial injection also had little effect on functional vasodilatation. These workers also observed the after-dilatation following vasoconstriction upon preganglionic sympathetic nerve stimulation; this vasodilatation also occurred during stimulation when alpha-blocking drugs such as phenoxybenzamine or tolazine had been used. Use of sympathetic beta-blocking agents, however, reduced or blocked the sympathetic after-dilatation. Their conclusions uphold the old view that parasympathetic vasodilator fibres innervate the vessels of the gland and that the sympathetic after-dilation is due to adrenergic action, but due to vascular beta-receptor stimulation.

Although Hilton and Lewis have associated vasodilatation in the submaxillary gland with increased output of kallikrein when the gland is stimulated by acetylcholine, adrenaline or noradrenaline injections or chorda tympani stimulation, Schachter, (1966) is reluctant to relate this causally in any significant way. Some of Schachter's opposition is probably blunted by Hilton's (1966) contention that the active peptide is probably formed in the interstitial fluid of the gland and not in the blood and that conclusions from intra-arterial saliva and intra-arterial desensitizing bradykinin injections are not valid arguments against what might be happening in a more intimate extracellular fluid-arteriole situation.

The controversy that exists between the proponents of vasodilator fibres and kinin mediated vasodilatation is further compounded by observations that many glandular organs of the guinea pig do not contain an active kinin-releasing enzyme (Bhoola, May May Yi, Morley & Schachter, 1962) and that in the rabbit's submaxillary gland both the secretion and vasodilatation which follow chorda lingual nerve stimulation are readily blocked by atropine (Morley, Schachter & Smaje, 1963).

The functional vasodilatation of the pancreas has also been studied with the view of implicating a kinin peptide as the cause. Using a similar approach to that of Hilton and Lewis (1955 b) in studying vasodilatation in the submandibular gland, Hilton and Jones (1963) investigated the functional vasodilatation in the pancreas. The output of kinin forming enzyme of the perfused gland (Locke's solution) was small at rest. Acetylcholine and pancreozymin,however, were both able to bring about a significant elevation. These findings have not yet been challenged.

The occurrence of increased vasodilatation in the forearm of man at the onset of sweating produced by body heating, has again suggested a role for bradykinin, which might be liberated as a result of sweat gland activity. Bradykinin-forming activity was shown to be present in sweat collected from hand and forearm by Fox and Hilton (1956). The studies of Roddie, Shepherd and Whelan

(1957) on the effect of atropine on the sweating and vasodilatation in the human forearm when the person is subjected to body heating, suggested that the situation in the forearm skin was analogous to that in the submaxillary gland when that gland is treated with atropine and the chorda lingual nerve is stimulated. The major vasodilatation in the forearm which follows the onset of sweating can be temporarily abolished by atropinisation of the forearm tissues. However, despite atropinisation the forearm blood flow eventually increases and atropine when injected at the height of heating vasodilatation, does not reduce the flow, even though the action of a subsequent dose of acetylcholine is completely blocked. Although intra-arterial atropine completely abolishes sweating, it does not completely prevent the "sweating" component of forearm vasodilatation.

In 1958 Fox and Hilton, using a technique of perfusion of subcutaneous tissue space with sterile saline, showed that when the subject was cool the perfusate showed small amounts of bradykinin-like activity, but did not contain any bradykinin-forming activity. When the subject was heated,however, and sweating developed, the bradykinin-like activity of the perfusion fluid increased up to five times the resting level. The bradykinin-like activity of the perfusate during the control period was attributed to the dilution of the interstitial protein. However,since

the protein content of the fluid did not change as the forearm vasodilatation developed, the increase in bradykinin-like activity could not be attributed to an increase in passage of protein across the capillary walls, but was due to sweat gland activity. They concluded that the active vasodilatation in the human forearm skin accompanying body heating is produced in the main, by bradykinin resulting from sweat gland activity.

There is much evidence which points to plasma kinin playing a part in inflammation. The striking features found in early stages of inflammation are pain, local vasodilatation, increased capillary permeability and the accumulation of leucocytes. It has been shown that bradykinin is one of the most potent agents able to produce these events.

Bradykinin or a very similar polypeptide is released from blister fluid, human inflammatory exudates and plasma, if the latter if first allowed contact with glass. This substance causes pain when applied to a catharidin blister base and has been called Pain Producing Substance (PPS). It is very likely to be bradykinin, it might be one of the physiological transmitters of pain and is likely to be a contributing factor in causing the pain of inflammation (Armstrong, Dry, Keele & Markham, 1953; Armstrong, Jepson, Keele & Stewart, 1957). The ability of bradykinin and kallikrein to cause increased capillary permeability

with a more rapid onset than histamine, and to produce accumulation of leucocytes has also been demonstrated (Bhoola, Calle & Schachter, 1960; Elliott, Horton & Lewis, 1960; Lewis 1960). Chapman, Ramos, Goodell and Wolff (1963) have perfused the subcutaneous tissue under the "flare" (produced by noxious stimulation of the skin) with isotonic saline. The perfusate collected during the onset of flare increased in its ability to relax the isolated rat duodenum and contract the rat uterus, indicating increased kinin formation.

Rocha e Silva and Antonio (1960) produced 'thermic oedema' in the rat's paw by immersion in water at 44 - 45° C and by coaxial perfusion of the subcutaneous space, found that at this temperature there was a sudden appearance of bradykinin in the perfusate. The conditions of the experiment were such that the participation of histamine formation was precluded. Thus a condition of inflammation and oedema was produced without the release of histamine and the response of the tissues to this mild thermal injury could be entirely due to bradykinin release.

Other evidence by Rocha e Silva and Rosenthal (1961) indicated that bradykinin, histamine and possibly other substances may be released in the skin when the thermal injury is more severe. Edery and Lewis (1963) concluded that the increase in kinin-forming activity in the lymph draining the dog's hind limb which

occurred when the limb was injured by severe scalding, ischaemia, mechanical pressure or freezing was due to a specific activation of the kinin-forming system in the interstitial fluid, brought about by the release of histamine.

The mechanism of the activation of the kallikrein-kinin system upon injury is not certain. The findings of kinin-forming activity in the lymph from injured limbs by Edery and Lewis (1963), have been criticised by other workers performing similar experiments (Jacobsen & Waaler 1965; Jacobsen 1966; Jacobsen & Waaler 1966). Jacobsen and Waaler confirmed that plasma kinins were formed on incubation of dog lymph with pseudoglobulin, but they ascribed this to the presence of kinin-forming activity in the pseudoglobulin and of substrate for this enzymatic activity in the lymph. On scalding an increase in lymph flow occurs and lymph content of total protein also increases. Their interpretation of the apparent increase in kinin-forming activity in the lymph of the injured limb is that it is due to increased content of substrate in the lymph for the kallikrein which is present in the pseudoglobulin. It is possible that histamine liberation plays a role in the change in blood vessel permeability upon this type of injury.

In a variety of conditions of shock, many potentially toxic substances have been suggested as possible mediators. That pro-

teolytic enzymes are activated in states of shock has been suggested for a long time (Roch e Silva, 1956) it is possible that a kinin-forming enzyme is among them. It is probable that the proteolytic activity occurs spontaneously in some states of shock as suggested by MacFarlaneand Biggs (1946), or it might be due to the entry into the organism of potent substances as might occur in shock states due to endotoxin, peptone, anaphylaxis, acute pancreatitis, haemorrhage, severe burns and barbiturate poisoning.

Beraldo (1950) estimated the amount of plasma kinin in the blood of dogs before and during anaphylactic and peptone shock. During shock, the plasma kinin increased, decreased or remained the same, and Beraldo concluded that plasma kinin activity bore no relationship to the severity of the shock. There is more evidence however of a causal relationship between anaphylaxis and plasma kinins from Brocklehurst and Lahiri (1962). They have shown that bradykinin appears in the blood of rat, guinea pig and rabbit when the sensitized animals are challenged with antigen. The perfusate from guinea pig lungs or skin during anaphylaxis was shown to have kinin-forming activity, however, when freshly drawn blood from sensitized guinea pigs was incubated with antigen there was no increase in bradykinin. It was thus shown that the activation of the kallikrein was occur-

ring in a tissue site, possibly the site of antigen - antibody reaction. The recovery of bradykinin-forming enzyme from perfused lung in the guinea pig is interesting, because it has been shown that bradykinin is a potent bronchoconstrictor agent in the guinea pig (Collier, Holgate, Schachter & Shorley, 1960). From these findings it suggests that bradykinin may have a role in the bronchoconstriction of anaplylaxis and perhaps asthma, since bradykinin in aerosol form has been shown to reduce the vital capacity of chronic asthmatics (Herxheimer & Stresemann, 1961; Collier, 1963).

However, another substance which is quite distinct from bradykinin, serotonin and substance P has been isolated from anaphylactic guinea pig lung and has been called SRS-A (slow-reacting substance in anaphylaxis) by Brocklehurst (Brocklehurst 1953, 1955, 1960). The actions of SRS-A and bradykinin have been compared on the guinea pig tracheobronchial muscle by Berry and Collier (1964) and it seems that there are distinct and separate receptors for these two substances on the bronchial muscle. It seems,then, that the pulmonary manifestations of anaphylactic shock may be due to one or both of these smooth muscle stimulating substances.

Further evidence implicating bradykinin in the vascular events of anaphylaxis comes from the work of Cirstea, Suhaciu and Butcul-

escu (1966). These workers have shown that a significant diminution of plasma bradykininogen is found in rabbits blood subjected to lethal anaphylactic shock, and associated with this, detectable levels of bradykinin appear in the blood.

The relationship between endotoxin, the release of bradykinin and the features of shock is tenuous in Beraldo's (1950) own conclusions. There is little evidence available at present to further the possible relationship,though Kobald, Lucas and Thal (1964) report that incubation of endotoxin with blood and various tissue homogenates releases several vaso-active substances including plasma kinin.

Vogt (1964) has put forward interesting positive evidence about plasmin being able to liberate bradykinin under certain conditions. Human plasmin will liberate plasma kinin from normal dog plasma globulin, but the liberation of the kinin is dependent on the conversion of kallikreinogen to kallikrein by plasmin i.e. plasmin liberates kinin only by this indirect mechanism. The intermediary involvement of kallikrein is further supported by the observation that after incubation of globulin with plasmin, there is a considerable decrease in the concentration of kallikreinogen. Like kallikrein, plasmin is also a proteolytic enzyme, derived from a precursor plasminogen, which is carried in the globulin fraction of the plasma.

So far there are no reports of plasminogen activation in vivo associated with production of bradykinin. However, activation of plasminogen has been described in a wide variety of conditions, through the intermediary of activators of tissue, body fluid or bacterial origin or even from psychic responses to mental or physical disturbances such as in persons awaiting surgery or even in normal people during air-raids (MacFarlane & Biggs, 1946).

Much speculation continues regarding the precise patho-physiology of acute pancreatitis. Both the cause and the mechanism of the changes which take place remain unknown. This is particularly disturbing because the present methods of treatment are not completely satisfactory and death rate in acute pancreatic necrosis in human being remains above 50% (Nugent, Henderson, Jonasson, Jones & Attendido, 1964). A popular theory is that trypsin, a pancreatic enzyme, is activated within the pancreas and precipitates a vicious cycle of autodigestion of the gland. Good evidence that trypsin is present in plasma in increased concentration in acute pancreatitis is given by Nardi and Lees,(1958) by their simple method of blood trypsin estimation. With the presence of active trypsin it is generally argued that trypsin further activates kallikrein from pancreatic and plasma kallikreinogen and that the kinins which result from the action of

kallikrein on plasma kininogen contribute to the vascular collapse. Hollenberg, Kobold, Pruett and Thal (1962) reported detection of a vasoactive material in the blood from patients with acute pancreatitis. They placed whole blood on an unspecified smooth muscle preparation and measured the response in terms of the tension developed. The evidence for this substance being bradykinin is inconclusive from their experiments.

Administration of Trasylol, a potent trypsin inhibitor and kallikrein inactivator, extracted from bovine parotid glands to patients with acute pancreatitis has met with varying amounts of success. Control studies on the usefulness of Trasylol in this condition have also been performed in dogs where the pancreatic necrosis has been induced chemically. The effect of Trasylol on the haemodynamic events has not been striking, though the overall mortality is usually reduced in the group which recieves Trasylol in the treatment (Forell, 1963; Nugent et al 1964; Grozinger, Artz, Hollis & Wesson, 1964; Haig & Thompson, 1964).

One of the best demonstrations of the participation of a plasma kinin in a disease state has been that of bradykinin in the carcinoid syndrome. The most distinctive and often the earliest symptom of the carcinoid syndrome is the acute, reddish cutaneous flush which starts with the face and neck and may ex-

tend to the chest, arms and legs. Colours range from bright red to violaceous. The flush usually lasts only a few minutes, but may persist longer and recur many times during the day. Periorbital oedema, hypotension, tachycardia, abdominal pain, diarrhoea and wheezing often accompany the flushing. Right sided cardiac involvement may appear late in the disease. The carcinoid syndrome is usually a slowly advancing disease and a patient who has developed the full complement of florid signs and symptoms may survive for many years. The puzzling array of manifestations in the syndrome and the protracted nature of the disease are some of the reasons why this condition has been the focus of much pharmacological and clinical investigation for more than a decade.

Pernow and Waldenstrom (1954) first demonstrated that patients with the carcinoid syndrome could have elevated blood levels of serotonin and histamine. These two pharmacologically active substances could account for many of the manifestations of the syndrome. However, the observation that the levels of serotonin in hepatic venous blood fail to increase in most patients after induction of typical flushes by injections of noradrenaline or adrenaline led to the questioning of the role of serotonin in flush production (Robertson, Peart & Andrews, 1962). Because catecholamines activate the flush mechanism in carcinoid patients, the

observations of Hilton and Lewis (1956) that catecholamines will release a kinin peptide from the perfused salivary gland suggested that such peptides might play a role in carcinoid flushes. Oates, Melmon, Sjoerdsma, Gillespie and Mason in 1964 demonstrated that hepatic venous blood showed elevated levels of a kinin peptide during the flushing attacks, this being due to the release of active kallikrein in the hepatic vein from carcinoid metastases in the liver. Later Oates, Pettinger and Doctor (1966) using more thorough purification procedures showed that this kinin peptide was indistinguishable from bradykinin.

Recently, Zeitlin and Smith (1966) have also implicated plasma kinins in the symptoms associated with the dumping syndrome and report the appearance of high levels of free kinin in the forearm venous blood of patients during the dumping peak. The levels noted by these workers seem exceedingly high (2·6 microgram/ml in one case), particularly in comparison with the levels of hepatic vein blood kinin reported by Oates et al (1964). Oates and his group report that the highest level of 1·2 μg/ml was found in hepatic vein blood during adrenaline provocation in a patient who also had right sided heart disease -- which is probably an index of the severity of the disease.

The findings of Zeitlin and Smith then would suggest that during the dumping attack there must occur an enormously accel-

erated production of plasma kinin in the periphery. It is difficult to calculate accurately what the total venous kinin content would be in a case that shows a concentration of 2·6 μg/ml in the forearm vein blood, but it is known from the studies of intra-venous bradykinin infusions (Fox, Goldsmith, Kidd and Lewis 1961) that infusion at the rate of 1 μg/Kg/min in man is enough to produce marked cardiovascular symptoms. The appearance of 2·6 μg/ml of bradykinin in a forearm vein would correspond to many fold higher intravenous infusion than 1 μg/Kg/min. The argument against the validity of such high blood levels of bradykinin is taken up again later in this thesis.

The rare condition of hereditary angioneurotic oedema is characterised by recurrent attacks of circumscribed non-inflammatory oedema with death frequently occurring from acute laryngeal oedema. It has now been shown that the inborn biochemical lesion is likely to be an inherited deficiency of serum inhibitor of plasma kallikrein (Landerman, Webster, Becker & Ratcliffe 1962). The increased capillary permeability is perhaps mediated through the production of plasma kinins.

d. THE SCOPE FOR STUDY

In reviewing the evidence that has emerged for the role of plasma kinins in physiology and pathology, two facts emerge.

1. The plasma kinins have been implicated in a wide variety of situations ranging from purely physiological roles as functional vasodilators in glandular tissue, to participation, with varying degrees of importance, in conditions of inflammation and states of general shock. Much of the evidence has come under criticism, so much so that perhaps only in the carcinoid syndrome is the evidence sufficiently complete to give the plasma kinins a definite standing as agents that can bring about striking functional alterations and perhaps contribute as aetiological agents in a disease process.

2. The controversy that has often arisen about evidence implicating the plasma kinins in some role has been associated with poor understanding of the structural and functional details of the kallikrein - kininogen - kinin - kininase system. Many experiments have been performed in artificial systems where the identity and significance of the reactants has not been under-

stood. Conclusions have been drawn and extrapolated from indirect evidence.

Even so, much of the evidence is striking and may eventually be substantiated when more precise techniques allow a more critical study in less distorted systems. The major difficulty that presents itself in the study of the role of kinins in in-vivo situations is that plasma kinin levels in blood or in tissue site are probably the resultant of processes of simultaneous production and rapid destruction. Any experimental procedure is very likely to favour one or both of the tendencies, with the result that true in-situ situation may become sufficiently distorted by the time the reactants are analysed. This, coupled with the minute levels of kinin that can be expected to be able to produce significant functional changes, has stood in the way, to a large extent, of the correlation of kinin level and functional changes. The hope for more fruitful study in kinin involvement depends heavily on the availability of a specific plasma kinin antagonist. Schachter (1964) has expressed this in a recent review: "the discovery of a specific antagonist would be the most valuable analytical tool in providing a more definite assesment of its (kinin) role."

The aim of the study reported in this thesis was to invest-

igate one body compartment, viz. blood in the human, to throw some light on the nature and significance of kinin metabolism in this area. At the time of commencement of the studies the occurrence of plasma kinins in human blood in normal healthy subjects was uncertain, and in fact in some current medical literature statements like "kinins....are not normally present in blood, but can be released in vivo" could be found (Ganong, 1963). This seemed a little extreme because the kinin precursor and the inactive kallikrein are both present in blood and blood is continually perfusing areas suggested as sites of plasma kinin turnover.

The first aim was to establish the presence of active plasma kinins in circulating blood in humans. No sensitive method for normal plasma kinin estimation was available and a large part of the time was devoted to modifying the method of Oates et al (1964), so as to be able to specifically measure the small levels of circulating kinin in human blood. With this method it was able to establish a range of levels in the blood of normal human subjects. It was not possible by the method used to distinguish between the nonapeptide (bradykinin) and the decapeptide (kallidin), but since the studies of Webster and Pierce (1963) had indicated that any kallidin that enters blood is acted on by an active aminopeptidase which converts the kallidin to bradykinin,

it was assumed that the kinin activity found in blood was due to bradykinin and assays of the plasma kinin were made by biological comparison with the synthetic nonapeptide. The possibility of the endecapeptide and other plasma kinins contributing to the kinin activity studied was ignored in these investigations. Their mode of release from plasma substrate is uncertain and suggests that their release under normal physiological conditions may not be significant and in fact extreme conditions, not usually encountered in the body, may be prerequisites for their release (Webster 1966).

For these reasons the plasma kinin studied in this thesis has been taken to be bradykinin (BK) and referred to by that name.

A study of arterio-venous differences in BK levels in a peripheral region was made, and this led to an examination of changes in blood BK levels associated with local vasodilator phenomena such as reactive hyperaemia, cold vasodilatation and vasodilatation produced by direct heating.

Studies of BK levels in peripheral blood were also made in patients with cirrhosis, the carcinoid syndrome and other non-carcinoid flushing states. Release of BK in the hepatic vein blood during carcinoid flushes had been reported by Oates et al, (1964), but there was no evidence at that time that these changes

also extended to the peripheral circulation. The hyperdynamic circulation in some severe cases of cirrhosis suggested a possible role for bradykinin, and the wide variety of cutaneous flushing phenomena (e.g. menopausal) which have no aetiological explanation,merited investigation from the point of view of kinin involvement.

Since one of the greatest obstacles in the investigation of plasma kinins has always been the ubiquitous presence of extremely active kininase, its activity in whole blood was investigated. This latter study led to an investigation of the effect of catecholamines on kininase activity and further, the effect of adrenaline blockade on this system.

The aims of study are presented in more detail in the introductory parts of the respective sections.

Part 2 METHODS

a. INTRODUCTION

b. BLOOD COLLECTION

c. EXTRACTION

d. BIOLOGICAL ASSAY

e. RESULTS

f. DISCUSSION

a. INTRODUCTION

In planning a suitable method for the estimation of normal blood BK levels in man cognizance had to be taken of the nature of the kallikrein - kininogen - kinin - kininase system as it occurs in the circulating blood. It is likely that with the presence of all these reactants the level of the active kinin present would be the result of the balance struck between concurrent production and destruction. To be able to estimate the true level precautions need to be taken to ensure that whatever turnover in the kinin system is occurring in blood is immediately arrested when the blood is sampled, and furthermore, that the collection and estimation procedures do not introduce any opportunities for artificial activation of the system.

The report of Fasciolo (1964) that kinin content of human blood might be in the range of 0 to 2 ng/ml, indicated that small levels were to be expected. Because of such small levels, the procedure of extracting bradykinin from blood should give high recovery values to enable as much as possible of the small endogenous amounts to survive. Also because of the small amounts

of BK involved, a biological assay procedure has to be used which is sufficiently sensitive to quantitate these small amounts but also specific enough to enable characterisation of the active substance from blood as BK.

Two promising methods for blood BK estimation were available in the published literature, that of Bina, Fasciolo and Carretero (1963) and that of Oates et al. (1964). The Bina method was based on the precipitation of blood proteins in alcohol and butanol extraction of the kinin; the biological assay preparation was the hindlimb of a dog perfused at constant flow through the femoral artery by a Dale-Schuster pump in which the measured response was the fall in perfusion pressure. They reported recovery of 53.7% ± 16% from dogs blood, but were unable to detect endogenous BK in most animals. The method of Oates et al. depended on the precipitation of blood in alcohol, ether extraction and adsorption of the kinin of the aqueous phase onto weakly acidic cation-exchange resin (IRC-50, 100-200 mesh). The final assay in this method was performed on the oestrus rat uterus. The identity of the extracted BK was further supported by the ability of chymotrypsin to inactivate it, and by the ability of the extracted substance to produce a characteristic bi-phasic blood pressure response on the nephrec-

tomised, vagotomised, pentolinium-treated rats. The immediate appeal of this method of extraction was that it promised high rates of recovery of BK (80 - 100%). As originally described it was to detect and estimate the high endogenous levels in the hepatic vein blood in flushing carcinoid patients; it was hard to predict whether this method of extraction could also be used for the much smaller normal endogenous levels. As will be recounted below, some important modifications had to be incorporated in the Oates' method before it could be sucessfully used in the present study. This modified method enables the measurement of blood BK levels in all normal subjects examined and is the method used for BK estimations in this work. The validity of these levels found is supported by recovery results and by the collateral evidence from other studies on BK metabolism in man reported in this thesis.

The methods of blood collection and subsequent extraction and bioassay are described in detail and important points of procedure are further amplified in appendices. Some of the experimental work which led to the development of the final form of the method of extraction is described in chronological order.

b. BLOOD COLLECTION

Collections of blood from an artery or vein were made through an all polythene - nylon system into nylon syringes. Any contact of blood with electronegative surfaces is avoided to prevent activation of the Hageman Factor which is a known activator of the plasma kallikrein (Margolis, 1963). To reduce the time for collection to a minimum, the largest cannula compatible with the size of the blood vessel was usually used. One of three types of methods of cannulation were used depending on the site or nature of the blood vessels (Fig. 3).

Arterial blood collections were always made from the brachial artery at the antecubital fossa, using a modified Seldinger technique (Seldinger, 1953), to cannulate the vessel. A 19-gauge short-bevel needle is inserted into the brachial artery after local infiltration with 2% (plain) Lignocaine (Xylocaine Astra), a length of nylon fishing line is then threaded through the needle into the vessel and the needle removed. A collecting catheter can then be introduced into the vessel over the nylon guide. The nylon guide is withdrawn when an adequate length of

the catheter has been threaded into the lumen of the vessel (Fig. 4). The collecting catheter is made from polyethene (Intramedic PE - 90/ S 36) or from nylon tubing of the same dimensions. The catheter is made as short as possible, to allow for approximately 5 cm intraluminally and approximately the same length extraluminally. The extraluminal end carries a Luer type polyethylene butt (Boots) fixed to it with an epoxy resin (Araldite, Ciba), into which can be fitted a three way plastic tap (Pharmaseal). This system can then be filled with heparinized saline (10 units heparin per 1 ml 0.9% w/v saline) until the blood collections are made. Immediately prior to collection, all dead - space contents is discarded by allowing the blood to flow freely from the catheter for 2 - 3 seconds. Nylon syringes ("Vandermic" Vann Bros. Ltd., London England) previously autoclaved and chilled in a deep - freeze were used for the blood collection (Fig. 5). Five ml Luer mount syringes were used uniformly. Syringes of this capacity were used to minimize the period available for change to occur in the blood before the destruction of enzymatic activity by the ethanol but at the same time to keep the number of syringes needed within manageable limits. In this way the transit time from blood vessel to alcohol was in the range of 5 to 10 sec. Six syringes were used

Fig. 3. Three types of cannulae used for blood collection (A, B, C).

A. Intracath (Bardic).

B. Braunula (B. Braun Melsungen).

C. Catheter used for the modified Seldinger technique cannulation (see text).

D. Plastic three-way tap which fits into butts of all three above cannulae.

E. A 5 ml Vandermic nylon syringe used for blood collection.

Fig. 4. In arterial cannulation, the needle (A) is first inserted into the vessel, it is then threaded with a nylon fishing line (B). Catheter (C) can be guided into the blood vessel over the nylon line after the needle has been removed.

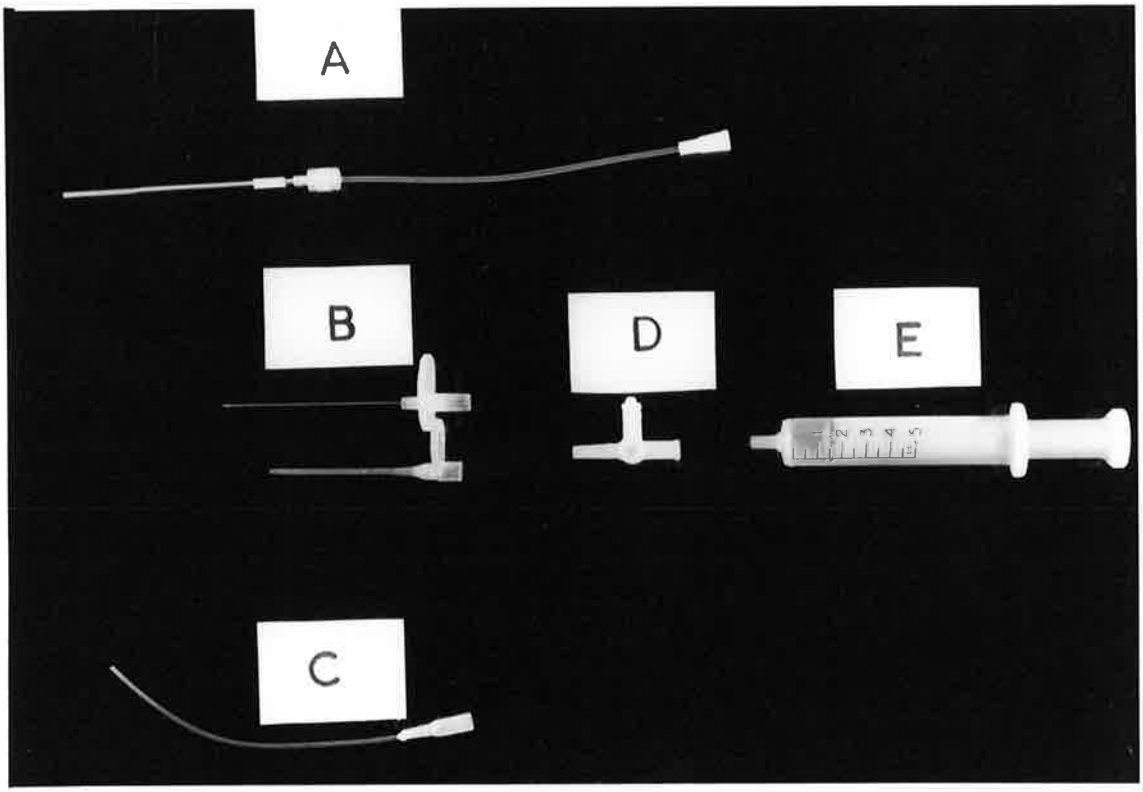


Fig. 3

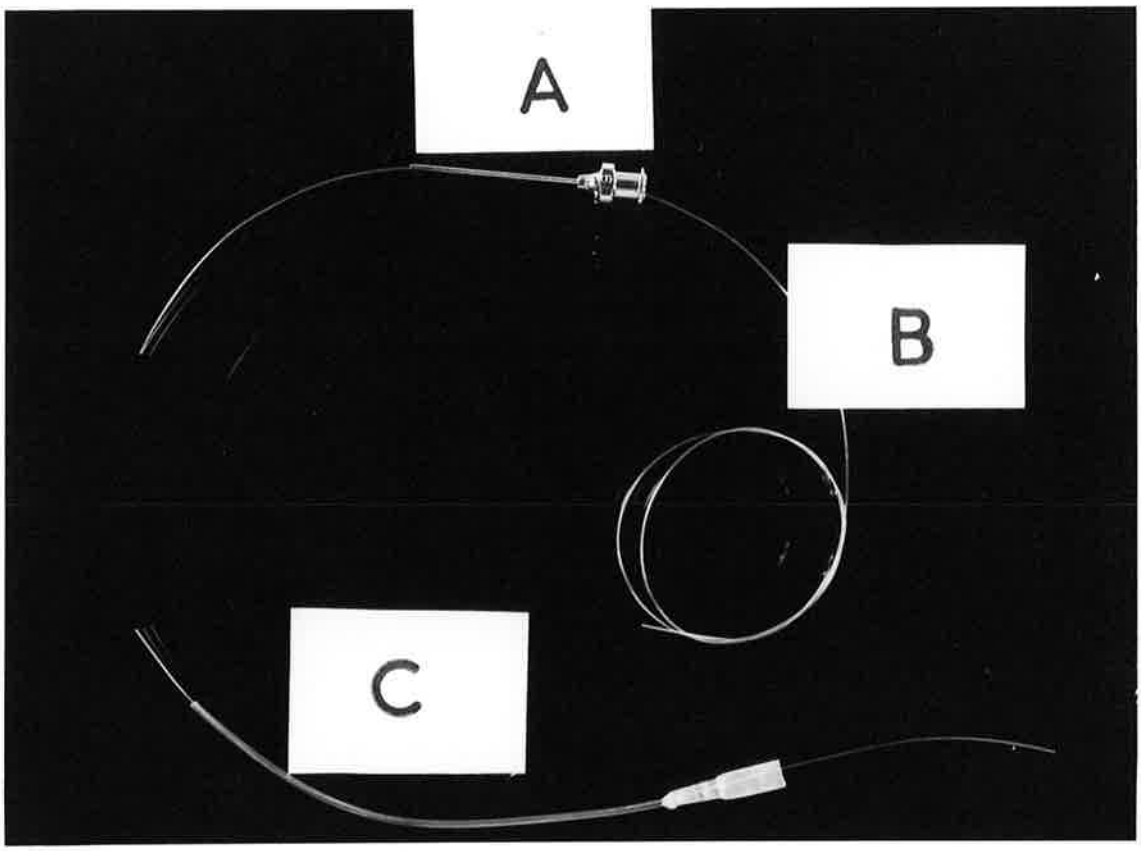


Fig. 4

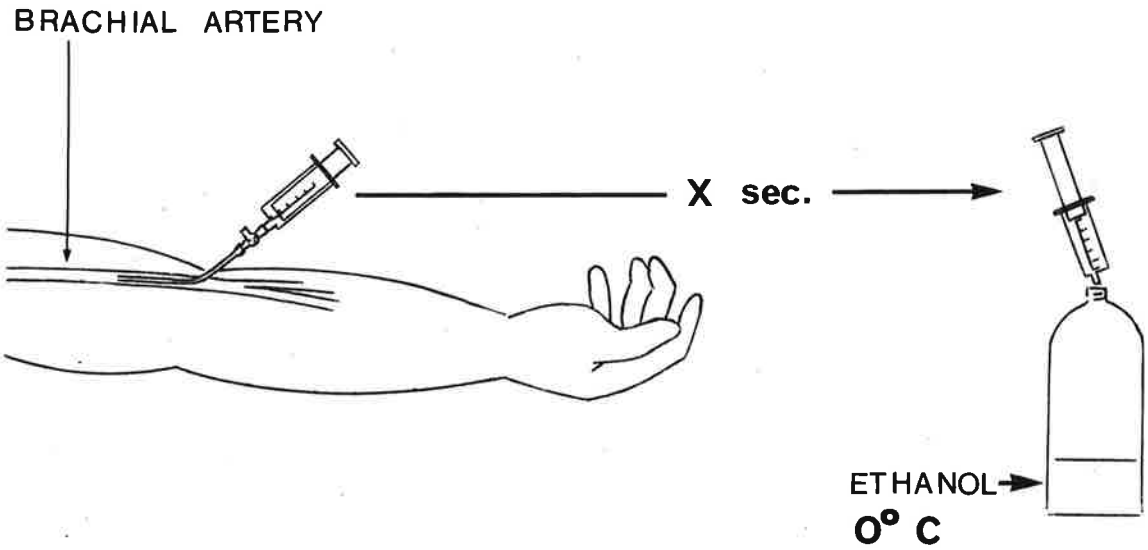


Fig. 5. Method of blood collection from the brachial artery. Blood is withdrawn into a 5 ml nylon syringe through an arterial cannula and plastic tap. Rapid transfer to nearby ethanol (0° C) is achieved in X sec. (X is in the range of 5 - 10 sec).

to collect the 30 ml of blood for each estimation and pooled into a flask containing 120 ml of 96% ethanol at 0° C. Prior chilling of the syringes is essential to suppress the activity of the kininase in the blood being collected, (Abe, Watanabe, Kumagai, Miwa, Mouri, Seki, Oikawa & Yoshinga, 1965). That this measure, together with the rapid transfer to alcohol was adequate in preventing kininase activity is shown by the good recoveries of internal standards. The necessity for freezing of the syringes is shown by the extremely rapid destruction of internal standards that occurs when syringes at 38° C are used (see Part 3). The exact amount of blood collected is determined by weighing the alcohol before and after the collection.

For venous blood collections the above technique of cannulation can be used but was not employed frequently. A polyethylene catheter (Intracath, Bardic) was extremely useful for the cannulation of an antecubital vein. For the cannulation of a lower forearm vein, to collect blood draining the hand, the modified Seldinger technique can be used after shaving the area, but a much more successful method was by the use of a Braunula (B.Braun Melsungen). In this way, the obstruction encountered from valves by a centrifugally directed catheter could be overcome because of the rigidity of the catheter and

the protruding metal tip, present during the insertion. Use of a Braunula is more traumatic, but much of the discomfort is avoided by the use of adequate local anaesthetic and prior local incision of the skin. The incision required is so small that no subsequent suturing is necessary. The rigid nature of the Braunula ensures that the catheter will not collapse or twist during the collection of blood. The sampling tip is distal to any site of trauma.

In determining the recovery of internal standards of BK from blood a known concentration of synthetic bradykinin was prepared in normal saline. A 0·1 ml amount of this solution was placed in the collecting syringe near the entrance. This volume contained known amounts so that the final concentration in the 5 ml of blood ranged from 250 ng/ml to 0·5 ng/ml and the recovery of BK from blood could be examined over a range of concentration. The small volume and the positioning of the saline solution in the syringe ensures complete mixing with the blood as the latter is drawn into the syringe, furthermore the small volume minimises the degree of dilution of the plasma.

A polythene bottle (500 ml capacity) which contains the 120 ml of 96% ethanol is kept in the deep freeze until the time of collection. Immediately after the collection of the 30 ml of

blood, the mixture is shaken and the bottle is stored again in the deep freeze until all the collections of the experiment have been completed.

c. EXTRACTION

The final form of extraction adopted includes Oates' et al. (1964) method up to the stage of elution of BK from the ion-exchange column. A technique which departs from Oates' method is then adopted which enables a salt free extract to be obtained which can be assayed directly on the superfused preparations. For the sake of continuity and completeness, Oates' method is described first, the subsequent subsections describe the way in which the modifications evolved and were adopted.

(a) "Oates' Method "

In the extractions of BK from blood, standard Quickfit glassware was used almost exclusively. All glassware with the exception of chromatography columns was siliconised (Silcote SF 114, Dow Corning) prior to use.

The mixture of blood and alcohol is filtered through a Buchner filter (containing a Whatman No. 2 filter paper) into a 500 ml Buchner flask. A Rud Browne Dynavac 2, rotary high vacuum pump was used to provide the necessary vacuum. The suspen-

sion is allowed to filter until the cake is dry but not cracked; at this stage a 120 ml 80% ethanol wash from the polythene collection bottle is added to the residue and allowed to filter through until the residue is dry and cracked. All blood samples are filtered immediately after the experiment, and the filtrates are then stored back in the deep freeze until the next stage of extraction.

The straw coloured filtrate is transferred to a pear shaped 1 L evaporating flask, 10 ml of octanol are added (to prevent frothing) and the contents are then reduced to 30 - 40 ml in a Buchi rotary evaporator at a temperature of 35 - 40° C. The concentrated solution is acidified with 1 ml of glacial acetic acid and twice extracted with 2 - 3 volumes of di-ethyl ether in a separating funnel. The ether phases are discarded. The aqueous phase, containing the BK is further reduced to approx. 10 ml in the rotary evaporator. The pH of this extract is adjusted accurately to 6·0 with 1 N NH_4OH. Varying amounts of precipitate may be found in the extract at this stage and they are removed by centrifugation. The precipitate and evaporating flask are washed with 0·1 M ammonium acetate buffer at pH 6·0 and the pooled volume of approximately 25 ml is applied to a column (Fig. 6) (1 cm x 5 cm) of Amberlite I.R.C. 50 (100-

200 mesh) (Appendix 2) equilibrated with the ammonium acetate buffer (Appendix 1). After the column has been loaded with the extract, it is washed through with 30 ml of buffer. Five ml of 1 N NH_4OH (Appendix 1) is applied to the column and the displaced buffer discarded. The peptide can now be eluted from the column with 10 ml of 1 N NH_4OH and 25 ml of 0·1 N NH_4OH in sequence. If the eluate is adjusted to near neutral pH with acetic acid (BK is unstable in alkaline pH) a salt solution of ammonium acetate results. If this eluate contains large amounts of BK, it can be diluted sufficiently to make the concentration of ammonium acetate sufficiently small, that it will not interfere with the assay on the rat uterus.

This in essence is the method of extraction of Oates et al. (1964) and the excess salt in the extract creates no drawback to the biological assay if sufficiently high concentrations of BK are present in the extract. However, the neutralised eluate will cause relaxation of the superfused rat duodenum because of the salt alone, this depression of duodenum tone persists even when the eluate is diluted 1:60. This degree of dilution is not feasible if normal blood BK levels are to be measured using superfused uterus and duodenum as assay tissues.

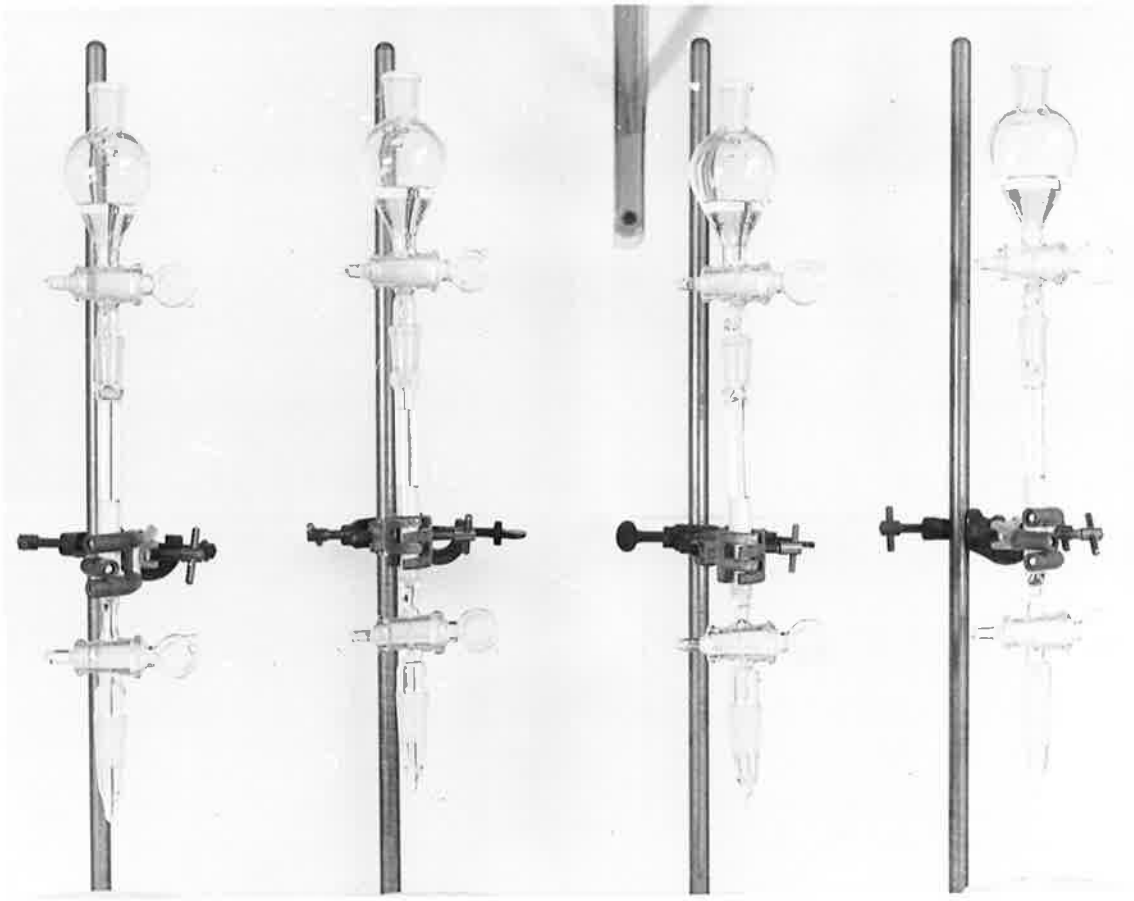


Fig. 6. Quickfit chromatography columns packed with Amberlite I.R.C. 50 100 - 200 mesh. Ammonium acetate buffer in detacheable reservoir. (See text and Appendix 2).

Using Oates' method as described above good recoveries could be obtained if large amounts of bradykinin were added to the blood, and the extract assayed on the superfused preparations after maximum possible dilution. Several attempts of assay of endogenous BK showed that the extract contained an uterus contracting substance although the duodenum gave fixed large relaxations that had no relationship to the BK concentration as gauged from the uterus responses. The need then became apparent of obtaining the extract sufficiently free of interfering salts, before both these tissues could be confidently used to estimate the concentration of small amounts of BK in the extract.

(b) Desalting Methods

(1) Ion Retardation Resin

Ion retardation resin AG 11A8 (Bio-Rad Laboratories) promised to be a useful agent to explore. AG 11A8 is polymerised acrylic acid inside Dowex 1; the result is styrene-divinylbenzene cross-linked, rigid polymer lattice with attached quaternary ammonium groups (strongly basic anion exchange groups) within which weaves a trapped, linear, relatively flexible acry-

lic polymer having carboxyl groups (weakly acidic cation exchange groups) (Bio-Rad Laboratories Technical Bulletin 113 Nov., 1963). The resin thus contains paired anion and cation exchange sites. When salts are added to this resin in the self-adsorbed form, the ions are adsorbed to the appropriate groups but without any ion exchange. The degree of adsorption depending on the type of ion. This resin had also been shown by the manufacturers to be able to separate a protein enzyme from its solution in $(NH_4)_2SO_4$ using water as eluting agent, the two substances appearing in the eluate as two distinct and separate peaks.

In an attempt to de-salt the BK extract a column 45 cm x 3·17 cm^2 of AG 11A8 50 - 100 mesh was prepared. Synthetic BK solution was prepared in 0·36 M CH_3COONH_4 , 0·36 M NH_4CL and 0·18 M $(NH_4)_2SO_4$, as all these salt forms could be conveniently obtained by appropriate neutralisation of the eluate from the Amberlite column.

Desalting of these BK solutions was tried on such a column using a small feed volume and eluting the substances with deionised water. The effluent was collected in 2 ml fractions in a fraction collector. The fractions were analysed for ammonia and BK content. Ammonia was detected by Nesslerization

of the samples and the concentration estimated in a Shimadzu spectrophotometer -- alternate tubes were bioassayed for BK content on the rat uterus. The possible potential of the ion-retardation resin was further explored by varying feed volumes, flow rates and pH of the feed solutions. These latter manipulations changed the behaviour of the column only little, in every case some degree of separation of the peaks of activity could be obtained, but there was always about 50% overlap of the splays of the elution peaks and the BK which appeared from a 5 ml feed was distributed through about 100 ml of effluent. For these reasons AG 11A8 was abandoned as unsuitable for desalting in this particular situation.

(2) SEPHADEX

Sephadex G25 medium mesh, which was the preparation with the smallest pore size available at the time, was also tried to assess whether this material would offer any salt retardation. A column of sephadex 50 cm x ·79 cm^2 was loaded with 1 ml of 1 /ml BK in 0·18 M $(NH_4)_2SO_4$ and eluted with deionised water. A minor but insignificant degree of salt retardation was observed when the eluates were analysed as for AG 11A8 chromatography. Sephadex G25 was then also abandoned.

(3) SUBLIMATION

Attempts were made to reduce the amount of CH_3COONH_4 in the BK - salt solution by sublimation, both by lyopholisation and by rotary evaporation at 40° C with a vacuum pump with a stated capacity of achieving 0·0001 mm Hg. The process was very slow and incomplete and the residue when redissolved still showed potent depressor effect on the rat duodenum.

Freeze-drying of a solution of BK in Elgastat* (5 ng/ml) in a siliconised flask gave an invisible residue at the completion of dryness. Rinsing of the flask with de Jalons solution (Appendix 1) for assay of the residual BK gave at the best only 50% recovery (after 10 min rinsing) -- further rinsing yielded more BK activity from the glass surface. In one such experiment where de Jalons solution had been unintentionally used to prepare the mixture for freeze-drying the residue was visible (salts from de Jalons soln.) and the solution of the residue showed 97% recovery as calculated from uterus and 140% as calculated from the duodenum. The apparent high recovery from the duodenum was ascribed to the depressor effect of the hypertonic solution.

Attempts to desalt the neutralised effluent from the Amberlite column were unsuccessful but indicated that if a solution

* Elgastat deionised water

of BK is to be reduced to dryness, extra precautions had to be taken to prevent major adsorbtive losses on the glass surface even though the latter is siliconised. The presence of a residue in the evaporating flask seemed to be desirable for maximum recovery.

(c) Final form of Extraction

Experiments were performed to test the stability of synthetic BK in NH_4OH at pH 11 over a period of time. The pooled effluent from the Amberlite column corresponds to a NH_4OH solution of 0·36 M and has a pH of 11. BK was maintained in such an NH_4OH solution at room temperature over a period of 1 hour and samples were assayed over this period of time to follow any destruction that might be occurring. It was found that no significant decay of BK occurred over this period. This indicated that the eluate from the Amberlite IRC 50 column could be pooled and without neutralisation evaporated to dryness in a rotary evaporator. This was subsequently done at 35 - 40° C. The ammonia from the solution is extracted in a few minutes and the 35 ml of eluate can be reduced to dryness in approximately 15 min.

The loss which occurs due to adsorption to glass surface when the extract is evaporated to dryness was overcome by fur-

ther treating the glass surface with hexadimethrine bromide and the use of casein to provide an inert residue.

The use of hexadimethrine bromide (Polybrene, Abbott) was suggested by the studies of Eisen (1964). Hexadimethrine bromide is a polymeric quaternary ammonium salt and has positive charges on its quaternary ammonium groups (Armstrong and Stewart, 1962) and its effect on the electronegative charges on the glass would contribute to the rejection of the positively charged BK molecule from the glass surface.

The work of Page and Bumpus (1961) on angiotensin had shown that no loss by adsorption occurred with crude angiotensin,probably because of the detergent action of peptide or protein impurities in the system; the losses encountered with pure angiotensin could be overcome by the use of 0·25% soluble casein added to the solution.

The siliconised evaporating flask is treated with hexadimethrine bromide by rinsing with a 1 mg/ml solution and drying in an oven at 40° C. Before the eluate is collected in this flask 1 ml of 0·25% casein solution (Appendix 1) is added to the flask. On evaporationto dryness, a residue is left on the walls of the flask which consists of casein, BK and a small amount of proteinaceous matter which has survived the purification procedure. The res-

idue rapidly dissolves on agitation in 10 ml de Jalons solution, which now contains 0·025% casein. A solution of 0·025% casein in de Jalons, when applied to the superfused uterus and duodenum, has no effect on either of these preparations.

When a solution of BK in Elgastat* is evaporated to dryness without casein in a flask which has been pretreated with hexadimethrine bromide, the recovery is variable but in such a pure system may only be 70 - 80%. Addition of casein increases recovery by 20%. The usefulness of casein is not quite so evident when recoveries of BK from whole blood are performed, because the blood extract itself contributes some amounts of protein material to the extract which acts in the same way as casein. However, the amount of protein that appears in the extract is variable. During the course of the work it became evident that if some recovery extracts were remarkable for their purity,as judged by colour and lack of turbidity, recoveries were often poor, if, however, casein was added routinely, persistently good recovery values were obtained. The use of casein has one other advantage, it forms an easily visible residue on the glass surface in the evaporating flask, and if evaporation is stopped as soon as there is macroscopic evidence of dryness, the residue is very easily redissolved in de Jalons. Complete taking

* Elgastat deionised water

up of the residue in de Jalons is thus readily visible -- this is an useful guide to the extent that rinsing has to be continued and always leads to good recovery of BK from the evaporating flask.

The final extract, in accurately measured 10 ml of de Jalons solution is now ready for assay. If the extract is not to be assayed that day, it is placed in a 50 ml polythene bottle which has also been treated with hexadimethrine bromide, and is stored in the deep freeze.

d. BIOLOGICAL ASSAY

The unique advantage of using rat uterus and rat duodenum for assay of plasma kinins has been discussed by Horton (1959). The uterus contracts and the duodenum relaxes to similar (nanogram) concentrations of the plasma kinins. Other biologically active substances also have an effect on these tissues, but the following considerations will demonstrate that these tissues when used in parallel provide a reliable and specific method for kinin assay.

Both vasopressin and oxytocin cause a contraction of the uterus and in very high concentrations a relaxation of the duodenum. This difference in doses of the two oxytocic polypeptides necessary to produce equiactive responses on the two tissues, makes them immediately distinct from plasma kinins. Gaddum (1955) has introduced the term "index of discrimination" and in this situation the index of discrimination of the oxytocic polypeptides from the plasma kinins is very high. Angiotensin in high doses will also cause a contraction of the uterus and in still larger doses a relaxation of the duodenum, but

the doses required and the high index of discrimination will easily distinguish between naturally occurring angiotensin and the plasma kinins. Substance P, acetylcholine, 5-hydroxy-tryptamine and histamine, all cause a contraction of the duodenum. The catecholamines will cause inhibition of the duodenum and uterus, but only in concentrations of 10^{-6} -- a concentration that is not encountered in normal plasma.

The technique of superfusion in assay of active substances on isolated plain muscle has been described by Gaddum (1953). The main advantage of superfusion over perfusion is that the active substance to be tested, can be applied directly to the surface of the muscle by stopping the flow of the superfusate. The test substance need not be diluted in a volume of perfusion fluid in the organ bath, enabling small amounts of low concentration of active substance to be assayed. For these reasons the technique of superfusion is very suitable for the assay of the small concentrations of BK to be expected in human blood.

The rats used were of Wistar strain of 150 G or more, treated 18 hours before with 0·1 mg stilboestrol subcutaneously (see Appendix 3). The rat is killed by a blow on the head and the abdomen is immediately opened and the horns of the uterus and

the duodenum removed and placed in de Jalons solution. The mesentery is carefully dissected from the organs and only the proximal 3 cm of the duodenum used. Lengths of cotton are fixed to each end of the tissues by one stitch,using a needle. These manipulations are carried out with the tissues in a Petri dish, submerged in the de Jalons solution and undue stretching and handling of the tissues with fingers is avoided. The tissues are then placed in double walled, glass organ baths (Figs. 7, 8, 9). The bottom tie is anchored in the organ bath, the top tie is fixed to the end of a frontal writing lever, thus suspending the tissue clear of the walls. The weight on the butt of the writing lever can be conveniently adjusted with plasticene, and the tissues are stretched by moderate tension. Both levers have a magnification of 1 in 10. The organ baths are adjacent and the recording levers from both tissues record excursions due to changes in tissue tone on the same smoked kymograph paper. The writing points and levers are so arranged that recordings of uterus are immediately above the recordings from the duodenum, so that immediate comparisons of responses are readily made. The water jackets of the organ baths are irrigated with water from an adjacent temperature controlled water bath at 31° C (Thermomix, B. Braun, Melsungen).

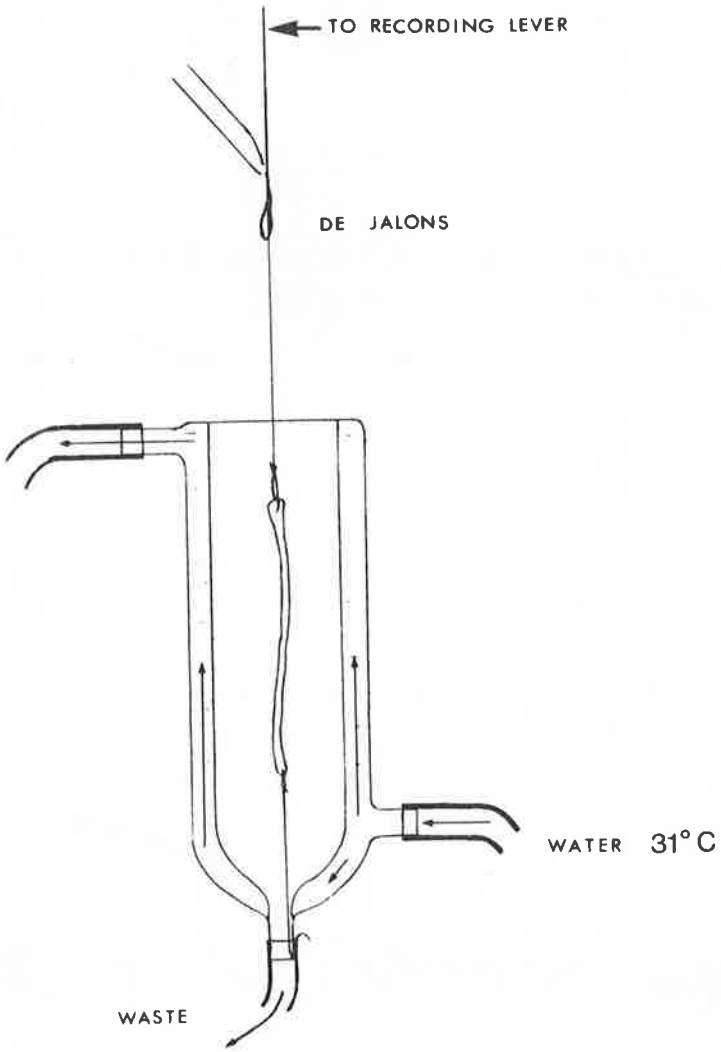


Fig. 7. Organ chamber, showing double glass wall whereby the wall of the chamber can be irrigated with water at a regulated temperature. The immediate environment of the tissue can be kept constant at the desired temperature. The superfusate (de Jalons solution) delivered to the top thread in a dropwise manner, bathes the tissue and is then lost in the waste.

Fig. 8. Biological assay apparatus. A thermostatically heated (Braun pump) water bath provides constant temperature for assay samples, spiral warming coils (for de Jalons superfusate) and the tissue chambers. Separate aspirator bottles for each tissue contain the superfusate. Delivery of de Jalons to the tissue can be regulated by separate taps and fine adjustment screw clips.
Excursions of the tissues are recorded by frontal writing levers on smoked kymograph paper.

Fig. 9. Close up view of organ chambers. Isolated rat duodenum at left and isolated rat uterus at right. Using adjacent organ baths enables records from both to be made and placed closely on the same kymograph tracing. See Fig. 10 for uterus and duodenum recordings.

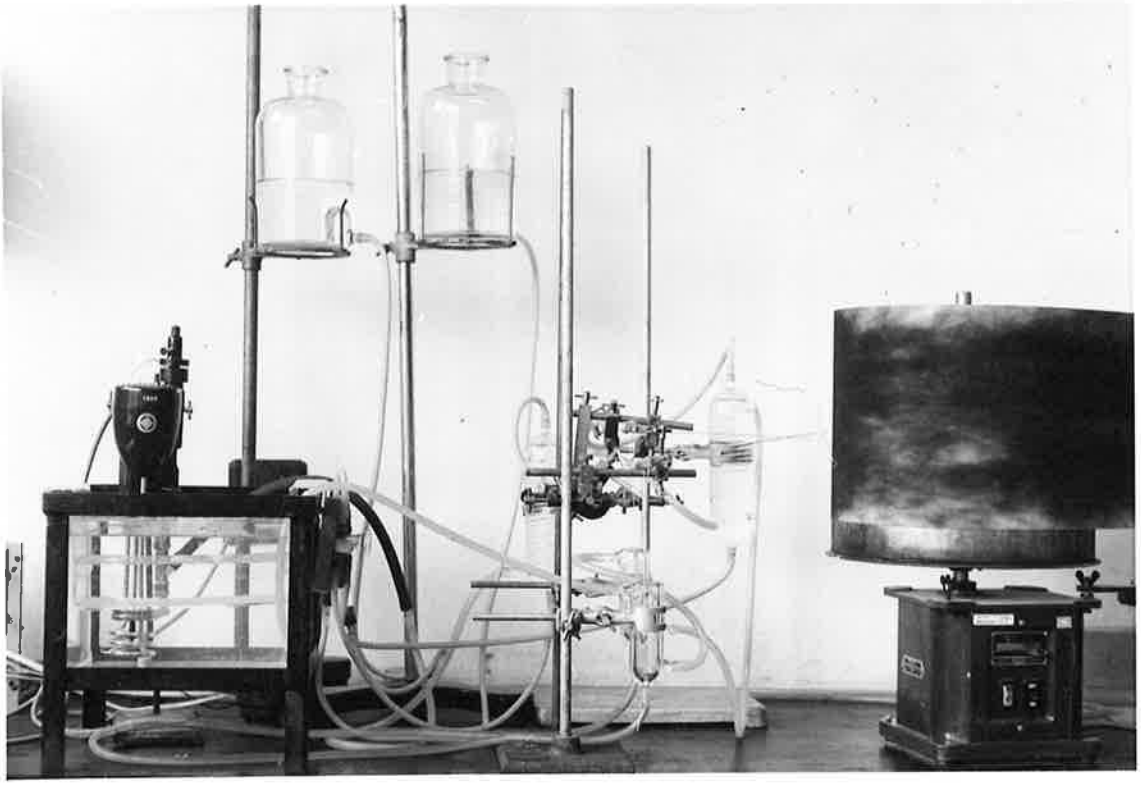


Fig. 8.

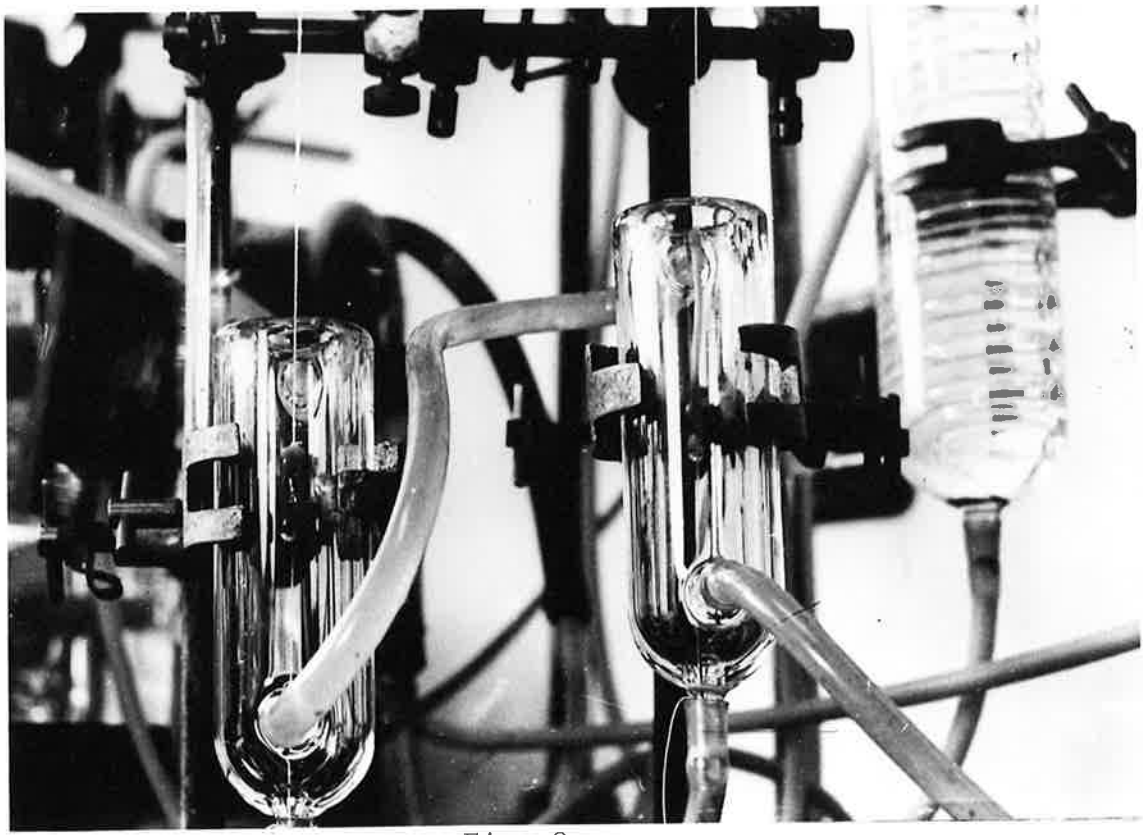


Fig. 9.

The superfusing fluid contained in aspirator bottles supplies de Jalons solution independently to the two tissues by gravity, passing first through a warming coil (31° C) and filter (glass wool) and then through a glass tube whose tapered tip rests against the thread suspending the tissue. The superfusate is delivered at approximately 30 d.p.m., sufficiently high above the tissue so that on falling, it breaks and bathes all sides of the tissue evenly. The delivery of the superfusate can be interrupted by a tap in the line to each preparation.

Both tissues require time to stabilise (usually 1 hour) and during this time progressive relaxation of the uterus and taking up of tone of the duodenum occur. The drum is allowed to rotate at a slow speed, so that these events will be displayed and also any spontaneous activity of either tissue will be clearly manifested. After an adequate period of stabilisation, the position of the writing levers is appropriately adjusted and counter balance applied so that maximum sensitivity (if it is required) can be obtained. The tissues are now ready for assay.

Standard solutions of BK, prepared with de Jalons solution, placed in test tubes with individual teat pipettes are maintained at 31° C in a rack in the common water bath. Doses are applied at 3 or 4 minute intervals. The time interval adopted

is maintained for the duration of the assay. Superfusion is stopped 30 sec. before a dose is applied, this allows all excess solution to drain off the tissue and is particularly necessary if doses of BK to be used are of small magnitude. A standard dose of 6 drops on each tissue is used. Such a relatively large volume is essential because although with superfusion response depends mainly on the concentration, with very small concentrations volume does contribute and with threshold doses a difference of two drops may decide between response and no response.

A contact of 30 sec. is allowed, after which the superfusion is turned on again. As often happens, the response of the uterus to a small dose commences after the superfusion is turned on -- this may constitute a non-specific trigger to the smooth muscle but in no way has it been found to invalidate the results, since the size of the response is still dose related. Before doses of the unknown are applied, it is well to confirm that the tissues are sufficiently responsive to react to the amounts likely to be encountered in the extract to be assayed. Since four-point bioassays have been used, it is necessary to establish the ability of both tissues to respond to two doses in a suitable ratio, (usually 2:1), with readable excursions and sufficient steepness of slope in the dose - response relationship to give acceptable

precision in the assay.

Responses to isolated tissues are often related in an approximately linear fashion to a log transform of dose over a limited range. The standard doses of BK are therefore chosen to fall in this range of the dose-response curve. Alternately, the tension on the tissues may be changed to achieve responses that fall in the linear part of the dose-response curve. Dose manipulations are done with the standards and the unknown need not be thawed out, until a suitable pair of tissues has been shown to function satisfactoraly.

The limit of sensitivity of the method is usually imposed by the uterus preparation. Considerable increase in sensitivity of this preparation can be achieved by treatment with chymotrypsin as suggested by Edery (1964). The uterus is primed with chymotrypsin prior to every application of the standard, until maximum sensitivity has been established and has become stable (Fig. 12, see also Appendix 3). When a satisfactory dose-response relationship for the two standard doses has been established for uterus and duodenum (with or without chymotrypsin for the uterus), assay of the unknown can be undertaken.

By appropriate dilution, one dose of the unknown is fitted to give a response between the two standards, the other concen-

tration of the unknown is then made up so that the ratio of the unknown concentrations is the same as that of the standards (Fig. 10).

The applications during assay are made in a 4 x 4 Latin square design. The assay of one sample takes one hour. A number of assays can be often done on the one preparation and it is particularly convenient if the samples are of the same order of potency. Although the use of chymotrypsin is advantageous in that its effect on the uterus increases the number of preparations that are sensitive enough for assay, it shortens considerably the period over which the uterus behaves reliably. Seldom can more than three consecutive four point assays be performed on a chymotrypsin treated uterus, as with repeated chymotrypsin applications the tissue loses sensitivity and eventually fails to discriminate between different doses. In recovery assays where the final concentration of BK in the blood is 10 ng/ml, great sensitivity of the uterus is not essential and the use of chymotrypsin in unnecessary,

Below is shown typical result from a 4 point assay of an extract of blood, where recovery of 10 ng/ml (a total of 300 ng added to 30 ml of blood) is estimated.

Fig. 10. Responses of uterus (contractions) in the top trace and duodenum (relaxations) in the lower trace, to (4 ng/ml & 2 ng/ml) doses of BK and to blood extract (u and $\frac{u}{2}$) which contains BK. The low and high doses of the standard and unknown are in the same ratio.

Fig. 11. The effect of an extract u, which contained serotonin contamination, in addition to BK, on the rat uterus and duodenum. Serotonin contracted both tissues. Addition of BOL* at a concentration of 10^{-6} G/L in the superfusate blocked the serotonin effect and unmasked the presence of BK. The effect of a standard dose of BK (1 ng/ml) was unaltered by the presence of BOL in the superfusate.

* BOL = 2 bromo- N,N Lysergamide bitartrate

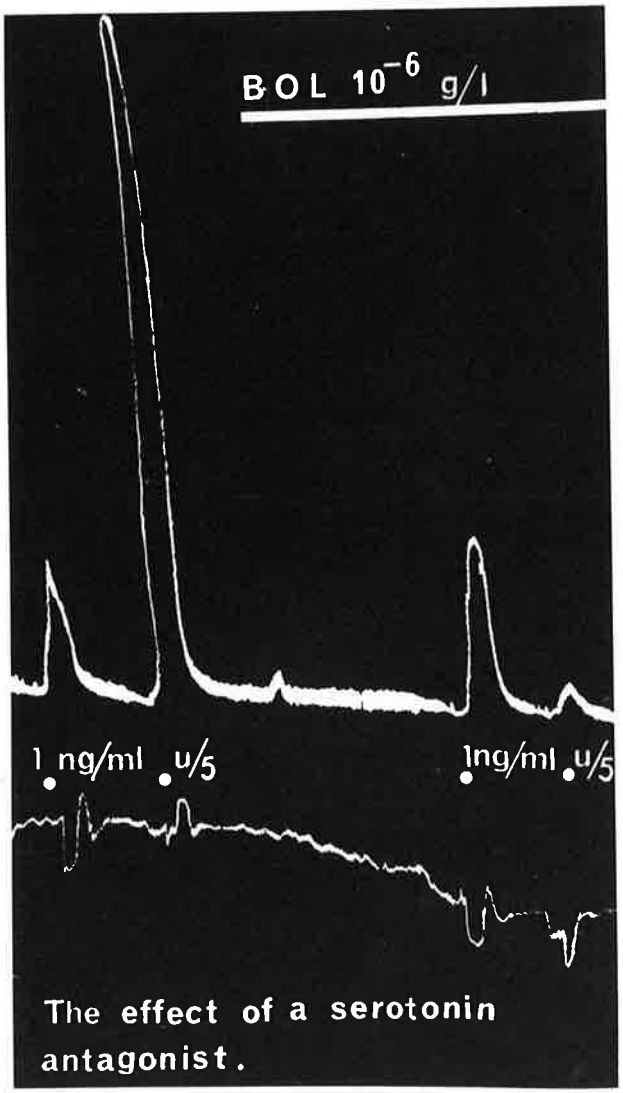

BOL 10^{-6} g/l
1 ng/ml
u/5
1ng/ml
u/5
The effect of a serotonin antagonist.

Fig. 11.

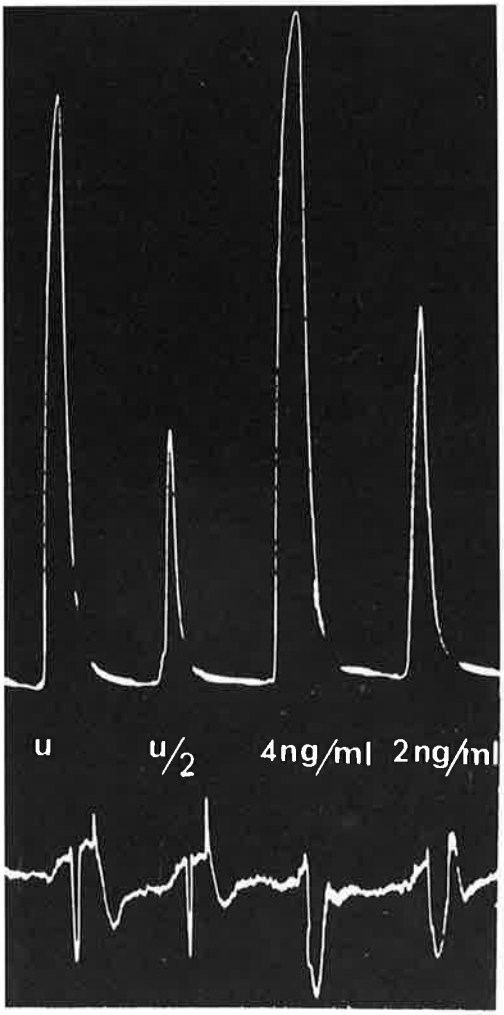

u
u/2
4ng/ml
2ng/ml

Fig. 10.

Fig. 12. Sensitisation of the rat uterus to BK by chymotrypsin. The preparation was primed with a solution of chymotrypsin (400 µg/ml) prior to every application of a dose of BK (2 ng/ml). Maximum sensitization is usually achieved in about 20 min, i.e. after about 6 - 7 doses. Priming has to be continued throughout the assay as sensitivity will begin to decline if priming is omitted for one or two doses. Note the depression of the base line occurring with the development of increased sensitivity.

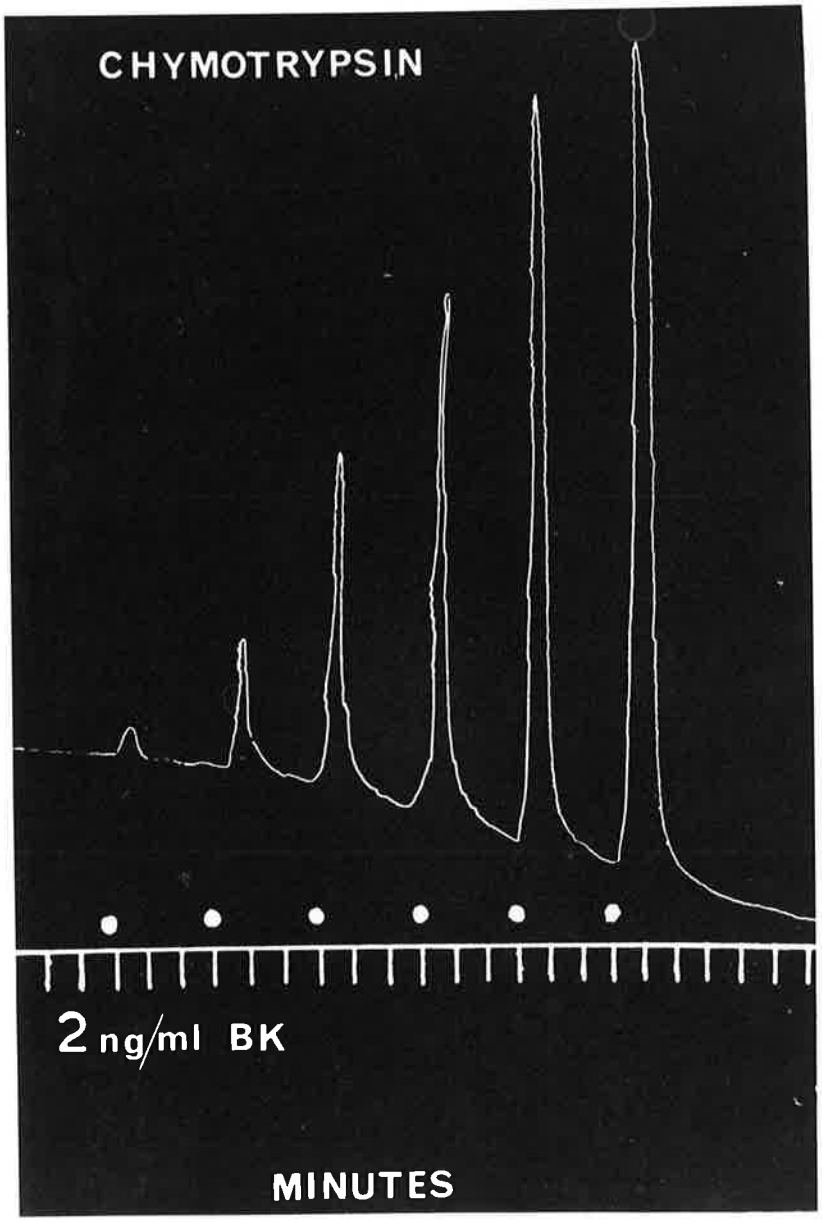


Fig. 12

Recovery assay;

Notations:

s_1 = low standard = 2 ng/ml

s_2 = high standard = 4 ng/ml

u_1 = low unknown = $\frac{1}{15}$

u_2 = high unknown = $\frac{1}{7 \cdot 5}$

These doses are applied to the uterus and duodenum in the order shown by the Latin square.

s_1	s_2	u_1	u_2
s_2	u_1	u_2	s_1
u_1	u_2	s_1	s_2
u_2	s_1	s_2	u_1

Inserting actual responses, measured in mm from the kymograph tracing:

Uterus

Sets	Contractions in mm.			
	s_1	s_2	u_1	u_2
1st	22	71	24	69
2nd	21	68	20	74
3rd	17	87	18	62
4th	13	77	26	71
Totals	73	303	88	276

Duodenum

Sets	Relaxation in mm.			
	s_1	s_2	u_1	u_2
Ist	23	26	25	22
2nd	22	35	22	26
3rd	20	33	25	37
4th	31	41	30	43
Totals	96	135	102	128

Calculation of BK concentration and thus recovery (Gaddum,1959).

In calculating the potency of the unknown the assumption has to be made that for both the tissues the relationship between response and log dose, over the range of doses employed in this assay, is linear. Since only two dose levels are used, there is no proof that this is so, but this can be ascertained with certain amount of confidence by prior testing of the tissues with various doses of standard prior to assay. It is also implicit that to calculate the relative potency of unknown to standard, the dose-response lines for the solutions are parallel. Divergence from parallelism can be tested for significance by analysis of variance (Appendix 4).

From the assay data in the tables above calculation of relative potency of unknown to standard can be made.

$$\log \text{Relative Potency} = \frac{\Delta \text{Resp}\ (u - s)}{\text{Estimated slope}}$$

(Resp = response in mm)

Δ Resp: can be calculated from two sources of information

viz, $u_2 - s_2$

and

$u_1 - s_1$

The average of these would then be

$$1/2\ (u_2 - s_2 + u_1 - s_1)$$

Estimated slope: $= \frac{\Delta \text{ Resp}}{\Delta \text{ log dose}}$

(individually for s and u)

$$= \frac{s_2 - s_1}{\log 2} \qquad = \frac{u_2 - u_1}{\log 2}$$

Average estimated slope $= \frac{(s_2 - s_1 + u_2 - u_1)}{0.3010} \frac{1}{2}$

Hence log Relative Potency (M) $= \frac{1/2 \ (u_2 - s_2 + u_1 - s_1)}{1/2 \ (s_2 - s_1 + u_2 - u_1)} 0.3010$

$$M = 0.3010 \frac{(u_2 - s_2 + u_1 - s_1)}{(s_2 - s_1 + u_2 - u_1)}$$

Applying this formula for log relative potency, the concentration and hence the recovery of bradykinin in the sample can be calculated.

From uterus:

$$M = 0{\cdot}3010 \times \frac{(276 - 303 + 88 - 73)}{(303 - 73 + 276 - 88)}$$

$$= \frac{0{\cdot}3010 \,.(-12)}{418} = -0{\cdot}0086$$

Potency in % = 100 x antilog $-0{\cdot}0086$

= 100 x antilog $\bar{1}{\cdot}9914$

= 98%

Concentration of u_2 = 0·98 x 4 ng/ml

Concentration of extract = 0·98 x 4 x 7·5 ng/ml

Total amount in extract = 0·98 x 4 x 7·5 x 10 ng

$$\% \text{ Recovery} = \frac{0{\cdot}98 \times 4 \times 7{\cdot}5 \times 10 \times 100}{300} \%$$

= 98 %

From duodenum

Using similar calculation, from the assay data

Potency in % (of u_2) = 99%

Also recovery = 99%

The average recovery from these tissue assays is then 98.5%.

Since the endogenous BK in the blood sample to which an excess exogenous has been added for recovery study forms such a small fraction, it can be ignored in this situation. When recovery estimations of smaller amounts are made e.g. 0.5 ng/ml, endogenous BK level must also be estimated so that appropriate allowance can be made in the determination of the recovery of added BK. In calculating the endogenous BK levels in blood the same mathematical approach is used, except that now the concentrations of the standard BK solutions are necessarily smaller.

TABLE 1

RECOVERY OF BK ADDED TO COLLECTION SYRINGE AT DIFFERENT LEVELS.

Amount added	% Recovery	Mean
250 ng/ml	88	
50 ng/ml	109	
10 ng/ml	96,90,106,99,92,97,99, 96,99,91,97,107,95,80, 92,105,105,80	96
1 ng/ml	100	
0·5 ng/ml	83,110,90,101,103	97

OVERALL MEAN RECOVERY 97%

e. RESULTS

Recoveries

As a test of the method of extraction and assay to measure blood BK levels, recovery experiments were performed covering a wide range of concentrations of internal standards added to the collection syringe. BK was added to the collecting syringes to give a final concentration varying from 0·5 - 250 ng/ml. The recoveries are shown in Table 1. The recovery values listed are the averages of parallel estimations on the uterus and duodenum (see Appendix 4). There is no difference in recoveries of BK over the range of concentrations used, and it is to be noted that the lowest concentration used is of the order of BK levels naturally occurring in the blood.

Arterial blood BK levels

The arterial blood BK levels found in 18 normal subjects are shown in Table 2. The blood was collected from some subjects specifically for normal blood BK estimations. In others, resting arterial blood BK estimations were made as part of an

TABLE 2

Arterial BK levels in 18 subjects. Columns 4 and 5 show results of assay on rat uterus and duodenum respectively. Column 6 shows the mean of these 2 levels.
Recovery values are mean of uterus and duodenum assays on blood to which BK was added in the withdrawal syringe.
* Averages of replicates used in calculation of the mean.
Replicates on P.A. and C.J. estimated on different occasions.

			BK level ng/ml			
Subject	Sex	Age	Uterus	Duodenum	Mean	Recovery
R.C.	M	19	0.02	0.03	0.03	110%
T.F.	M	19	0.07	0.07	0.07	90%
* P.A.	F	27	0.07	0.11	0.09	82%
* R.R.	M	20	0.09	0.11	0.10	
			0.10	0.12	0.11	96%
			0.09	0.11	0.10	
N.P.	M	20	0.11	0.14	0.12	99%
H.M.	F	45	0.11	0.15	0.13	92%
P.S.	F	40	0.14	0.14	0.14	90%
R.A.	M	35	0.13	0.17	0.15	(not done)
L.T.	F	34	0.14	0.16	0.15	91%
R.S.	M	20	0.17	0.15	0.16	101%
* B.K.	M	20	0.12	0.18	0.15	97%
			0.14	0.18	0.16	
B.McG	F	44	0.16	0.20	0.18	107%
D.F.	M	25	0.17	0.18	0.18	103%
* P.A	F	27	0,21	0.29	0.25	99%
* J.R	M	36	0.23	0.33	0.38	
			0.19	0.30	0.25	95%
J.Y.	F	21	0.37	0.36	0.36	86%
* A.B.	M	23	0.52	0.65	0.59	92%
			0.50	0.77	0.63	
G.S.	M	44	0.60	0.90	0.75	80%
* C.J.	M	21	0.70	0.90	0.80	105%
* C.J.	M	21	0.70	1.2	0.95	105%
	Mean		.22 ng/ml	.28ng/ml	.25 ng/ml	97%
	S.D.		.19 ng/ml	.28 ng/ml	.23 ng/ml	

experiment. In all instances, the collections were made from the subject lying on a couch and after a period of quiet rest for approximately 30 min. The mean level from 18 subjects is 0.25 ng/ml (SD 0.23 ng/ml). The age of the subjects ranged from 20 to 40 years; no age or sex difference was found.

In C.J. the levels at an interval of 9 months were very similar but in subject P.A. who was a young woman with vasomotor instability, there was a marked difference in the two estimations made 3 weeks apart (see Discussion Part 5).

DUPLICATE VARIABILITY

Two sequential BK estimations were made on 7 subjects. Four of these were subjects presented in Table 2 (R.R.,B.K.,J.R. & A.B.); three others were subjects studied subsequently, but in whom duplicate estimations were also made, either on arterial or venous** blood; these latter are included in the table below for convenience and the pooled results are used for the estimation of duplicate variability of the method of extraction and assay. It is shown that when the average of estimations by uterus and duodenum is used, duplicate variability is only 5.5% ($\pm$ 4.9% as 95% confidence limits).

DUPLICATE VARIABILITY

	Uterus BK ng/ml	Diff	Duodenum BK ng/ml	Diff	Average BK ng/ml	Diff
R.R.	0.09 0.10	11%	0.11 0.12	9%	0.10 0.11	10%
L.C.*	0.13 0.17	31%	0.17 0.13	31%	0.15 0.15	0%
B.K.	0.12 0.14	17%	0.18 0.18	0%	0.15 0.16	6.6%
J.R.	0.23 0.19	21%	0.33 0.30	10%	0.28 0.25	12%
M.H.**	0.36 0.32	12%	0.32 0.36	12%	0.34 0.34	0%
A.B.	0.52 0.50	4%	0.65 0.77	18%	0.59 0.63	7%
L.H.*	1.07 1.19	11%	1.07 1.01	6%	1.07 1.10	2.8%
Mean		15.3%		12.3%		5.5%
S.E.		± 3.3%		± 3.7%		± 2.0%
95% confidence limits (t_6 = 2.45)		15.3 ± 8.29%		12.3 ± 9.07%		5.5 ± 4.9%

* Patients with the carcinoid syndrome (see Part 5).

Two important facts emerge from an inspection of the results of normal blood levels as estimated by uterus and duodenum assays. The mean for duodenum results from the 18 subjects is 0.28 ng/ml c.f. 0.22 ng/ml for uterus; also the scatter of the duodenum assays is greater then for uterus assays viz. SD for duodenum assays is 0.28 ng/ml and for uterus assays is 0.19 ng/ml. These findings can be accounted for by a consideration of the behaviour of the duodenum as an assay tissue. Since the change in length of the duodenum is smaller than of the uterus for a given dose of BK, duodenum assays are characterised by a smaller slope of the dose response curve. This gives less precision and inevitably greater scatter. The duodenum is also very sensitive to non-specific impurities which when present in the extract can cause some relaxation of the tissue independant of the concentration of BK in that extract. The bias towards higher assay values from the duodenum is most likely due to impurities in the extract which have escaped the purification steps.

More information about the divergence of the results was obtained by statistical analysis of data from recovery experiments and from endogenous BK estimations. The assays of re-

covery BK (10 ng/ml) and endogenous BK are distinguished by the fact that a much greater dilution of the recovery extract is made prior to assay. The dilution may be 5 - 15 fold greater and higher standard concentrations are usually employed. Both these factors would tend to minimize the effect of any impurities present in the extract.

The statistical analysis (Appendix 4) shows that there is no significant difference between uterus and duodenum assays in the recovery estimations, and there is no significant divergence from parallelism of the standard vs unknown dose-response lines for both tissues.

The difference between endogenous BK levels as estimated by uterus and duodenum assays are significant at the 5% level and a mean difference of 30% is found. There is no significant deviation from parallelism for the standard and unknown dose-response slopes for the uterus, although the deviation is significant for the duodenum for the group data. It seems then that the impurities present in the extract effect only the duodenum and are not influencing the assays on the uterus. Regression analysis of the bias of the duodenum assays as compared to the corresponding uterus values shows no trend over the range of values of BK concentrations assayed in normal subjects. There is a constant bias of + 30% ($\pm$ 10% as 95% confidence limits).

f. DISCUSSION

The merits of this method of estimation of blood BK levels are:

1. Recoveries of internal standard averages 97%. This order of recovery is maintained over a wide range of internal standards, from 250 ng/ml to 0·5 ng/ml.

2. The final extract of endogenous BK is sufficiently pure to allow assay (in some cases with little or no dilution of the extract) on superfused preparations.

3. The method is sensitive enough to enable the estimation of BK levels in all normal subjects so far studied.

4. The use of uterus and duodenum provides maximum sensitivity and specificity and any interfering substances can be readily detected (Fig. 11).

5. Use of chymotrypsin on the superfused uterus can increase its sensitivity to doses of BK as low as 5 pcg/ml in some preparations. This sensitivity is exceptional, but one to 50 pcg/ml can be frequently obtained.

General and specific drawbacks are:

1. Large amounts of blood are required for each individual estimation.
2. The method is cumbersome and time consuming.
3. The method of blood collection requires arterial or venous cannulation.
4. The final blood extract still contains impurities as shown by its effect on the duodenum, but this error is constant and the drawback is offset by the gain in specificity.

The achievement of a salt free extract depended upon the realisation that BK will remain stable in pH 11 NH_4OH for a period of time sufficient to remove the ammonia by rotary evaporation. The closer to purity that an extract approaches the greater is the risk of losing BK by adsorption to glass surfaces in the final steps of purification, especially when the extract is evaporated to dryness. This has been minimised by the use of hexadimethrine bromide to treat the glass surface of the evaporating flask, and the use of small amounts of casein to provide and inert residue.

Although the assay method will not distinguish between the plas-

ma kinins, for reasons stated in Part 1 the major kinin peptide present in blood is probably BK. Endogenous BK levels are too small to allow any chemical characterisation from the extracts, such as by paper electrophoresis or thin layer chromatography.

The slightly higher results given from duodenum assays do not pose a serious problem. It is important to emphasize the implications and consequences of the discrepancy between the uterus and duodenum assays. The presence of impurities in the extract for assay would effect the duodenum in two ways:

(a) they cause the duodenum to relax out of proportion to the BK content in the extract,

(b) since the effect of dilution in the low dose of the unknown is different on BK and impurities, the dose-response (D-R) line of the unknown assumes a slope which deviates form parallelism from the (D-R) line of the standard.

Analysis of variance was performed on results (Table 2)

that showed the most marked discrepancy between values from the two tissues. It showed that in the individual assay, the divergence from parallelism is not significant and does not prohibit an estimation of a relative potency of the extract by the method used. However, when group data are tested by a paired t test, it is shown that some of the discrepancy (30%) that exists between the uterus and duodenum assay results is due to a divergence from parallelism within the duodenum assays. Regression analysis shows that the bias produced by the combination of (a) and (b) (above) is constant over the range of levels assayed in normal blood, and duodenum assays are valid as long as this bias is recognised. In other words variations in blood BK levels over a normal range can be reliably estimated on the duodenum.. Although the mean blood BK level calculated from duodenum assays is higher by 30% than the mean level estimated with the uterus, no correction factor can be validly used to treat individual assay results. With 95% confidence limits any duodenum estimation will lie in the range of $\pm$ 10% of a value 130% higher than the value obtained by uterus assay. The point may be raised that uterus estimations only should be used as a true measure of blood BK levels. However, greater accuracy is achieved by averaging the values

from the uterus and duodenum. This is exemplified by inspecting recovery values (Table App. 1) and the decrease in variability in sequential replicates when the values from the two tissues are averaged.

Some comment is needed regarding the normal levels presented here as compared with normal levels reported by other workers. Allwood and Lewis (1964) report an average BK level in venous blood collected from the forearm of 32 ng/ml, with similar levels in arterial blood, using a method of extraction and assay which gives recoveries ranging from 6 - 80%. Fasciolo (1964) using the method of Binia et al. (1963) reports values ranging from 0 - 2 ng/ml. in femoral artery and basilic vein blood; Abe et al. (1966) report kinin levels of 0 - 2 ng/ml using a method which gives recoveries of added BK of approximately 50%. This latter range of levels is derived from 12 estimations in eleven of which zero levels were detected. One must agree with the conclusions of Webster and Gilmore (1965), that the high levels encountered in studies such as those of Allwood and Lewis may have been due to activation of plasma kallikrein during the withdrawal of blood samples.

In a more recent report from Carretero, Nasjeletti and Fasciolo (1965) values of kinin in arterial and venous blood of

0 - 4.0 ng/ml have been found, with an average of 1.2 ng/ml; one wonders whether the collection of blood through needles may have caused some generation of BK in the collection process. According to Margolis and Bishop (1963), 1.5 micrograms of BK can be released from 1 ml of plasma by kallikrein which has been activated by the Hageman Factor. Only a small amount of BK released from such a plasma reserve could produce marked elevations of plasma BK levels. Carretero's et al. findings that venous and arterial blood do not differ significantly in kinin content lends support to such possibility, for as will be shown later in this thesis, there is normally a higher arterial than venous BK level (Part 3).

It is interesting to draw a comparison between the levels of BK in arterial blood 0.25 ng/ml (SD 0.23 ng/ml) and the levels of adrenaline and noradrenaline. The blood levels of these catecholamines as reported by Vendsalu (1960) are adrenaline 0.23 ng/ml ($\pm$ 0.02 ng/ml) and noradrenaline 0.31 ng/ml ($\pm$ 0.02 ng/ml). It is interesting to note that two very pharmacologically active substances occur in such similar concentrations.

Part 3. PHYSIOLOGICAL CHANGES IN BLOOD BK LEVELS IN MAN

a. INTRODUCTION
b. SPECIAL METHODS
c. RESULTS:-
    (1) Intra-arterial and intravenous BK infusions.
    (2) Arterio-venous BK differences.
    (3) Direct heating.
    (4) Reactive hyperaemia.
    (5) Cold vasodilatation.
    (6) Sympathetic nervous activity, and blood BK.
d. DISCUSSION
e. SUMMARY

PHYSIOLOGICAL CHANGES IN BLOOD BRADYKININ IN MAN

a. INTRODUCTION

The establishment of the presence of bradykinin in arterial blood in all normal subjects so far investigated, indicated that the kallikrein-kininogen-kinin-kininase system in the body must be in a state of turnover. It leaves one to wonder then what the possible significance of these small concentrations might be.

Since blood contains an inactive form of the kinin forming enzyme kallikrein (Werle, 1955), the substrate bradykininogen (Diniz & Carvalho, 1963) and an extremely active kininase (Erdos & Sloane, 1962), the concentration of the peptide at any time may be determined by some physiological control mechanism acting on one or more of the four reactants in this system. On the other hand the presence of BK may simply represent an aimless process of generation and destruction. In other words, the presence and the level of concentration of BK in the blood may have some physiological significance or it may be entirely fortuitous. Even if the turnover in blood is of no significance,

another possible source of blood BK is a leakage away from tissues where BK is thought to be generated and to function as a vasodilator e.g. salivary gland (Hilton & Lewis, 1955 a, b; 1956) skin, (Fox & Hilton, 1958) or the pancreas (Hilton & Jones 1963), and the level of tissue production may be largely or entirely responsible for the extent of its occurrence in blood. The effluent venous blood BK levels from these sites during their activation have not been studied but there is evidence that during sweating there is increased concentration of BK in the interstitial spaces (Fox & Hilton, 1958) and also increased BK concentration in interstitial fluid following tissue injury in animals (Rocha e Silva & Antonio, 1960; Rocha e Silva & Rosenthal, 1961). Lymph from traumatized hind limbs of dogs has also shown evidence of changes in kinin metabolism e.g. increased kinin-forming activity (Edery & Lewis, 1963 and increased kininase activity (Jacobsen & Waaler, 1966).

The difficulties in interpreting lymph and interstitial fluid studies are obvious. Lymph collection is a very slow process and by the time it is analysed for kinin content, all of the latter may have disappeared, particularly if injury is associated with increased kininase activity. Collection of interstitial fluid entails the grossly abnormal conditions during perfusion of the region with physiological solutions and involves the risk

of kallikrein activation by dilution. The assay of blood draining certain regions for kinin content,as an index of kinin turnover in that region, has certain advantages, the chief one being that blood which has perfused the particular region is readily available from the draining vein and in most instances rapid collection of adequate amounts of blood can be made.

The studies of Allwood & Lewis (1964) seemed to indicate that any correlation between hyperaemic states and BK involvement would be difficult or impossible since they found that infusions of large amounts of synthetic BK into the brachial artery produced little or no change in BK levels in the effluent venous blood. Since some criticism can be levelled against their methods of BK assay and on the validity of their BK levels in human blood (see Part 2), it was necessary to repeat such infusion studies to see whether the present method of BK assay could detect changes in blood BK levels when small doses of infused BK were producing moderate haemodynamic changes. This was done by assaying effluent venous blood for BK content during the infusion of BK into the brachial artery. Similarly BK was infused intravenously, and changes in arterial BK levels were estimated during the changes in blood pressure that occurred as the result of the infusion.

Following these preliminary studies, the hand was chosen as

a peripheral site across which studies of arterio-venous differences in endogenous BK levels could be made. The purpose of this was to investigate any participation of a peripheral site in modifying blood BK levels. Firstly by an appraisal of the arterio-venous (A-V) differences in BK levels that exist in resting conditions and secondly during hyperaemic states in the hand. Sampling of arterial blood entering and venous blood leaving the hand is relatively easy and measurements of blood flow are readily made. The hand also represents a fairly homogenous vascular bed which dilates in response to various physiological stimuli which may possibly act through the production of BK (Burch & De Pasquale 1962; Rocha e Silva, 1963). The hyperaemic states investigated were those produced by direct heating, cooling, and by ischaemia. During these latter studies it was also noted that painful procedures, associated with reflex sympathetic discharge, caused a fall in arterial blood BK. In one subject the effect of sympathetic blockade with bethanidine on blood BK levels was investigated. The realisation of the ability of the sympathetic nervous system activity to lower BK levels led to a comparison of arterial and venous blood in terms of kininase activity. (See Part 4 for action of catecholamines on blood kininase activity).

b. SPECIAL METHODS

The methods of blood collection and blood BK estimation have already been described.

BK infusions were made through an intravenous or intra-arterial catheter by a mechanically driven syringe.

Blood pressure recordings were made with a Statham transducer (P 23 AC) and Grass Polygraph (Model 5D) from the intra-arterial cannula used for sampling.

Hand blood flows during direct heating and reactive hyperaemia were measured by venous occlusion plethysmography of the conventional type (Greenfield, 1954). Cold vasodilatation was produced by immersion of the hand in a stirred calorimeter containing water at an initial temperature of 0° C (Greenfield & Scarborough, 1949). The onset of the hyperaemia was associated with an abrupt increase in heat elimination.

An estimate of kininase activity of blood being sampled was obtained by incubating a mixture of blood and BK for a known period of time, then halting the process by the injection of the mixture into ethanol (0° C). The amount of BK which had survived the contact with blood was estimated using the usual method of extrac-

tion and assay. A 500 ng amount of BK in a volume of 0·1 ml was placed in the collecting syringe and the syringe and its contents were warmed to 38° C in a water bath. Five ml of blood were then collected into this syringe from the artery or vein to give a final concentration of BK of 100 ng/ml of blood. Because of the small volume of the substrate placed near the nozzle of the syringe immediate effective mixing occurred upon blood entering the syringe. In view of the high activity of the kininase, the incubation period was reduced to a minimum, the lower limit being set by the speed at which 5 ml of blood could be withdrawn into the syringe and its contents injected into ethanol. A seven sec. transfer time could be uniformly employed in all experiments and served as the common incubation time for comparison of kininase activities. To improve accuracy, four such collections were made and pooled for each estimation. The BK which had survived a seven sec. incubation period was expressed as the per cent survival. The per cent survival value is thus distinct from the per cent recovery, the former being a measure of kininase activity whereas the latter is a gauge of the recovery of an internal standard.

c. RESULTS

(1) INTRA-ARTERIAL AND INTRAVENOUS INFUSIONS

BK was infused into the brachial artery in 2 subjects. Blood flow in the forearm was measured by venous occlusion plethysmography and blood was collected through a catheter introduced centrifugally into a vein from the antecubital fossa on the side of the infusion. The results of the experiments on the subjects C.J. and I.K. are shown in table 3. Increasing doses led to successively larger increments in forearm blood flow. Infusion of 50 ng/min in each subject resulted in an increase in venous blood BK and the 100 ng/min dose led to a further rise. Figure 13 shows diagramatically the results on the forearm blood flow and effluent venous BK levels from the three intra-arterial doses in subject C.J. Although the small dose 25 ng/min in C.J. caused an increase in forearm blood flow of 76%, there was no accompanying change in the effluent venous BK level. In this same subject, the 50 ng/min infusion, which was the lowest intra-arterial dose to cause a detectable increase in venous BK level was given into 70 ml of blood flowing to the forearm per minute. Therefore the maximum rise in concentration which could be produced would be about

0·7 ng/ml, but the actual level is certain to be much less than this because of considerable destruction of BK in the blood and its loss along with other small molecular solutes by diffusion into the tissues. The observed rise in venous BK level of one tenth of this estimated maximum might then be expected. In subject I.K. a similar calculation shows an observed rise of one eighth of the estimated maximum when 50 ng/min are infused into the brachial artery.

In one experiment BK was infused intravenously in doses of 50, 100, 200 and 400 ng/KG/min. Before and during each infusion, blood was collected from the brachial artery for BK assay. Fall in blood pressure in response to intravenous BK is variable, but the subject studied (J.Y.) was sensitive and reacted to 50 ng/Kg/min with tachycardia and hypotension and with this intravenous dosage, a rise in brachial arterial blood level from 0·36 to 0·45 ng/ml was detected. Increasing the dose led to increases in arterial blood levels as shown in Table 4 and Fig. 14. The small increase observed in the arterial BK level from intravenous infusions can probably be accounted for by the same factors as the venous blood changes during intra-arterial infusions.

TABLE 3

I.A. dose BK ng/min	SUBJECT C.J.		SUBJECT I.K.	
	% Increase in forearm blood flow	Venous BK ng/ml	% Increase in forearm blood flow	Venous BK ng/ml
Control	0	0.55	0	0.74
25	76	0.55	-	-
50	100	0.62	54	0.93
100	116	1.27	277	1.50

Changes in forearm blood flow and forearm venous blood bradykinin levels during infusions of bradykinin into the brachial artery in 2 subjects.

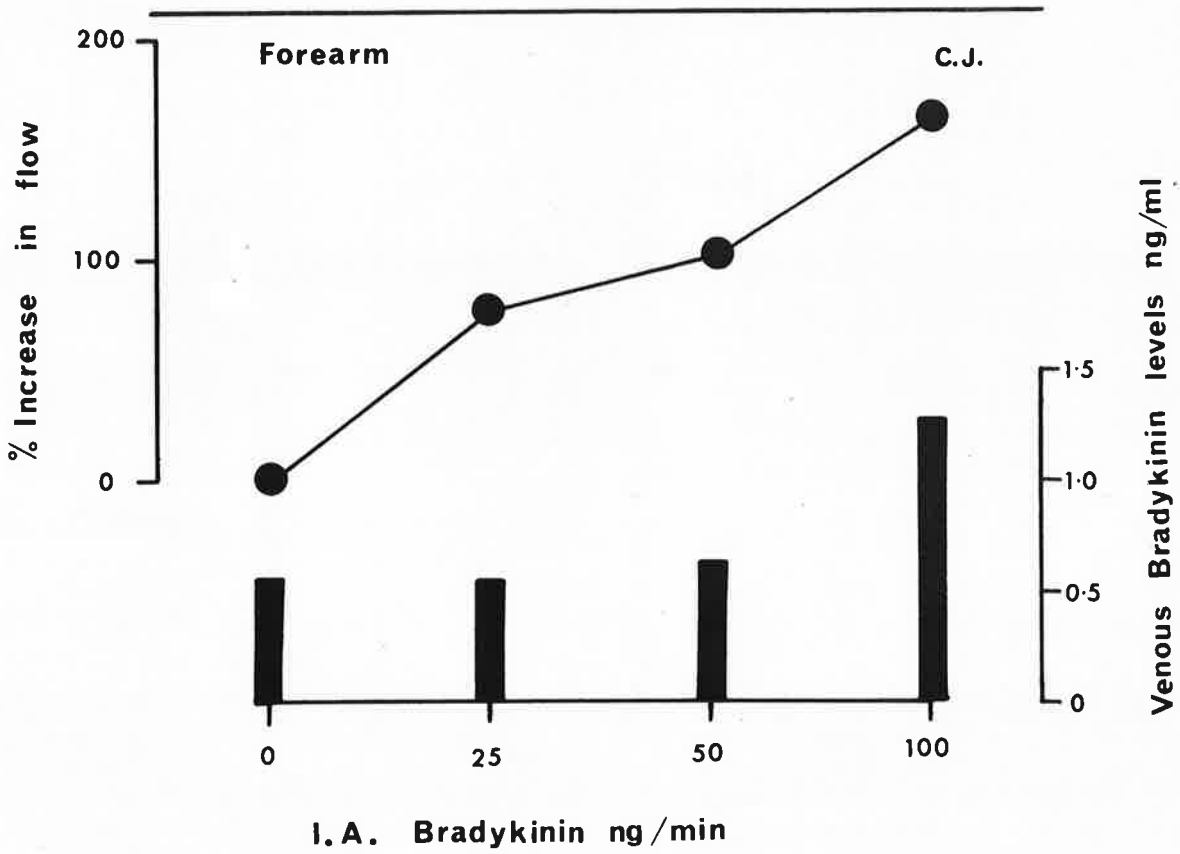


Fig. 13. BK was infused into the brachial artery (subj. C.J.) in doses of 25, 50, and 100 ng/min. Blood flow in that forearm was measured by venous occlusion plethysmography and changes in flow are expressed as per cent increase above control blood flow. BK levels estimated in effluent venous blood from that forearm during the infusion are shown by the solid vertical bars.

TABLE 4.

Dose ng/Kg/min	% fall in Mean B.P. during infusion	Arterial blood BK (ng/ml)
Control	-	0.37
50	7.1	0.45
100	11.5	0.48
200	18.8	0.60

Changes in blood pressure and brachial arterial blood bradykinin levels during intravenous infusions of BK in one subject J.Y.

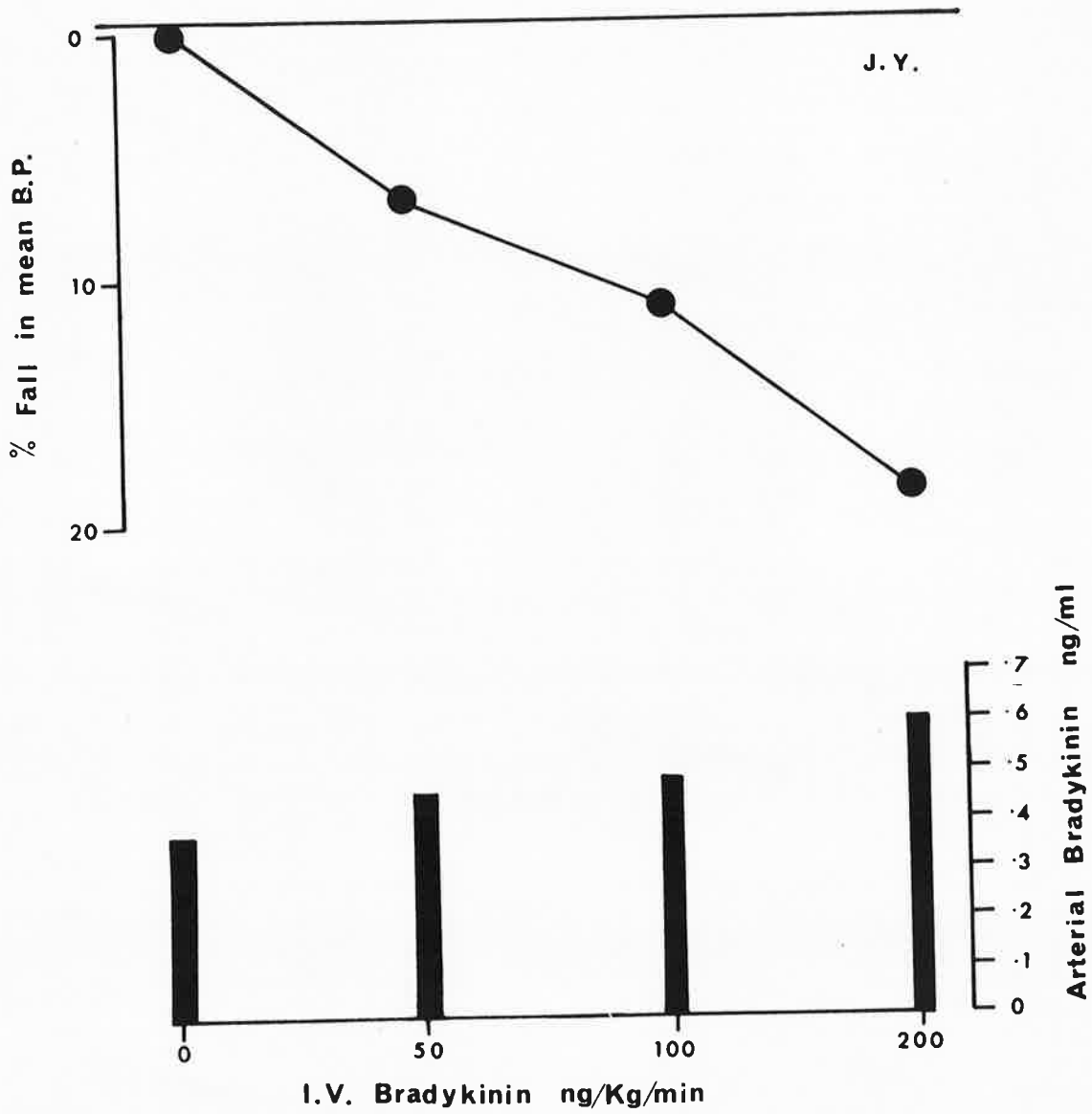


Fig. 14. BK was infused intravenously in doses of 50, 100 and 200 ng/kg/min. in subject J.Y. The resultant per cent falls in mean blood pressure ($D + \frac{S-D}{3}$) are shown. The solid vertical bars show the changes in arterial BK levels.

(2) ARTERIO-VENOUS (A-V) BK DIFFERENCES

Bradykinin estimations were made on blood withdrawn simultaneously from the brachial artery and a vein draining the forearm or hand on the same side. The venous catheter was introduced centrifugally into the forearm vein, so that the tip of the catheter was as close to the wrist as possible. Where such a vein could not be cannulated, blood was taken from an ante-cubital vein. The blood flow through the hand is extremely labile and although in most instances adequate venous outflow could be obtained at ambient temperature of 21° C, in some the hand had to be warmed slowly in the hand plethysmograph (to 38° C in one case) to obtain adequate blood flow for venous sampling. In one case, however, blood could easily be collected when the hand was at 17° C and blood flow was only 3 ml/100 ml of tissue. Eight sets of A-V estimations were performed in six normal subjects and the results are shown in Fig. 15. In almost all instances the arterial blood BK was higher than the venous; venous blood from the hand at 17° C, however showed a level which was higher than the arterial.

To exclude the possibility that venous blood BK estimations gave falsely low results, a comparison was made of the recovery of synthetic BK from arterial and venous blood. In eight such

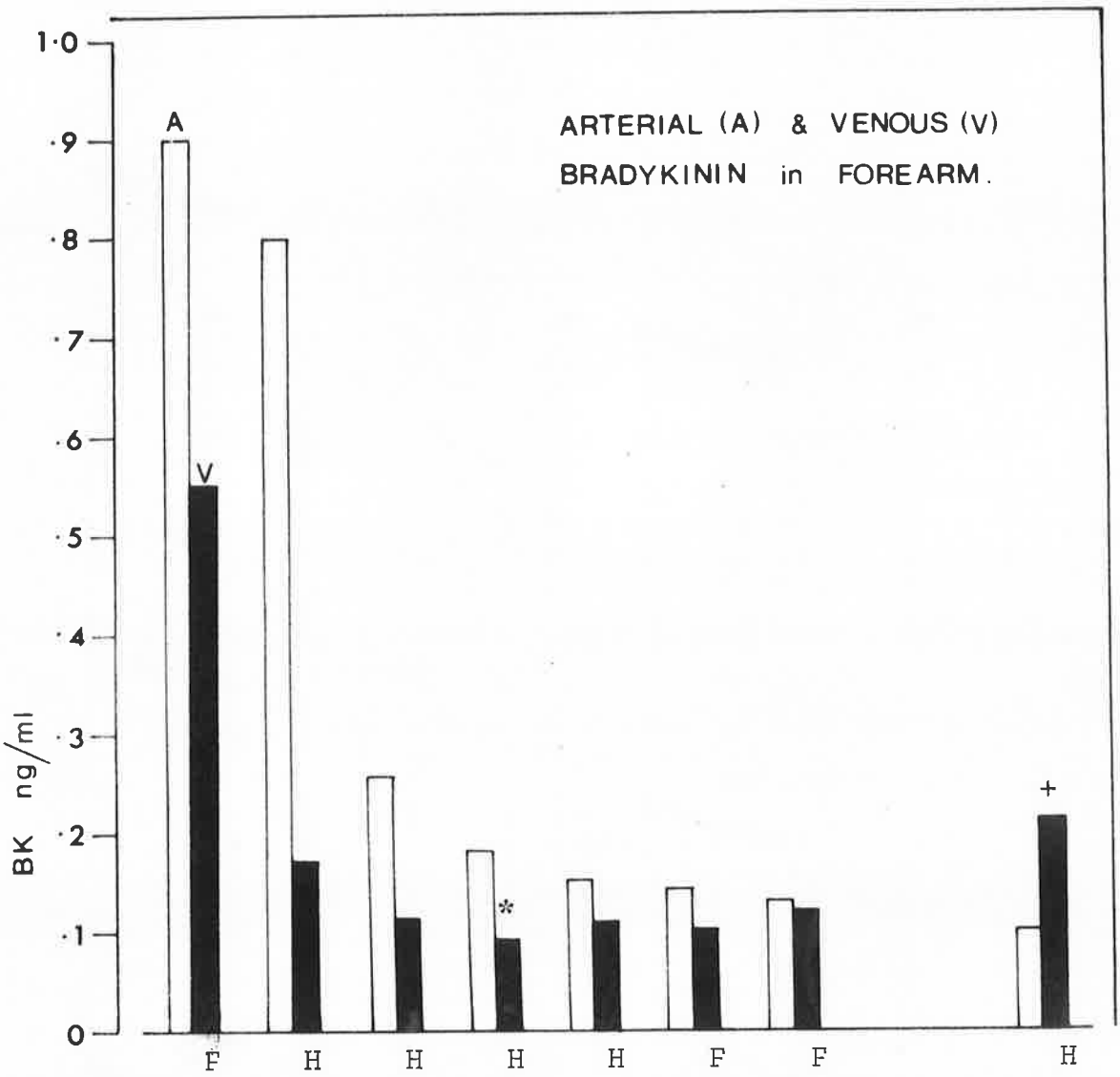


Fig. 15.

BK levels (ng/ml) in blood collected simultaneously from the brachial artery (A) and a vein (V), (an antecubital vein (F) or a vein draining the hand (H). Eight sets of estimations in 6 subjects.

*Hand at 38° C.

+Hand at 17° C.

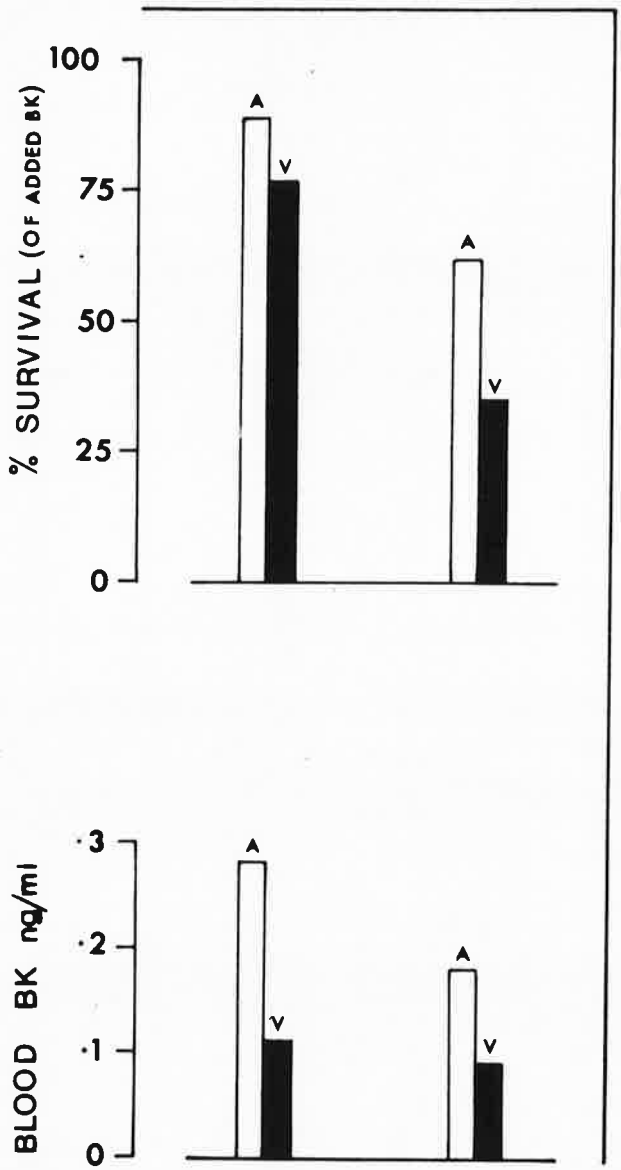


Fig. 16. A comparison of arterio-venous differences in endogenous BK levels (ng/ml) and kininase activity (% survival after 7 sec incubation) in two subjects. Arterial (A) and venous (V) blood was collected simulatan-eously from the brachial artery and a vein draining the hand on the same side.

Amount of BK added	% Recovery
10 ng/ml	105, 101, 99, 97, 97, 94, 92
1 ng/ml	100

Table 5. Recovery of bradykinin from venous blood. Bradykinin added to collecting syringe. Mean recovery 98%.

estimations on venous blood, the mean recovery was 98% (Table 5). This compares very well with the mean recovery of 97% from arterial blood (Table 1).

Kininase activity was also compared in arterial and venous blood in two subjects who showed a higher arterial than venous BK level. It was shown that in both cases venous blood had a greater capacity for the destruction of BK. Figure 16 compares the difference in BK concentrations in arterial and venous blood with the kininase activity in blood from the same artery and vein in these two subjects.

(3) DIRECT HEATING AND VENOUS BLOOD BRADYKININ

The effect of direct heating of the hand in a plethysmograph on the BK level in the venous blood leaving that hand was studied in three subjects. In all three a rise in BK occurred when the temperature was raised from 33° C to 45° C. One subject experienced moderate pain in the hand at this temperature, the others experienced only slight discomfort. Figures 17 - 19 show the effect on the hand blood flow and hand venous BK of direct heating. Arterial blood was also sampled throughout each experiment to follow any general changes in circulating BK. The temperature

in the plethysmograph was raised or lowered as quickly as possible, and the recording of blood flow and sampling of venous and arterial blood were carried out after a 10 - 15 min period of stabilization at the new temperature. In Fig. 17 (subject B.K.), raising the temperature from 33° C to 45° C increased the venous BK level from 0·12 ng/ml to 0·18 ng/ml and a reversal of A-V BK difference was noted. When the plethysmograph temperature was returned to 33° C there was a lingering elevation of blood flow in the hand associated with a continued elevated BK level. Blood flow continued to settle over a period of two hours. When it had finally reached the pre-heating level of flow, the BK had also returned to pre-heating level. During the whole experiment arterial blood BK showed minimal changes.

In subject C.J. (Fig. 18) flows were recorded and blood was sampled at an intermediate temperature of 40° C as well. This was done for two reasons, first to see whether in this subject, who had also shown a high arterial blood BK level on a previous occasion, speeding up of the circulation through the hand would cause an increase in the venous BK level; second, to see whether the elevation of venous BK upon heating was a gradual and temperature dependent process. With the hand at 40° C and a considerable elevation in flow, the venous BK level decreased from 0·17 ng/

ml to 0·15 ng/ml. Elevation of the plethysmograph temperature to 45° C produced a rise in the BK level to 0·26 ng/ml. Returning the temperature to 33° C reduced the hand blood flow within 15 min and by then the BK level had also fallen to the pre-heating value. When this subject experienced moderate pain at 45° C the associated sympathetic discharge was minifested by a constriction of hand blood vessels in the opposite control side and a decrease in arterial blood BK was noticed.

In subject R.R. (Fig. 19) the arterial blood BK level remained constant throughout the entire experiment at 0·11 ng/ml, but the venous blood level rose from 0·11 ng/ml to 0·13 ng/ml* when the temperature was raised to 45° C. In this subject because of a very well-draining venous catheter, venous blood BK level could also be examined at a very low rate of flow found when the plethysmograph temperature was at 17° C, and the hand was being perfused at the rate of 3 ml/100 ml of tissue/min. Arterial blood entering the hand still contained 0·11 ng/ml BK but the venous blood leaving the hand showed a doubled concentration of 0·21 ng/ml.

The rise in BK levels in venous blood from the hand associated with elevation of the local temperature to 45° C is summarised in Table 6.

* $p < 0.05$

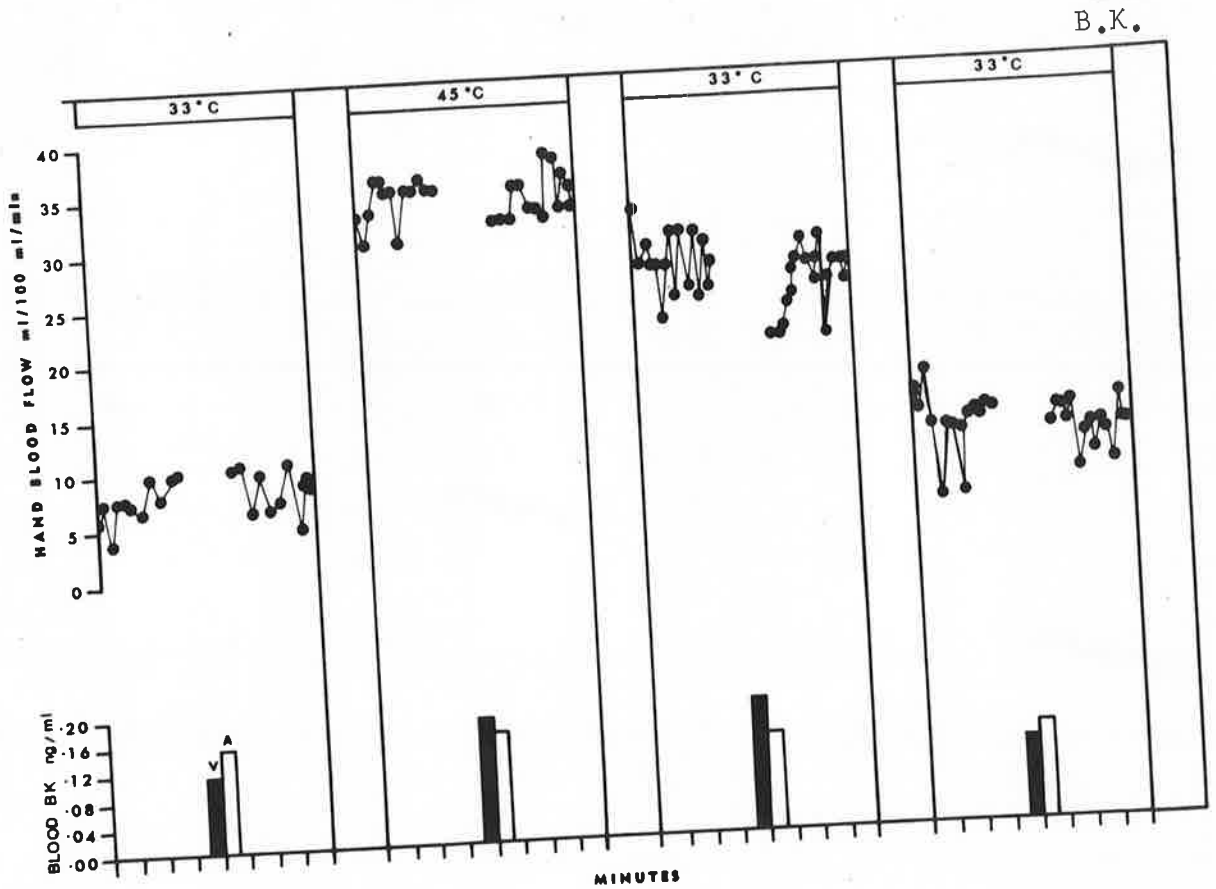


Fig. 17. The effect of direct heating of the hand on the arterial and hand venous BK levels (ng/ml) and hand blood flow (ml/100 ml/min). A reversal of the A-V BK difference was found at 45° C and the higher venous BK level was maintained during a slow return of hand blood flow to pre-heating level. The change in hand blood flow induced by heating to 45° C persisted for 2 hours.

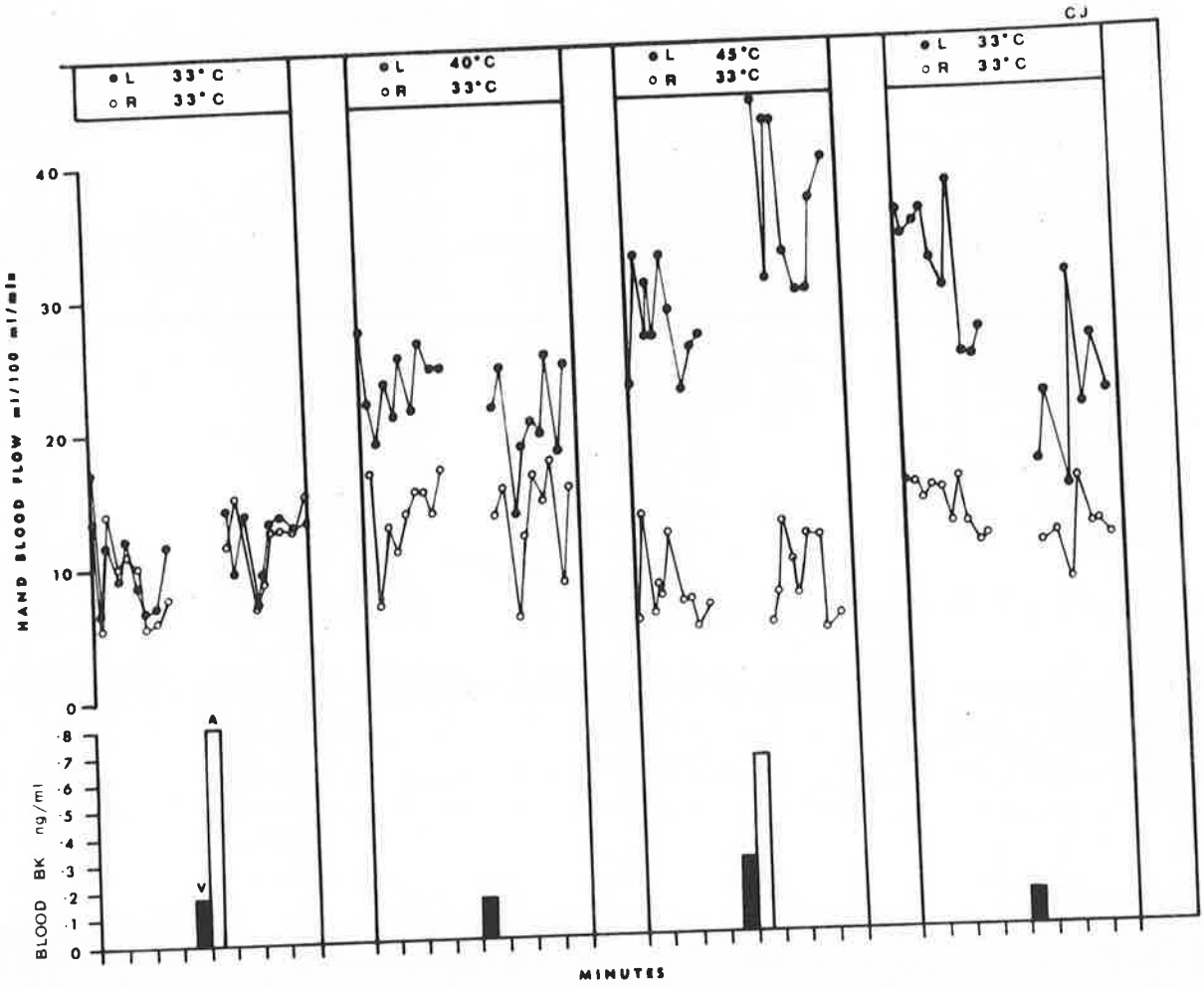


Fig. 18. The effect of direct heating of the L hand on the arterial and hand venous BK levels (ng/ml) and blood flow (ml/100 ml/min) through L (●) hand. Blood flow was also measured in the R hand (o) which was maintained at a temperature of 33° C. There was a rise in BK in the effluent venous blood from the L hand when the temperature was raised to 45° C. The occurrence of pain in the heated hand was accompanied by a vasoconstriction in the control hand. A fall in arterial BK level from 0·8 ng/ml to 0·64 ng/ml occurred together with the sympathetic discharge.

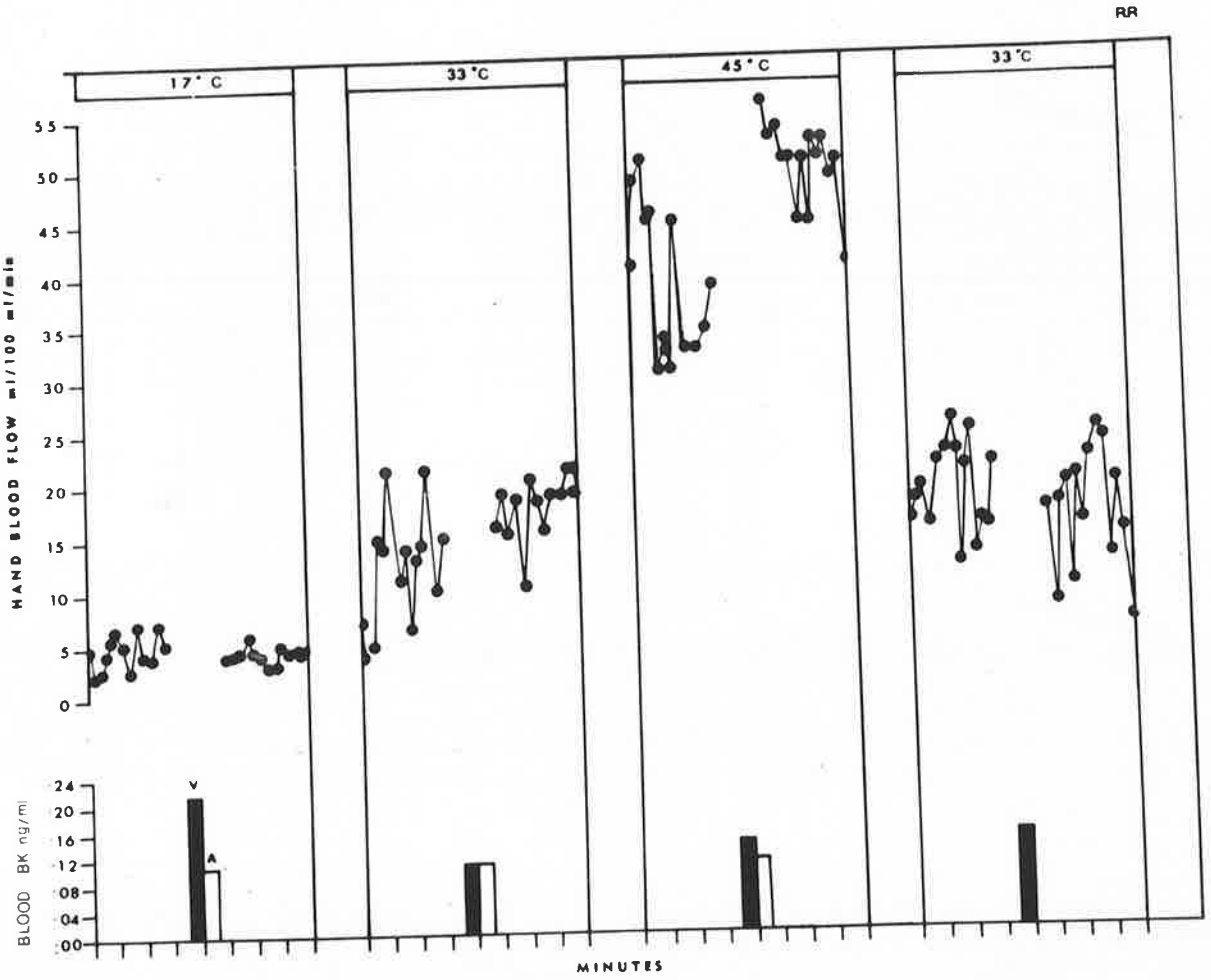


Fig. 19. The effect of direct heating of the hand on the arterial and hand venous BK levels (ng/ml) and hand blood flow (ml/100 ml/min) over a range of temperatures from 17° C to 45° C. There was a high venous BK level and a low flow at the low temperature. A fall in BK level occurred at 33° C followed by a rise at 45° C. Fifteen min after reduction of temperature to 33° C, blood flow was approximately back to pre-heating (33° C) value, but the effluent venous BK level was still elevated.

SUBJECT	Venous BK ng/ml	
	33° C	45° C
B.K.	0·12	0·18
C.J.	0·17	0·26
R.R.	0·11	0·13

Table 6. Increase in venous blood bradykinin levels in three subjects when the hand temperature was raised from 33° C to 45° C.

(4) REACTIVE HYPERAEMIA AND VENOUS BLOOD BK

In two subjects hand blood flow and changes in BK levels in venous blood from the hand were studied before and during reactive hyperaemia. This was produced by the release of a 10 minute arterial occlusion at the wrist with a cuff inflated to 200 mm Hg. Hyperaemia was induced three times in succession with adequate periods of rest between each. During the second run, no flows were recorded but venous blood was collected at times corresponding to periods before, during and after the reactive hyperaemia. Figure 20 shows the hand blood flow and venous BK levels in one of the subjects (R.S.). BK level fell from 0.48 ng/ml to 0.35 ng/ml and was still low when the period of high blood flow had passed three minutes later. In the other subject (N.P.) who had smaller initial level of 0.12 ng/ml, the level during and after hyperaemia was 0.11 ng/ml (Fig. 21).

(5) COLD VASODILATATION AND VENOUS BLOOD BK

In one volunteer, the onset of cold vasodilatation about nine minutes after the immersion of the hand in water at 0° C was noted by the subject as an easing of the severe pain in the hand and was

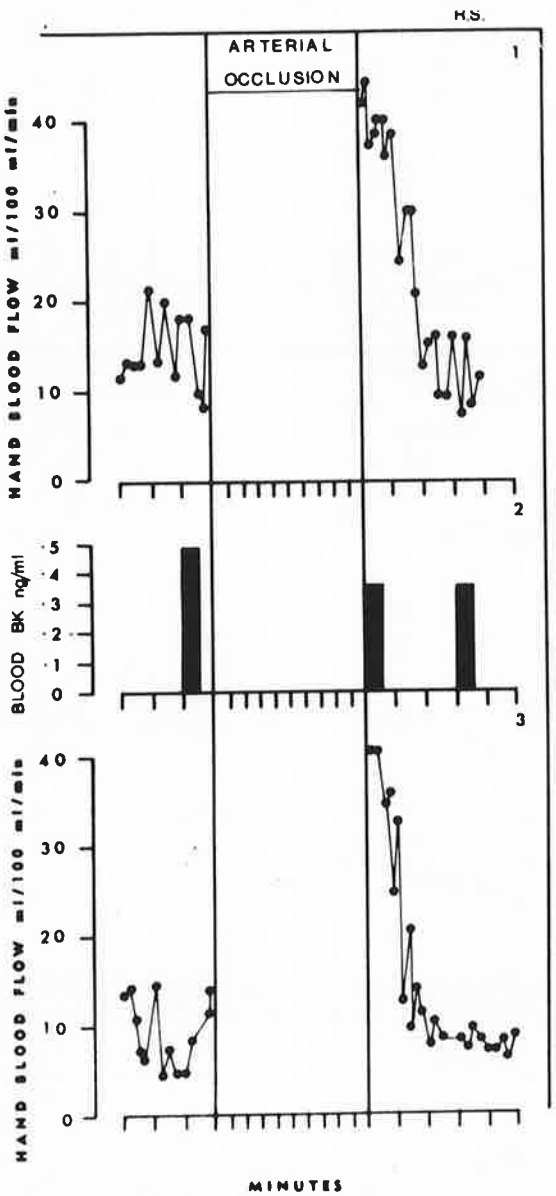


Fig. 20. Reactive hyperaemia was induced three times in succession (1,2,& 3)in one hand. Hand blood flow was measured in 1 & 3 only and hand venous blood BK levels were estimated before and after the second occlusion at times shown in above figure.
(Subject R.S.)

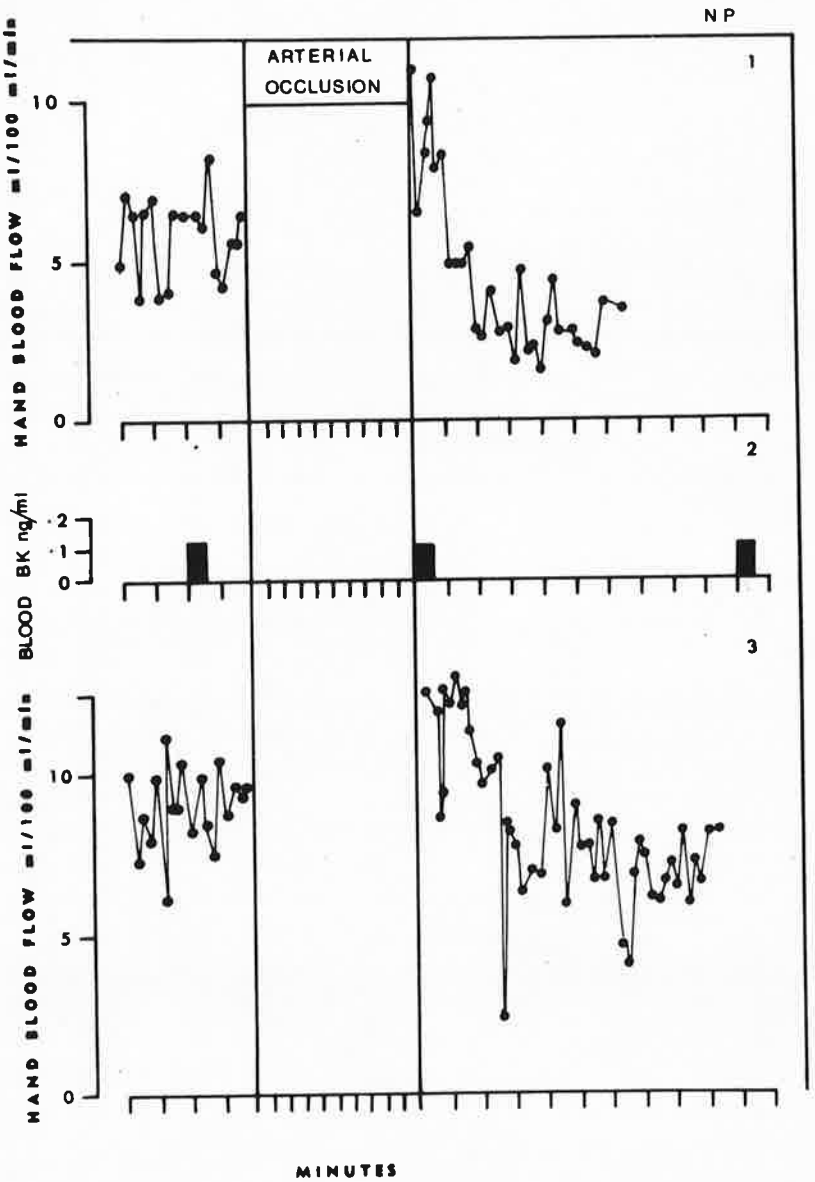


Fig. 21. Reactive hyperaemia was induced three times in succession (1,2, & 3) in one hand.Hand blood flow was measured in 1 & 3 only and hand venous blood BK levels were estimated before and after the second occlusion at times shown in above figure. (Subject N.P.)

also evident as an increased elimination of heat from the hand into the calorimeter. Venous blood was withdrawn through a centrifugally directed catheter with the tip at the wrist, first when the hand was immersed in water at 28° C and again at the onset of vasodilatation before which event there was virtually no blood flowing in the vein. Figure 22 shows the considerable fall in venous blood BK which occurred during the hyperaemia. Immersion of the hand in ice water is a very painful procedure and is associated with very intense sympathetic discharge. It was demonstrated in another subject that during the entire period of immersion there was a complete shutdown of flow in the control hand which was maintained at 32° C.

(6) THE EFFECT OF SYMPATHETIC NERVOUS ACTIVITY ON ARTERIAL BLOOD BRADYKININ

The fall in arterial BK level in subject C.J. associated with pain in the heated hand (45°) and manifestation of increased sympathetic nervous activity in the control hand at 33° C (Fig. 18) was unexpected. In another subject arterial BK level was estimated before and during the application of ice to the neck, which is a recognised method of causing a general-

ised sympathetic discharge (Parks, Skinner & Whelan, 1961). With this stimulus there was virtual disappearance of BK from the arterial blood. In a third subject generalised sympathetic blockade was carried out with intravenous bethanidine. This was achieved with a dose of 0·5 mg/Kg administered over a period of 8 min. Blockade was confirmed by the absence of vasoconstriction in the hand upon application of ice to the neck and the elimination of the systemic blood pressure overshoot which normally follows the performance of the Valsalva manoeuvre. Arterial blood BK was estimated before and after the blockade and showed a 7·5 fold increase in BK level. The results of sympathetic stimulation and blockade are summarised in Fig. 23.

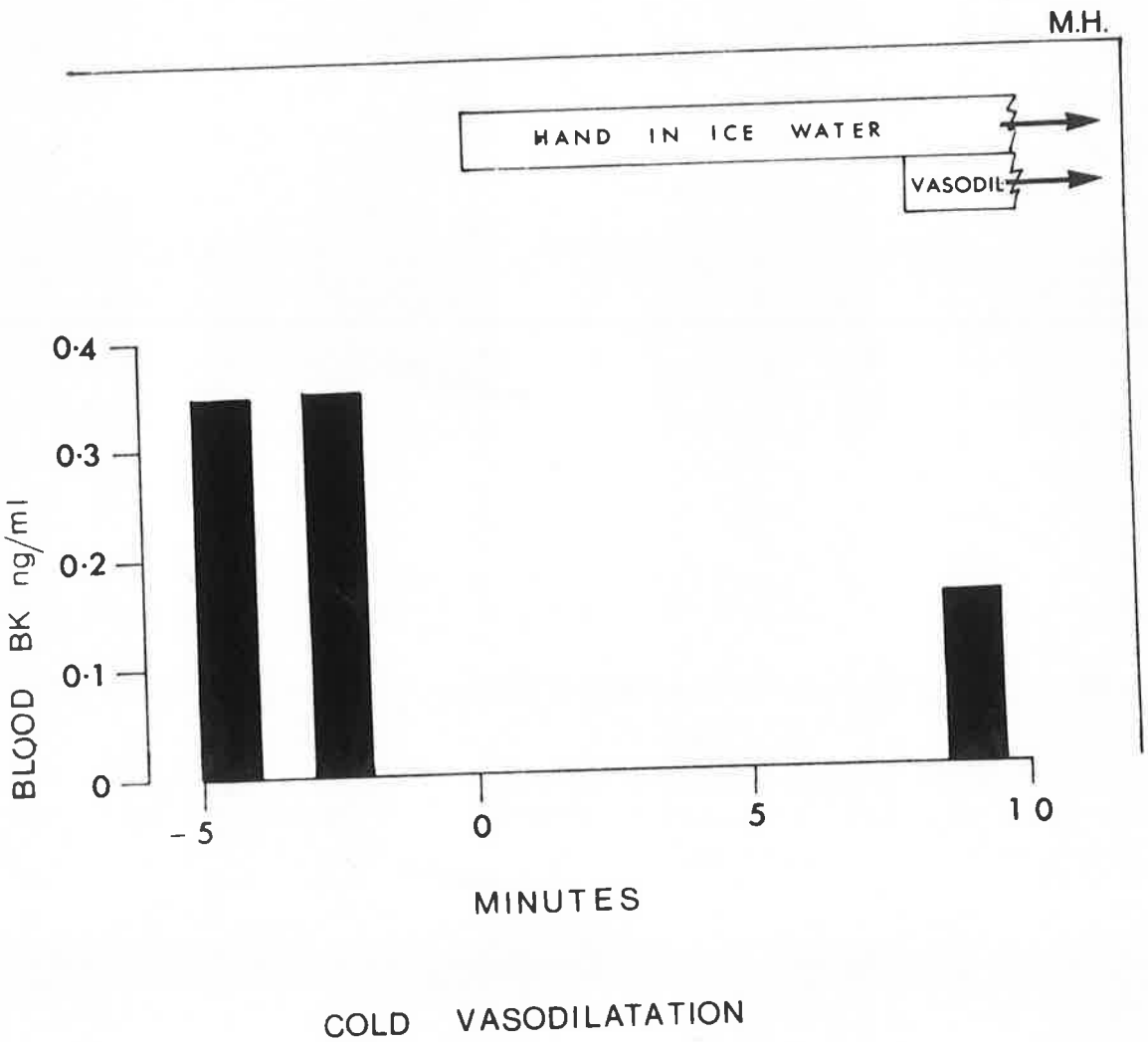


Fig. 22. Cold vasodilatation in the hand induced by immersion of the hand in a calorimeter at an initial temperature of 0° C. Onset of vasodilatation occurred after nine minutes. The decreased venous BK level estimated at the onset of hyperaemia is compared with venous BK levels from the hand at 28° C.

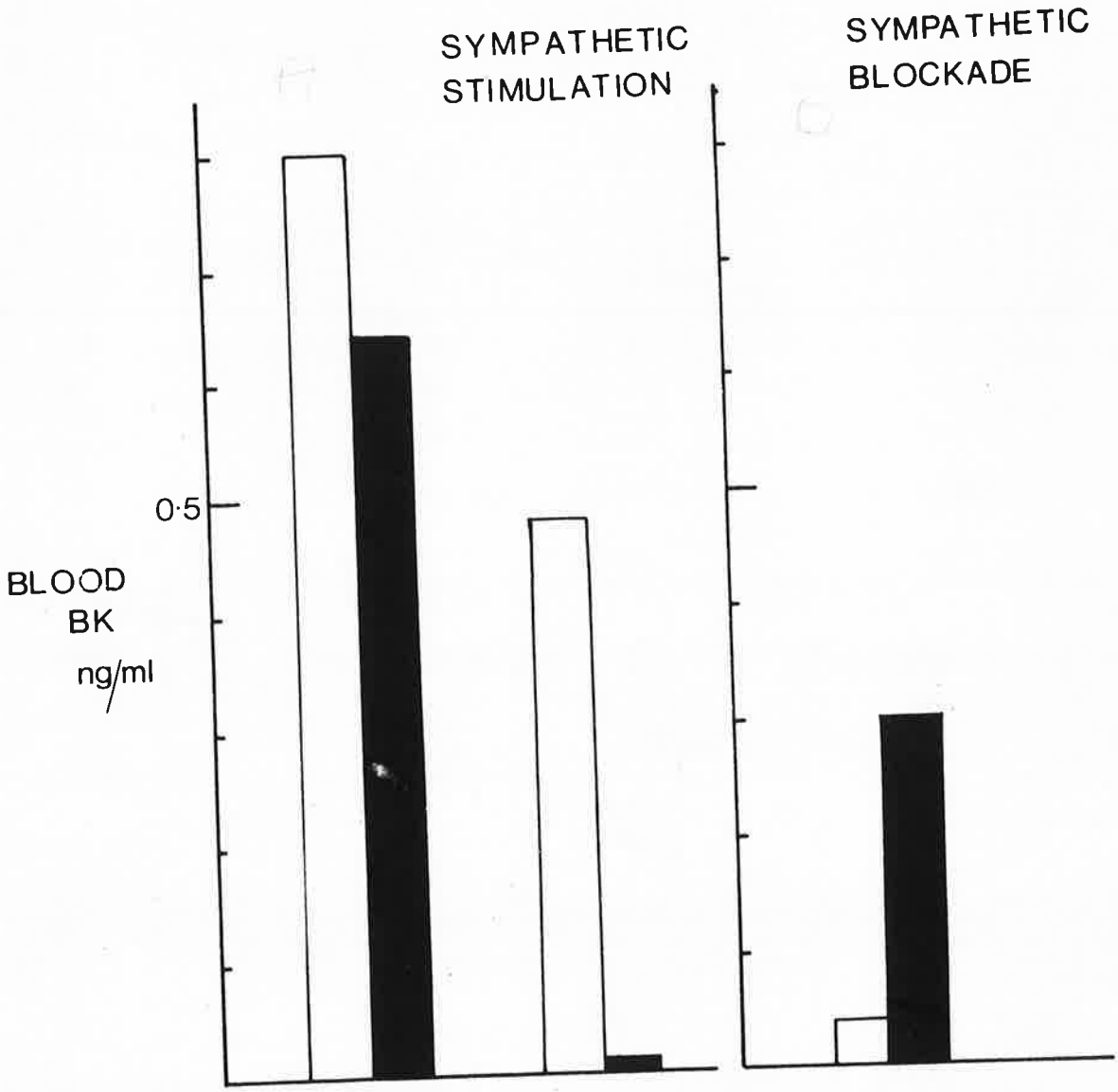


Fig. 23. Effect of (a) sympathetic stimulation, and (b) sympathetic blockade on arterial blood BK. A moderate fall in arterial BK level from 0·8 ng/ml to 0·64 occurred with pain associated with heating of the hand in one subject. A fall from 0·48 ng/ml to 0·03 ng/ml occurred in another subject when ice was applied to the neck. Sympathetic blockade was achieved with I.V. bethanidine and arterial blood BK rose from 0·04 ng/ml to 0·3 ng/ml.

d. DISCUSSION

The intravenous and intra-arterial BK infusion studies show that it is possible to detect changes in blood BK levels when infusions of small doses produce moderate circulatory changes. The loss of the infused BK across a vascular bed such as the forearm is of the order of 90% which indicates the large degree of clearance of BK by peripheral tissue.

The fact that such increases in blood levels can be detected suggests that with the present method of extraction and assay one should detect changes in endogenously produced blood BK if the latter is producing haemodynamic alterations. This of course depends on the assumptions that either BK generation occurs intravascularly or sufficient amounts of BK pass into the lumen of the vessel from some extravascular site of production. It is conceivable also that if BK does play a part in the regulation of vascular calibre in response to physiological or pathological stimuli, it may do so entirely from an interstitial, extravascular site and no evidence of such BK participation would be obtained by studying its levels in the blood.

Allwood and Lewis (1964) were unable to produce detectable

changes in forearm venous blood BK until the infusion dose into the brachial artery was raised to 10 μg/min. Comment has already been made (Part 2) of the extremely high endogenous blood BK levels reported by these workers and one wonders whether their methods are to blame for the negative infusion results.

That a difference exists in the estimated BK levels between arterial and venous blood (c.f. Allwood & Lewis, 1964: Carretero et al. 1965) is not due to different recoveries from venous and arterial blood and must be accepted as a true difference. If the venous blood leaving the hand has a higher BK content than the arterial blood entering it, then it must be due to a generation in the peripheral site. The interpretation of lower venous levels found in most resting subjects is not clear cut because of the numerous factors affecting the rate of production and disappearance of BK from the blood. If disappearance of BK from the blood is very rapid this may mask any loading which might be occurring at the same time. The depletion of arterial BK as it passes through a peripheral region such as the hand is probably due to two main factors: destruction of the peptide by the blood kininase and the loss by diffusion into the interstitial spaces where it would be destroyed by tissue kininases.

It has been shown that only about one tenth of the BK infus-

ed into the brachial artery can be recovered from the venous blood draining that extremity. This extent of clearance was observed with a small intra-arterial dose of 50 ng/min and the augmented venous levels resulting were still within the physiological range of blood BK levels. The order of difference in arterio - venous BK levels calculated from infusion studies was not observed in any of the subjects in whom endogenous A-V BK differences were studied (highest A-V ratio was 4), and indicates that the blood content must be replenished from the periphery.

In one subject (R.R. Fig. 19) where the rate of blood flow through the hand was low and where the hand temperature was 17°C (in contrast to the other cases where hand temperature was 21 - 38° C) a remarkably higher BK level was observed in the venous blood. This could be the result of the slow rate of flow enabling more BK to be picked up from the periphery, aided by the diminished kininase activity at this temperature (Abe et al. 1965). The effects of temperature and rate of blood flow on the eventual venous BK level cannot be dissociated in these experiments, since temperature and blood flow were closely correlated variables.

When the temperature of the hand was raised to 45° C there

was a distinct rise in venous BK levels above the arterial levels in two subjects and the production of BK must have greatly outstripped the destruction. In subject C.J. (Fig. 18) an A-V reversal could not be demonstrated, possibly because of the high arterial level and also because of the increased sympathetic activity which would have operated against BK elevation. A temperature of very close to 45° C must be critical for the acceleration of BK production. This is supported by the findings of Rocha e Silva (1960) in the production of " thermic oedema " in the rats paw. Carretero et al. (1965) failed to detect any increase in BK levels in effluent venous blood when the hand was heated to 40 - 45° C by immersion in a bath. The negative results are not suprising if the actual temperature did not reach 45° C. Further, they sampled venous blood from the antecubital vein which would mean a great dilution of any vasoactive substance that may originate from the hand. Since the blood BK levels are of such small magnitude and the largest increment on heating in the present experiments was only 53% (in subject C.J.) the method of blood BK estimation of Binia et al. (1963) which was used may not have been sufficiently sensitive to detect the changes.

The falls in BK levels of the venous blood in reactive hyper-

aemia and in cold vasodilatation are in sharp contrast to direct heating results. Factors contributing to the fall could be the hyperaemia itself, but, more significantly, the increase in sympathetic nervous activity which occurs with these procedures. Increases in plasma noradrenaline concentrations during reactive hyperaemia have been reported (Abrams, Barker & Butterfield 1965), and support other observations of increased sympathetic nervous activity during this event. The results of experiments on reactive hyperaemia and cold vasodilatation do not suggest that BK is a mediator of these hyperaemic states, but it is possible that whatever factors are operating to cause blood BK levels to fall with sympathetic discharge could cause venous blood levels to be low, despite normal or even elevated tissue levels. The ability of increased sympathetic nervous activity to lower circulating BK levels and the rise in arterial BK with generalised sympathetic blockade suggests a role for the sympathetic nervous system as a general or local influence on BK metabolism. In a subsequent section of this thesis it will be shown that catecholamines are directly responsible, acting through an influence on the kininase.

e. SUMMARY

1. Using the method of blood BK estimation described in Part 2, it was possible to demonstrate that I.V & I.A. BK infusions which produce moderate haemodynamic changes also cause an increase in blood BK levels.

2. Arterial and venous BK levels were compared in the forearm. Arterial levels were usually higher, the ratio of ~~differences~~ *levels* ranging from 4 - 1.

3. Changes in blood BK in venous blood from the hand were studied during hyperaemic states in the hand produced by warming, cooling and ischaemia.

a. Increases of BK up to 53% in effluent blood from the hand occurred when the hand was warmed to 45°C.

b. BK levels fell in the effluent blood in association with reactive hyperaemia and cold vasodilatation.

4. An increase in sympathetic nervous activity caused a fall in arterial BK level of 20% in one case and virtually complete disappearance in another. Generalised sympathetic blockade with bethanidine brought about a 7.5 fold increase.

5. The ability of increased sympathetic nervous activity to lower blood BK level, may possibly contribute to the decreased venous BK levels found in cold vasodilatation and reactive hyperaemia where an increase in sympathetic nervous activity occurs as a result of the pain and discomfort associated with these procedures.

PART 4. THE EFFECT OF CATECHOLAMINES ON BRADYKININ METABOLISM IN BLOOD

a. INTRODUCTION

b. SPECIAL METHODS

c. RESULTS

1. Arterial blood BK levels during I.V. adrenaline.
2. Arterial blood kininase activity during I.V. adrenaline.
3. Blood kininase activity:
   (a) In vitro adrenaline.
   (b) In vitro phenoxybenzamine.
4. Kininase activity in normal arterial blood.

d. DISCUSSION

e. SUMMARY

INTRODUCTION

In addition to factors which influence the production of bradykinin in vivo, the peptide levels are also influenced by the activity of kininases. Erdos and Sloane, (1962) have characterised the kininase in human plasma as a carboxypeptidase which is a metalloenzyme that inactivates bradykinin by the hydrolysis of the peptide bond between the C-terminal arginine and phenylalamine in the peptide chain. Because of the activity of this enzyme, the half-life of BK in plasma is short and has been variously estimated as 0.3 - 0.4 min (Saameli & Eskes, 1962), 1 - 2 min (Bishop & Margolis, 1963), 0.27 $\pm$ 0.013 min (M^{c}Carthy, Potter & Nicolaides, 1965), and 17 sec (Ferreira, S.H., & Vane, J.R., 1966).

There is evidence that this enzyme plays an important part in terminating the action of BK in vivo, because when it is inhibited by such chelating agents as ethylenediamine tetraacetate and dimercaptopropanol, the hypotensive effect of the peptide is enhanced and prolonged (Erdos & ~~Sloane~~ Wohler, 1963). There is also evidence that kininase activity may be changed in some disease states in man; decreased in asthmatics (Sicuteri, Fanciulacci &

Anselmi, 1963), and increased in patients with hyperthyroidism and liver disease (Watanabe, Abe, Kumagai, Mouri, Seki & Yoshinaga, 1966), although other workers claim that it may be decreased in liver cirrhosis (Erdos, Wohler, Levine & Westerman, 1965). The latter discrepancy may be due to a measurement of a different carboxypeptidase (Watanabe et al. 1966). Serum of pregnant women, particularly in their last trimester, shows a high carboxypeptidase activity (Erdos et al. 1965).

The evidence for a role of catecholamines on BK metabolism until now, has suggested that adrenaline or noradrenaline cause an activation or release of kallikrein thus producing local or generalised increases in BK concentration.

1. The cat submaxillary gland is stimulated to secrete saliva by sympathetic stimulation or by infusion of noradrenaline and such secretion is associated with release of kinin-forming enzyme (Hilton & Lewis,1956).
2. In the carcinoid syndrome, the infusion of adrenaline may cause release of kallikrein from metastatic deposits (Oates et al., 1964).
3. Rocha e Silva (1963) suggests that catecholamines released during thermal or other local injury may activate proteases in interstitial spaces which in turn release BK from the precursor protein.

However, in contrast to this, it has been described in Part 3 of this thesis that there is a fall in arterial BK levels associated with increased sympathetic discharge, and when total sympathetic blockade is achieved with I.V. bethanidine a 7.5 fold increase in BK level in the arterial blood is observed, In Part 5 results will be presented that flushing produced in patients with the carcinoid syndrome by the adrenaline provocation test (Peart, Robertson & Andrews, 1959) is associated with an increase in peripheral arterial blood BK levels. However, when equivalent doses of adrenaline were given to normal subjects a constant lowering of arterial BK level occurred.

Rocha e Silva, Corrado and Ramos (1960) also made observations in experiments on cats which suggested that catecholamines may influence the activity of injected BK. Previous injection of phenoxybenzamine, apresoline, chlorpromazine and reserpine strongly delayed the recovery from hypotension provoked by a standard small dose of intravenous BK. Cocaine had the opposite effect of shortening the time of recovery. The intramuscular injection of cocaine to a cat, 30 min before an I.V. injection of BK produced a quicker recovery to normal levels, if this same cat was further treated with phenoxybenzamine a further dose of BK showed that the cocaine effect had not only been eliminated, but that there was again a strong potentiation of the

hypotension produced by bradykinin. It is now known that cocaine specifically prevents the uptake of noradrenaline by tissues, thus increasing the amount of noradrenaline available for combination with adrenergic receptors (Muscholl, 1961).

Graham and Katib (1966) have given evidence however, that the in-vitro potentiation of BK on smooth muscle by halogenoalkylamines (e.g. phenoxybenzamine) is due to an ethanolamine derived from the parent compound. The Ethanolamine is inactive against noradrenaline and on this basis the possibility that halogenoalkylamines inhibit a kininase through some adrenergic blocking mechanism seems to be dismissed by these workers.

Since there was preliminary evidence that catecholamines do influence circulating BK levels, more detailed investigations were undertaken to elucidate to which pharmacological action of catecholamines this effect on the kinin system could be attributed. It has been discussed previously (Part 3) that the level of BK found in blood at any time is probably the resultant of simultaneous processes of production and destruction (Fig. 2) so that a fall in BK level such as the one observed with adrenaline infusion could result from either a decrease in formation or an increase in destruction. It was decided to look at the latter possibility first, because it is difficult to devise a way to examine the possibility of decreased formation and the

observations of Rocha e Silva et al. (1960) hinted that the actions of catecholamines and their blocking agents might be to influence the survival of BK.

Changes in blood kininase activity were studied when adrenaline was infused I.V. and when adrenaline was added to whole blood in vitro. Evidence is presented that adrenaline accelerates BK breakdown and that the effect is blocked by the alpha-adrenergic blocking agent phenoxybenzamine. An appraisal is also made of the possible half-life of BK in normal whole blood.

METHODS

The first 3 subjects in whom the effect of I.V. adrenaline on arterial BK levels was examined were suspected to be suffering from the carcinoid syndrome. All were ambulant and engaged in their normal occupations, all had in common histories of sporadic flushing unrelated to any specific stimuli. They also shared some or all of such non specific symptoms as episodic weakness, sweating, vomiting and throbbing sensations in various parts of the body. All had some degree of emotional instability and in all the provisional diagnosis of the carcinoid syndrome was eventually dismissed (one had a laparotomy to finally exclude the diagnosis). The rest of the group were healthy medical students. The subjects for all other experiments described in this section were healthy medical students.

The methods for blood collection, BK assay, I.V. infusions, and blood pressure recording, have been described previously.

Drugs;

Adrenaline hydrochloride (D.H.A.)

Noradrenaline (Levophed; Winthrop)

Ascorbic acid (Baxter)

L - epinephrine bitartrate (crystalline (Sigma))

Phenoxybenzamine (Dibenylene; Smith Kline & French)

Solid adrenaline (L - epinephrine bitartrate) was used in in vitro experiments where sterility was not essential.

The method for estimating kininase activity during a seven second incubation has already been described (Part 3). In the present experiments the method was extended to study the degree of BK destruction over longer periods of time viz.multiples of 7 sec (or for convenience of 7.5 sec) i.e. over 15, 30, 45, and 60 sec, depending on individual experiment. When the mixture of blood and BK had to be incubated for longer than 15 sec, the syringe containing the mixture was returned to the water bath (38° C) and kept there until the set time had elapsed.

The dose of adrenaline infused was 0.1 μg/kg/min. Such a dose is usually just sufficient to produce slight tachycardia and widening of the pulse pressure. Adrenaline was diluted in saline (0.9% w/v) containing ascorbic acid (1 : 50,000).

When kininase activity was studied in the presence of adrenaline and phenoxybenzamine, the following reactants were placed in the 5 ml collecting syringe (in a volume of 0.1 ml saline and adjusted uniformly to pH 4.95) to give a final concentration

in 5 ml of blood of:

	BK 100ng/ml	Ascorbic acid 1 µg/ml	Adrenaline (base) 2.8×10^{-8} M 5 ng/ml or Noradrenaline (base) 4.6ng/ml (2.8×10^{-8} M)	Phenoxybenzamine (base) 50 ng/ml (1.6×10^{-7} M)
a	+	+		
b	+	+	+	
c	+	+	+	+

This enabled a comparison of kininase activity in blood when the only difference in the incubates was the presence or absence of catecholamine and the alpha-adrenergic blocking agent, phenoxybenzamine.

RESULTS

1. ARTERIAL BLOOD BK LEVELS DURING I.V. ADRENALINE

The effect of I.V. adrenaline on arterial blood BK levels was measured in six subjects. Prior to the blood collections the subjects were lying quietly on a couch and were receiving an I.V. infusion of saline (0.9% w/v) through a cannula in an ante cubital vein, at a rate of 2 ml/min. After a period of stabilisation of approx 30 min a control collection of arterial blood was made through an intra-arterial cannula in the brachial artery on the opposite side. Adrenaline was then substituted for the saline and infused at the same rate in a dose of 0.1 μg/kg/min. A collection of arterial blood was made again when haemodynamic responses to adrenaline occurred, usually during the third or fourth minute of the adrenaline infusion. The falls in arterial blood BK levels resulting from the adrenaline infusion are shown in Table 7. The mean fall of 61.4% was highly significant ($p < 0.001$).

TABLE 7

EFFECT OF ADRENALINE INFUSION ON BLOOD BK LEVEL

BK concentration ng/ml			% Fall
Pre-infusion	During infusion	Difference	
*0.18	0.13	0.05	28
*0.15	0.08	0.07	47
*0.25	0.03	0.22	88
0.03	0.01	0.02	67
0.07	0.03	0.04	57.1
0.16	0.03	0.13	81
	Mean ± SE	0.089 ± 0.0072	61.4
	P †	< 0.001	

† Paired t-test

* Denotes subjects who had vasomotor instability and in whom the diagnosis of carcinoid syndrome was excluded.

2. ARTERIAL BLOOD KININASE ACTIVITY DURING I.V. ADRENALINE

Arterial blood was collected before and during I.V. adrenaline infusions as described above, and kininase activity in whole blood estimated. Comparisons of per cent BK survival in blood under the two conditions were made on the basis of 7 sec incubation periods and results in three subjects are shown in Table 8. Increased kininase activity was demonstrated in all three cases with a mean fall of 20.8% in per cent survival of BK.

In two of the three subjects incubations of blood - BK mixtures were also continued for 1 min and 5 min and the per cent survival of BK estimated in an attempt to establish the velocity and characteristics of the enzymatic action involved. Exposure of BK to blood for 5 min showed that less than 1% of the amount added was present in the incubate. Such an amount probably represents a situation where the substrate had been exhausted at some indeterminate time before the 5 min. Since 60 sec incubations in these two experiments still showed approximately 25% survival, it was decided that the best appreciation of the time - course of BK destruction would come from incubation periods confined to times shorter than one minute. In a third subject

(R.S.) Fig. 24, a comparison of kininase activity was made before and during adrenaline infusion when survivals were estimated in incubates at 7, 15 and 30 seconds. The % survival vs log time plot for the control samples in this case extrapolates quite well through 100% and such a result would indicate that kininase activity in an adrenaline poor system approximates a first order reaction.

The presence of adrenaline which had been infused or which had been added <u>in vitro</u> to a mixture incubated in a closed system changes the survival - log time plot in such a manner that on extrapolation it shows a survival of considerably less than 100% at zero time. Per cent survival vs log time plots for 7 and 60 second incubates in the preceeding 2 subjects showed the same pattern as in the case of subject R.S. (Fig. 24). The possibility will be discussed later that the appearance of the plot of kininase activity when adrenaline is present in the system may be explained by feed back inhibition.

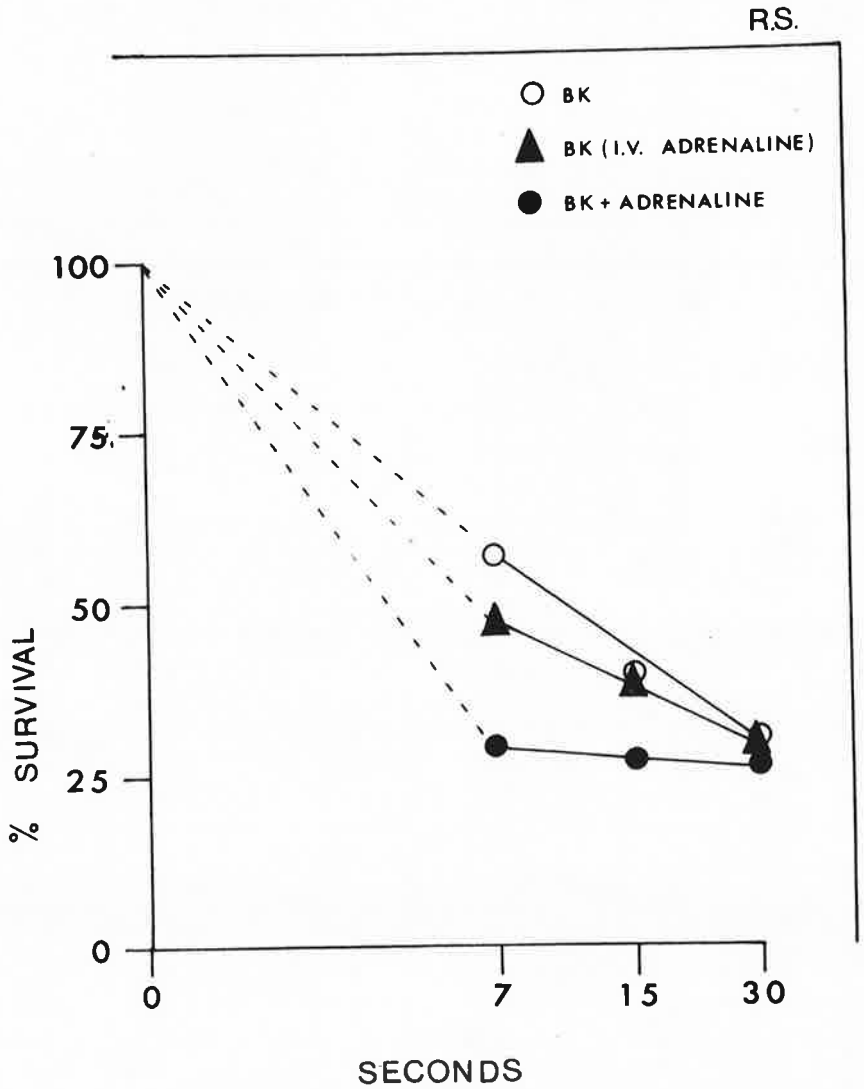
TABLE 8

PERCENT SURVIVAL OF BK AFTER 7 SEC. CONTACT WITH BLOOD

EFFECT OF ADRENALINE INFUSION

Pre-infusion (A)	During infusion (B)	Fall (A - B)	% Fall $\frac{A-B}{A} \times 100$
46.0	41.0	5.0	11
54.5	36.0	18.5	34
56.5	47.0	9.5	17
	Mean	11.0	20.8

Fig. 24. The time course of BK destruction in arterial blood in vitro. A mixture of BK and blood (100 ng/ml) was incubated for 7, 15 and 30 sec, the per cent BK remaining in the incubate was estimated by the usual method of extraction and assay, and expressed as % survival. The plot for BK destruction in the control incubates approximates an exponential time course, since it does not differ significantly from a straight line between 100% survival (datum point for zero time) and per cent survival at 30 sec. In the incubations of blood and BK when exogenous adrenaline was present, there is a marked deviation from a straight line, and the interrupted lines for these incubates indicate the minimum acceleration that may have occurred.



IN VITRO DESTRUCTION OF BK BY BLOOD

Fig. 24

3. BLOOD KININASE ACTIVITY: IN VITRO ADRENALINE, IN VITRO PHENOXYBENZAMINE

3 (a) IN VITRO ADRENALINE

The effect of adrenaline on kininase activity in vitro was studied on blood drawn from eight normal subjects. Paired incubations were made in each experiment, one incubate containing blood, BK and ascorbic acid, the other in addition, containing adrenaline. At least three incubations were performed in each experiment over different periods of time. Using zero time as datum point, four points can thus be obtained for the survival - time plot. The shortest incubation period which was uniformly possible in all subjects was seven seconds. The per cent survivals of BK after 7 sec contact with blood are shown in Table 9. In six out of the eight experiments decreased survival of BK was observed in the incubates which had added adrenaline; in two there was no change. Adrenaline caused a mean fall of 25.1% $\pm$ 6.99% in the per cent survival. Plots of BK survival against incubation time are presented for seven of the eight experiments (Figs 24 - 30). In four of the control incubations

TABLE 9

PERCENT SURVIVAL OF BK AFTER 7 SEC. CONTACT WITH BLOOD.

EFFECT OF 5 ng/ml ADRENALINE ADDED IN VITRO.

	Without adrenaline (A)	With adrenaline (B)	Difference (A-B)	% Difference $\frac{A-B}{A} \times 100$
J.H.	53.5	33.0	20.5	38.3
L.K.	26.0	26.0	0	0
R.H.	46.0	42.0	4.0	8.7
G.F.	61.0	43.0	18.0	29.5
B.K.	51.0	51.0	0	0
C.J.	64.0	37.0	27.0	42.2
A.B.	41.0	27.0	14.0	34.1
R.S.	56.5	29.5	27.0	47.8
		Mean $\pm$ SE	13.8 $\pm$ 3.98	25.1 $\pm$ 6.99
		p†	$.02 > p > .01$	$.02 > p > .01$

† Paired t-test

(Figs. 24, 25, 26, 29) the plot of survival against time showed an exponential appearance. The deviations in the plots of the other control incubations are uniformly in the same direction (except B.K., see below). The additional presence of adrenaline can produce such a deviation or exaggerate an existing one. It is seen that where adrenaline produces an acceleration of the destruction of BK, such acceleration is most evident with the shortest incubation times, and in most cases the difference has disappeared when the 30 sec incubates are compared.

In subject J.K. where adrenaline did not effect survival, at 7 sec already 74% of the BK added had been destroyed, the 15 and 30 sec incubates showed no decrease in % survival:

Subject L.K.

		7 sec	15 sec	30 sec	
% survival	Control	26	25	26	
	Adrenaline	26	25	25	

This equilibrium which is reached is seen in cases where the initial velocity of kininase is accelerated by adrenaline (subjects J.H., C.J., A.B., R.S., B.K., and to a lesser extent in G.F., and R.H.). In subjects R.H. and B.K. after a period of equil-

ibrium a nett destruction of BK is again evident. It is likely that such equilibrium which follows an accelerated phase of activity, presents an end-product inhibition of the enzyme. It is not due to a balance struck between exogenous BK destruction and endogenous BK production, because it can be shown that incubation of whole blood in nylon syringes at 38° C for a period of up to 5 min produces no recognisable amounts of BK in the incubate.

The results of experiments on subjects R.H., B.K., and C.J. need additional comment.

In subject R.H. the apparent ineffectiveness of adrenaline to accelerate BK destruction in the 7 sec incubate is probably due to an inadequate amount of ascorbic acid in the incubate. In this experiment a concentration of 1:100,000 was used as suggested by Gaddum et al. (1949), and such a concentration seems too small to prevent the oxidation of adrenaline in the stock solution. When ascorbic acid concentration of 1:1000 (1// g/ml) was used in subsequent experiments adequate preservation of adrenaline was evident.

Subject B.K. was studied on two separate occasions, on the first occasion (Fig. 28 a.) the plot of survival vs time was unusual in that it showed a lag in the onset of BK destruction, suggesting an inhibition of the destruction process; on a sub-

sequent occasion (Fig. 28 b) the control incubation produced a different plot which was identical to the one with adrenaline. It is likely that on this second occasion the enzyme had been fully activated by endogenous adrenaline as suggested by the ability of phenoxybenzamine to block this activation.

Subject C.J. (Fig. 26) was of special interest because on two previous occasions arterial blood BK levels had been 0.8 ng/ml and 0.95 ng/ml (Table 2), these levels presented the highest concentrations which had been encountered in normal subjects. The BK destruction rate in this subject was in accord with expectations: it can be seen from Fig. 26 that a 50% destruction was achieved only after 40 sec which represents the longest ½ life of BK in all subjects studied.

In subject J.H. (Fig. 25) the effect of equimolar concentrations of adrenaline and noradrenaline on kininase activity was compared. In the doses used (5 ng/ml adrenaline and 4.6 ng/ml noradrenaline) the two catecholamines produced an identical degree of acceleration.

The relationship between adrenaline concentration and kininase activity was not explored, but in subject R.S. (Fig. 24) the effect on kininase activity of an adrenaline infusion (100ng/kg/min) is compared with the enzyme activity when larger concen-

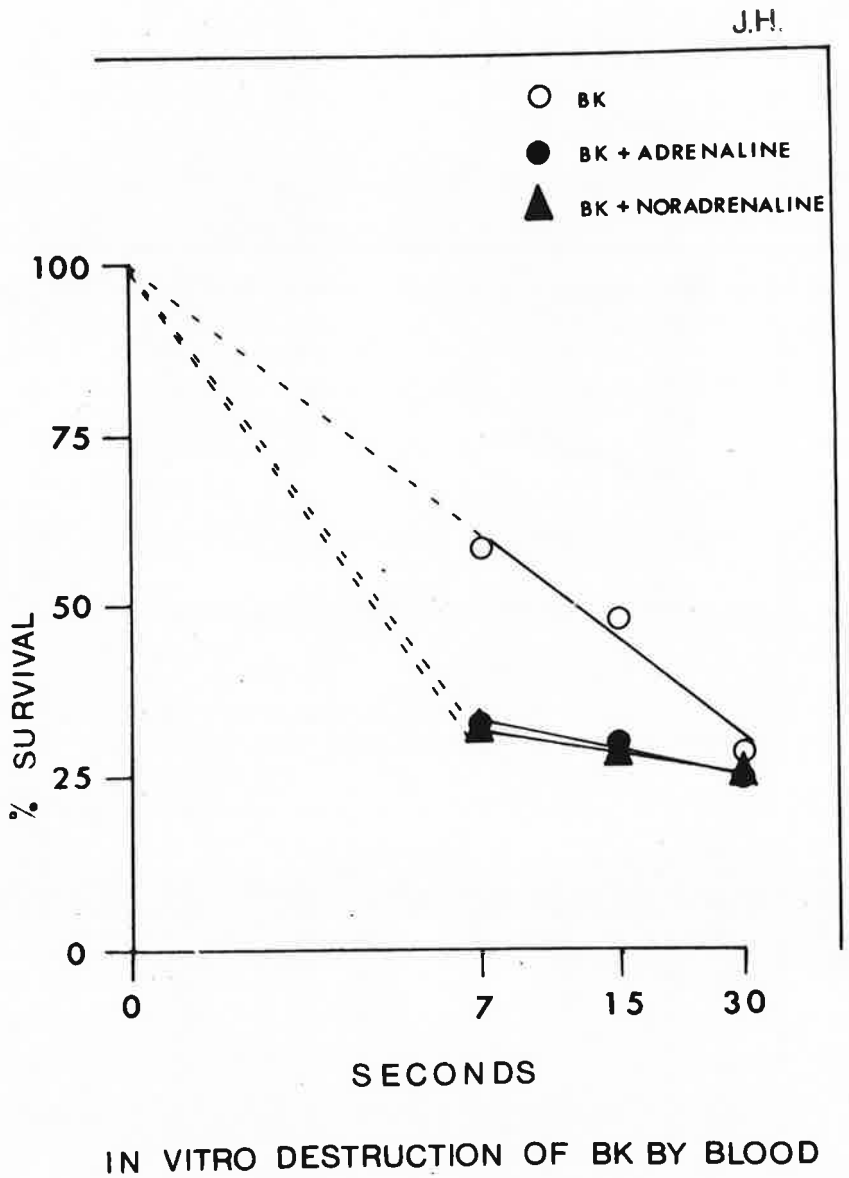


Fig. 25. The time course of BK destruction in arterial blood in vitro. (See also legend for Fig. 24). The BK in the control incubate undergoes an exponential decay. There is an equally marked acceleration produced by the presence of equi-Molar (2.8×10^{-8}) concentrations of adrenaline and noradrenaline.

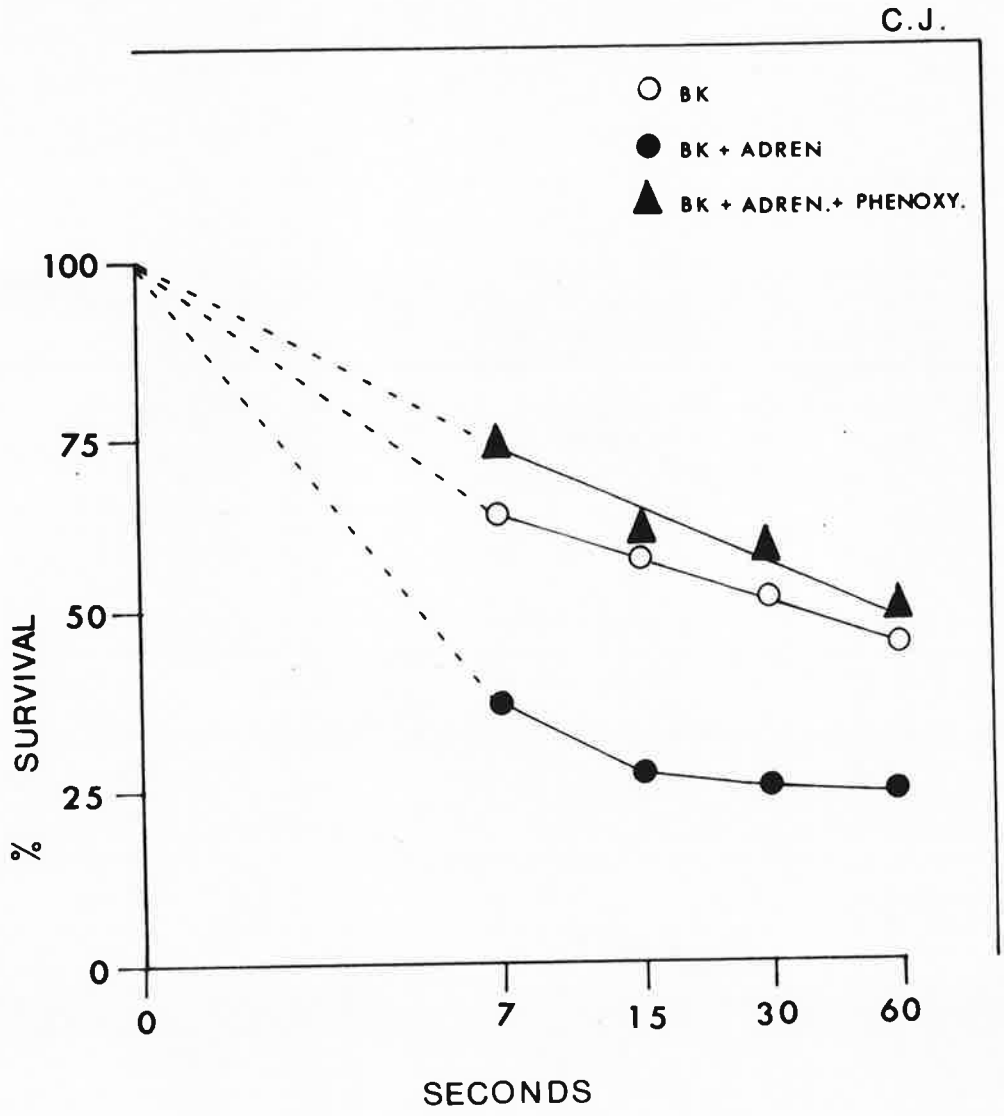


Fig. 26. The time course of BK destruction in arterial blood in vitro. (See also legend for Fig. 24). A marked acceleration occurred in the incubate which contained adrenaline. The additional presence of phenoxybenzamine, prevented the acceleration and slowed the destruction rate beyond that observed in the control incubate. The half life of BK in the control blood of this subject was longer (40 sec) than that found in blood of any other subject studied.

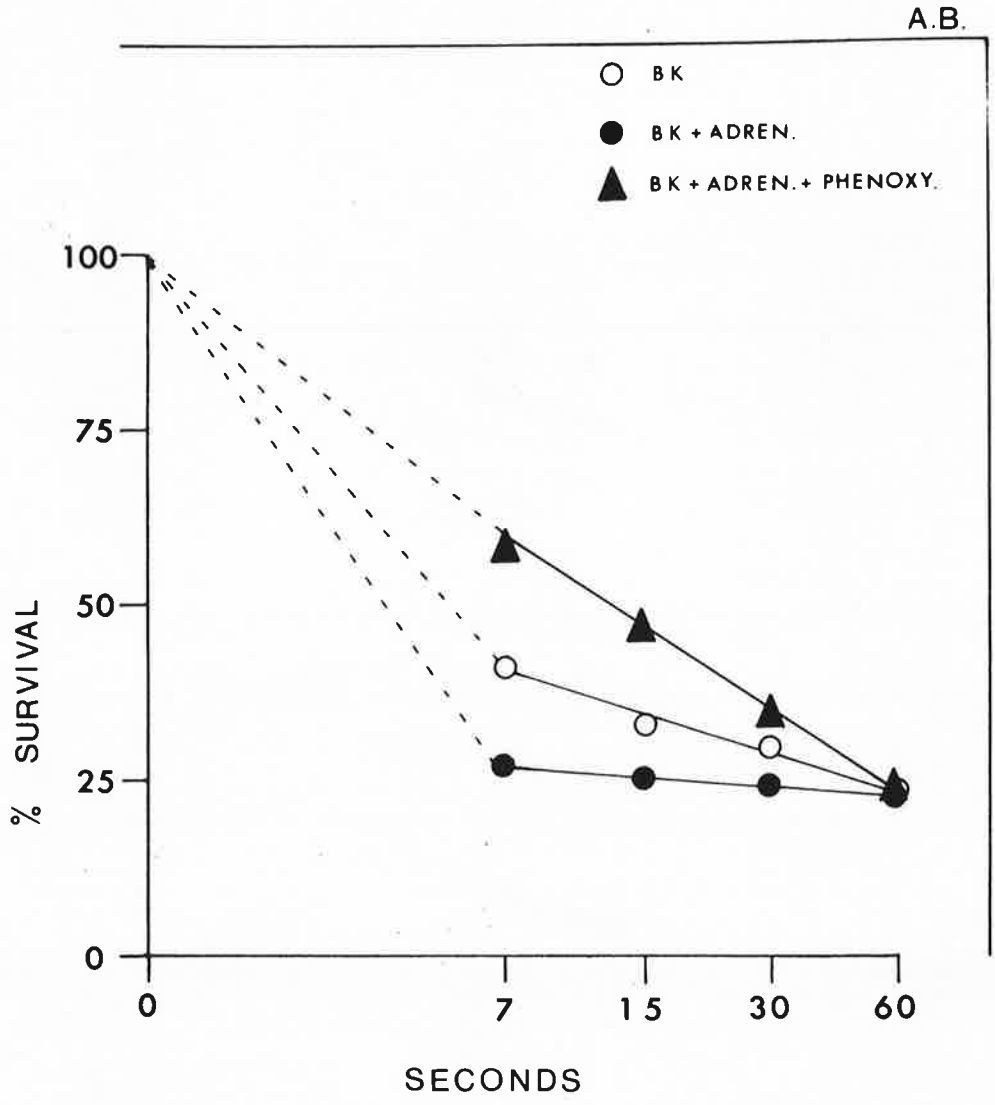


Fig. 27. The time course of BK destruction in arterial blood in vitro. (See also legend for Fig. 24). The plot from the control incubations deviates from an exponential course and the deviation is increased by the presence of exogenous adrenaline. The presence of phenoxybenzamine abolishes the acceleration and the deviation in the control incubate.

Fig. 28. In subject B.K. the kininase activity of arterial blood in vitro was studied on two occasions (two month interval).

(a). The survival-time plot shows an apparent delay in the onset of BK destruction. (See text for discussion).

(b) The control and adrenaline-containing incubates shows an acceleration which could be blocked with phenoxybenzamine. The survival-time plot for the incubations containing phenoxybenzamine is identical to the plot for (a).

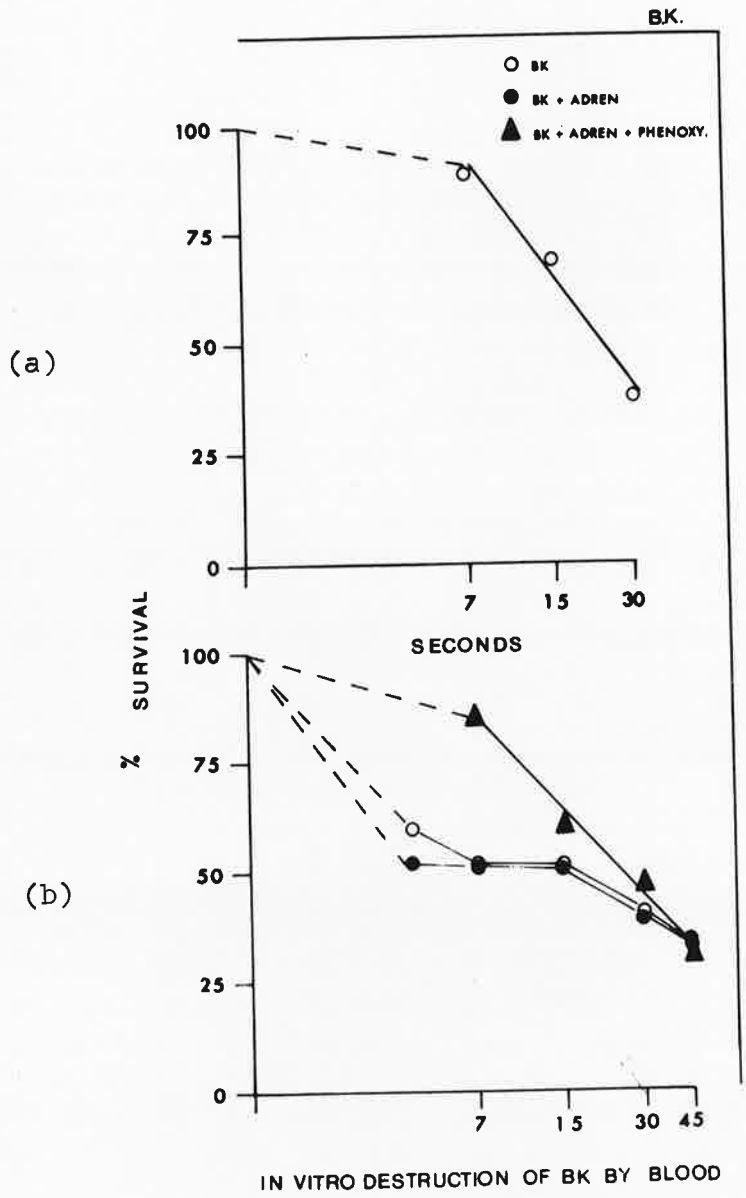

B.K.
BK
BK + ADREN
BK + ADREN + PHENOXY.
(a)
100
75
50
25
0
7
15
30
SECONDS
% SURVIVAL
(b)
100
75
50
25
0
7
15
30
45
IN VITRO DESTRUCTION OF BK BY BLOOD

Fig. 28

Fig. 29. Time course of BK destruction by arterial blood in vitro. (See also legend for Fig. 24). After an initial acceleration of kininase activity in the incubates containing adrenaline (7 and 15 sec), the destruction rate approximated that of the control incubations (30 and 45 sec).

Fig. 30. Time course of BK destruction by arterial blood in vitro. (See also legend for Fig. 24). The presence of adrenaline produced very little acceleration, the probable cause of the failure of adrenaline is discussed in the text.

Fig. 29

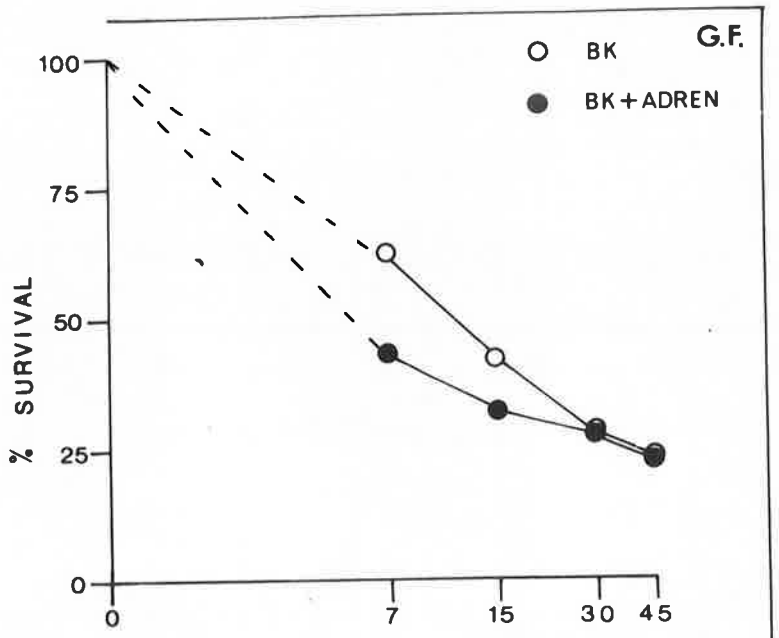
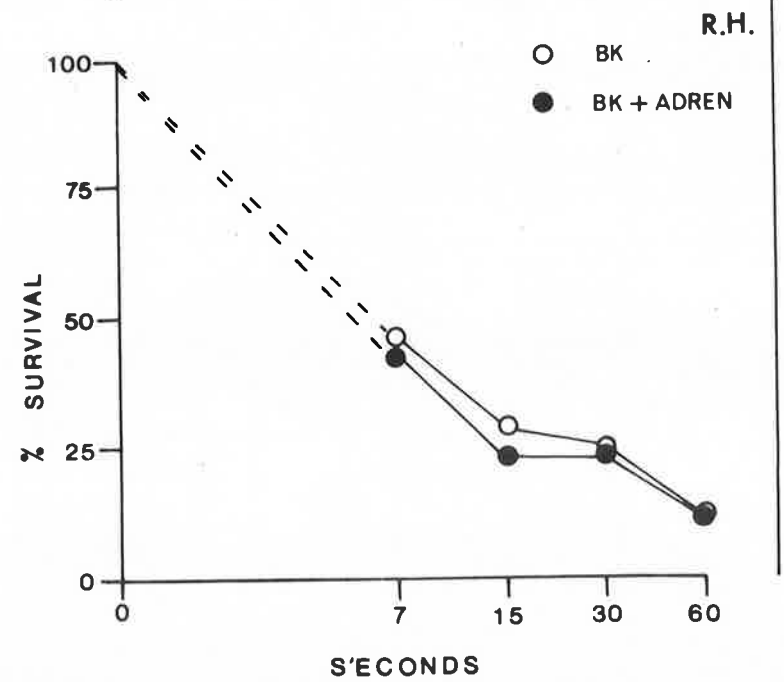

G.F.
BK
BK + ADREN
100
75
50
25
0
% SURVIVAL
0
7
15
30
45

Fig. 30


R.H.
BK
BK + ADREN
100
75
50
25
0
% SURVIVAL
0
7
15
30
60
S'ECONDS

trations were present when adrenaline (5 ng/ml) was added to blood in vitro.

3 (b) IN VITRO PHENOXYBENZAMINE

In three (B.K., C.J. & A.B.) of the eight subjects described, the effect of phenoxybenzamine on the adrenaline - induced acceleration was determined. The method was similar to that used to demonstrate the effect of adrenaline in vitro except that a third incubation was carried out in addition. This contained phenoxybenzamine 1.6×10^{-6} M in addition to the adrenaline, ascorbic acid and BK. The concentration of phenoxybenzamine was approximately 5 times the molarity of adrenaline. In subject B.K., no further acceleration was produced with adrenaline, but presence of phenoxybenzamine slowed the destruction rate and gave a survival time plot which was identical to the one which had been obtained on a previous occasion in this subject. The unusual appearance of the plot in this subject has been commented on.

In subjects A.B. and C.J., the phenoxybenzamine not only blocked the acceleration induced by exogenous adrenaline but also decreased the destruction rates beyond that found in the control

blood. The activity of kininase in incubates with phenoxybenzamine in these subjects was such that destruction rates followed an exponential course, with the semi-log plot extrapolating back through 100% survival at zero time.

The possibility that adrenaline was having a direct chemical effect on BK or that the presence of adrenaline in the extract (if any survived) was affecting the estimation of BK recovery on the tissues had to be considered and excluded. Two solutions of BK were prepared (pH 4.95), one contained in addition adrenaline in a ratio to the BK identical to that existing in the blood incubates. After the pH of each was adjusted to 6.0, both samples were chromatographed and assayed biologically on the rat uterus and duodenum. The recoveries obtained were:

93% (with adrenaline)

91% (without adrenaline)

In kininase activity experiments, the amount of adrenaline added to the incubate is such that the final extract when diluted for assay contains 0.1 - 0.2 ng/ml adrenaline, assuming that all that was added had survived. In the control experiment just mentioned it was shown that an assay solution containing that order of adrenaline concentration has no effect on the two tissues.

The possibility that phenoxybenzamine in the concentrations used was potentiating the action of BK on the tissues also had to be excluded. This was done by estimating the recovery of BK from a blood extract which contained phenoxybenzamine. The extract was obtained from a mixture of blood, ascorbic acid and phenoxybenzamine in the manner used for BK extraction from blood. An internal standard of BK was then added to this extract before assay and its recovery estimated. Recovery was 92%.

In this experiment and in the other assays of extracts containing phenoxybenzamine, the final concentration of the adrenergic blocker in the sample diluted for assay is of the order of 1.5-3.0 X 10^{-9} M. The potentiation and antagonism of the effects of BK on the isolated guinea pig vas by halogenoalkylamines and their derivatives occure in concentrations of 10^{-5} M (Graham & Katib, 1966); it is unlikely that phenoxybenzamine in the former concentration would have any direct effect on the tissue.

4. KININASE ACTIVITY IN NORMAL ARTERIAL BLOOD

The kininase activity exhibited by arterial blood from eight normal subjects in whom incubations were preformed for 7, 15 & 30 sec periods is shown in Fig. 31.

In three of the eight subjects already more than 50% of the substrate had been hydrolysed by the 7 sec period and in two, where the plots are very flat 50% destruction would have been achieved at a time very close to zero. It is probable that in cases like the latter, activation of kininase by endogenous catecholamines has been achieved. No estimation of mean half life of BK in blood can be made from these experiments, but from the subjects so far studied, the longest half life observed was in Subj. C.J. (40 sec), the shortest, as deduced from Fig. 31 is likely to be less than 3 sec.

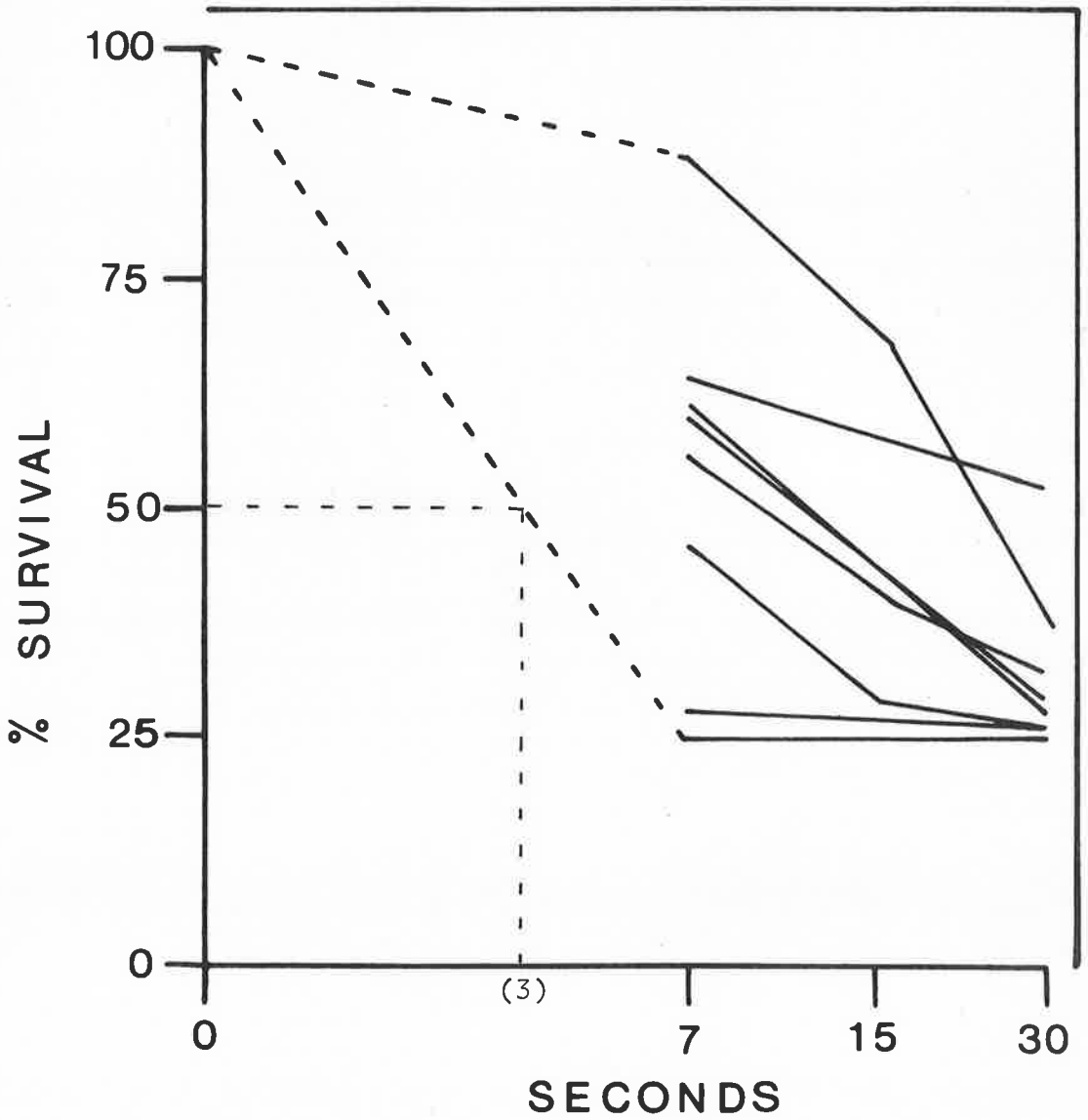


Fig. 31. The time course of BK destruction in arterial blood in vitro, in eight normal subjects.
BK was incubated in whole blood (100 ng/ml blood) for periods of 7, 15 and 30 sec.
A large variation in kininase activity is evident. The shortest half life of BK in whole blood is equal to and probably less than 3 sec. The longest half life of 40 sec was found in subject C.J. (see Fig. 26).

DISCUSSION

The in vitro experiments show that the decrease in BK levels in blood which follows adrenaline infusion is not due to circulatory or extravascular metabolic changes resulting from the infused catecholamine, but must be due to an action of the catecholamine on some determinants of kininase activity in blood. It is unlikely that the adrenaline infusions in the doses used produce changes in pH and electrolyte concentrations sufficient to effect kininase activity (Aarsen & Kemp, 1963). The effect of pH on differences in kininase activity in the incubates is excluded by the fact that the substrate for control, adrenaline and phenoxybenzamine studies was in solutions with pH uniformly at 4.95 and the 0.1 ml aliquot at pH 4.95 would change the pH of all incubation mixtures (5 ml) to the same degree. Red blood cells are also rich in a carboxypeptidase (Erdos, Renfrew, Sloane & Wohler, 1963) which might be liberated by environmental changes and thus contribute to the plasma kininase activity. However, Mashford (1967) in preliminary studies, using a modified method of Abe et al. (1965) for assay of kininase activity in blood, has shown that noradrenaline will accelerate kininase

activity in cell free plasma.

The manner in which catecholamines accelerate bradykinin hydrolysis is uncertain, but the fact that this acceleration can be blocked by a specific adrenergic blocking agent raises the intriguing possibility that the action of the catecholamine is by means of a specific "receptor" mechanism.

There are several documented examples of catecholamines modulating enzyme activity. Sutherland and Robison (1966) have discussed the action of catecholamines in relationship to cyclic 3' 5' -AMP. There is evidence that in a wide variety of tissues the formation or accumulation of 3' 5' - AMP is stimulated by catecholamines. The mononucleotide is formed from ATP by the enzymatic action of adenyl cyclase, which in turn is susceptible to activation by adrenaline. The activation of adenyl cyclase by adrenaline, and the resultant production of cyclic 3' 5' - AMP and active phosphorylase, might be the events linking adrenaline and its metabolic effects.

Tyrosine hydroxylase is a highly specific enzyme for converting tyrosine to dopa and this enzymatic action may represent a rate limiting step in the production of noradrenaline. One of the known inhibitors of tyrosine hydroxylase is noradrenaline itself and it is an interesting hypothesis that noradrenaline released as a result of sympathetic nervous activity may retard further

noradrenaline production by a mechanism of end product inhibition (Undenfriend, 1966).

The early observations of MacFarlane and Biggs (1946) that activation of fibrinolysin occurs as part of a general "alarm reaction" suggested a role for the sympathetic nervous system in the activation of a proteolytic enzyme. This possibility was further supported by Pohala, Yen and Singher (1962). The studies of Holemans (1965) however offer the possibility that the effect of catecholamines and other vasoactive drugs to enhance fibrinolysis is by the resultant readjustment of the peripheral circulation and the entry of plasminogen activator into the blood stream from the vessel walls of new circulatory beds.

Although the locality and structure of the adrenergic "receptor" in cells is unknown, the interaction between catecholamines and receptors is receiving greater attention at a molecular level. Properties of catechols which may be important are: a) their ready participation in redox reactions;

b) their ability to form chelates;

c) their ability to engage in hydrogen bonding and

d) their marked reactivity in the monoanionic form in acyl, alkyl and phosphoryl group tranfer reactions (Belleau, 1966). It is probable that the resultant transformations and ionic re-

distributions which result from catechol - "receptor" binding may be at the basis of their biological effects. It is tempting to speculate that similar mechanisms are involved in the adrenaline - carboxypeptidase reaction as in adrenaline - receptor interaction.

There are also several features of carboxypeptidase enzymes which are associated with enhanced or diminished activity and which may relate to its activation by catecholamines.

(a) The enzymatic activity of human carboxypeptidase N is dependant upon Co^{++}, since if the cobalt ion in the metalloenzyme is chelated by ethylenediamine tetraacetate its activity is inhibited (Erdos et al.,1963). Chelation of the catechol ring with magnesium has been proposed by Senoh, Tokuyama and Witkop (1962) as a mechanism in the methylation of catechols by catechol - o - methyl transferase and Belleau (1966) has extended this by suggesting that chelation may be a key factor in reaction between catechols and bioreceptors. It is possible that the catechol may chelate with Co^{++} in the carboxypeptidase and thus change the activity of the enzyme.

In addition there is evidence that the amino acid precursors of catecholamines are linked with carboxypeptidase action.

(b) When the aromatic aminoacid phenylalanine is linked to the carboxypeptidase derived from swine kidney, its enzymatic

activity is enhanced and it releases C-terminal arginine from the substrate at a faster rate (Erdos & Yang, 1966).

(c) Another aromatic amino acid, tyrosine,is at the active centre of bovine pancreatic carboxypeptidase B and is essential for activity since alkylation of the tyrosine residue decreases the enzymatic activity by 90% (Plummer & Lawson, 1966).

A study of the plots of % survival of BK against log time in a mixture of whole blood shows that adrenaline and noradrenaline induced acceleration is most evident at the shortest incubation period (7 sec) and in most cases it seems that the degree of destruction seen at this time had probably been achieved much earlier on , but with the present method, it is impossible to study events at times less than 7 sec and it is likely that the difference in destruction rates manifest at 7 sec period is an underestimation of the degree of acceleration that actually occurrs.

If the initial activity has been rapid as seen in most cases with in vitro adrenaline and in some control incubates, an "equilibrium" period is usually evident at about 75% destruction of BK. In a closed system, where the end products of the reaction are confined to environment of the reaction, this equilibrium is most likely to be explained in terms of feedback inhibition (Dixon & Webb, 1964). This possibility is supported by

the observation of Erdos (1962) that arginine is a potent inhibitor of the enzyme hydrolysis of bradykinin, showing the same relative degree of inhibition as the potent chelating agent ethylenediamine tetraacetate exhibits in equimolar concentrations (3×10^{-3}). Since arginine is the C-terminal amino acid which is released on BK hydrolysis, a relatively high concentration of this amino acid would be rapidly achieved in the incubation mixture.

Phenoxybenzamine is closely related chemically to the nitrogen mustards and in large doses may be toxicto the tissues, however, the toxicity of the pharmacological blocking drugs is approximately 1,000 times less than that of the nitrogen mustards and pharmacological doses are safely separated from toxic doses (Nickerson, 1949). The specificity of the adrenergic blocking action of phenoxybenzamine is greater than that of the other classes of adrenergic blocking drugs, but in common with other halogenoalkylamines, phenoxybenzamine has slight antihistaminic activity (Nickerson & Harris, 1949), otherwise it is ineffective against other pharmacological agents on smooth muscle (Nickerson,1949). The pharmacplogical behaviour of phenoxybenzamine is probably due to an irreversible alkylation of an alpha receptor component accomplished by the pharmacologically active ethyleniminium ion derived from the halogenoalkylamine (Triggle, 1965). Such an

alpha receptor component may be a sulphhydryl, amino or carboxyl group (Harvey & Nickerson, 1954), but because of the limited knowledge of the carboxypeptidase molecule no further speculation of the site or nature of bonding is justified.

Although the studies presented here can not give a precise concept of mechanisms involved, the implications are interesting and of potential importance. The link of kinin metabolism with catecholamines and the sympathetic nervous system imposes a regulatory mechanism on the plasma kinin activity and adds credence to many speculations regarding the plasma kinins as factors in the regulation of peripheral or regional circulation. There is also indirect evidence that the activation of kininase demonstrated in the blood may also occur in the tissues, for Jacobsen and Waaler (1966) have shown that lymph from injured areas in animals has increased kininase activity.

Further reciprocity exists between bradykinin and the sympathetic nervous system and has been well documented. Bradykinin releases adrenaline from the adrenal medulla. Such a mechanism is potentially of physiological significance, for Feldberg and Lewis (1964) estimate that one molecule of bradykinin may release up to 50 molecules of catecholamines. These studies have been extended by Lewis and Reit (1966) to show that BK may also stimulate sympathetic ganglia. Benetato, Haulica, Muscalu,

Bubuianu and Gelesanu (1964) in cross circulation studies in dogs, have shown that BK also stimulates the sympathetic nervous system through a central mechanism.

Cognizance of the many points of interaction between the hypotensive plasma kinins and the sympathetic nervous system may offer a more comprehensive understanding of the observed pharmacological effects of BK on the cardiovascular system. The bradykinin released catecholamines may thus contribute to the poorly sustained circulatory responses to BK infusions (Bishop, Harris & Segel, 1965; Coffman & Javett, 1963) by promoting a more rapid destruction of the administered BK. It may also help to explain the decreasing venous blood BK levels in the forearm during the infusion of BK into the brachial artery as described by Allwood and Lewis (1964).

E. SUMMARY

1. I.V. infusions of adrenaline in normal subjects in doses which produce threshold pharmacological effects, cause a fall in arterial BK levels. In 6 subjects a mean fall of 61.4% was observed. The fall produced by I.V. adrenaline is highly significant ($p < 0.001$).

2. Kininase activity of whole blood was estimated by determining the amount of BK destroyed on incubation of blood and synthetic BK for a set period of time.

3. During I.V. adrenaline infusion, arterial blood increased its ability to destroy BK by 20.8%, when comparisons were made from 7 sec incubation periods.

4. In paired incubates, the addition of adrenaline to blood in vitro in amounts of 5 ng/ml of blood, increased hydrolysis of BK by 25.1% $\pm$ 6.99%.

5. The additional presence of adrenergic blocking agent phenoxybenzamine in the incubate is able to block the exogenous adrenaline, and probably also blocks the acceleration existing from endogenous catecholamines.

6. The half-life of BK in arterial blood in man is very short and variable. The lower limits can not be calculated with the present method. The range encountered in these experiments is:

$$3 \text{ sec} \geqslant \text{BK } t_{1/2} < 40 \text{ sec}$$

7. Catecholamine modulation of enzymatic activity is known in the case of adenyl cyclase and tyrosine hydroxylase, the modulation of carboxypeptidase N can only be speculated on in terms of the known properties of the catechols. The blocking effect of phenoxybenzamine may be due to an irreversible alkylation of a "receptor" component such as a sulphhydryl, amino or carboxyl group.

8. The ability of catecholamines to accelerate the hydrolysis of BK by kininase, places kinin metabolism under a potent general controlling mechanism.

Part 5 — THE CARCINOID SYNDROME
ADVANCED LIVER CIRRHOSIS
MENOPAUSAL FLUSHING

a. INTRODUCTION

b. SPECIAL METHODS & SUBJECTS

c. RESULTS

d. DISCUSSION

e. SUMMARY

a. INTRODUCTION

(A). The carcinoid syndrome is a clinical entity with interesting biochemical and pharmacological implications.

The cause of the syndrome is a neoplastic tumour classically arising from the small intestine. Since numerous cases of carcinoid tumours arising from sites outside the small intestine have also been reported, Williams and Sandler (1963) have proposed a more unified concept of tumour genesis by suggesting that these tumours can arise from different embryonic divisions of the gut. In this way, tumours originating in extra-intestinal sites such as bile ducts, pancreas, ovaries, thyroid and bronchi may be considered to have a common embryonic cell origin.

The tumour and its metastases usually exhibit only low grade malignancy, hence the term "carcinoid" (Oberndorfer, 1907), and the tumour in the disseminated stage may allow survival for as long as 20 years (Mengel, 1966).

The tumour became associated with a clinical syndrome following the observations of Thorson, Biorck, Bjorkman and Waldenstrom, (1954). Large quantities of serotonin are found in the tumour tissue (Lembeck, 1953) and in peripheral blood (Pernow & Waldenstrom, 1954) in the carcinoid syndrome with excess of the

serotonin metabolite 5 - hydroxyindole acetic acid (5 H.I.A.A.) in the urine (Page, Corcoran, Undenfriend, Sjoerdsma & Weissbach, 1955). After the demonstration by Page and McCubbin (1953) that intravenous injections of serotonin in man could produce a flush, it seemed likely that the spontaneous flushes in the carcinoid syndrome were produced by this agent. However, recently evidence has accrued that the carcinoid syndrome is more complicated chemically than was initially thought. Although the implication of increased production of serotonin in the syndrome is well established, the unsuitability of this amine to explain the vascular manifestations has been remarked by many authors (Robertson et al., 1962; Sjoerdsma & Melmon, 1964), and interest has recently been taken in the possible role of the plasma kinins as mediators of the carcinoid flush.

Oates et al. (1964) were able to detect kallikrein in the metastases in patients with the syndrome; and the hepatic venous blood during flushing contained large amounts of a peptide indistinguishable from bradykinin. Oates et al. (1966) extended these observations by identifying the kinin in the hepatic venous blood as the nonapeptide, bradykinin. Since intravenous injections of BK can mimic the flushing and other cardiovascular events of spontaneous and adrenaline induced flushes (Oates et al., 1964), this peptide is eminently suited to play an import-

ant role in the manifestations of the syndrome.

The role of serotonin in flushes cannot be entirely discarded since Peart, Andrews and Robertson (1961) have demonstrated release of serotonin during flushing in one patient and Melmon, Sjoerdsma and Mason (1965) have shown that an increase of urinary 5 - H.I.A.A. may occur associated with flushing. Other substances may also be liberated from the tumour and contribute to the final picture, and Hallwright, North and Reid (1964) have reported finding adrenocorticotrophic and melanocyte - stimulating activity in extract of a pancreatic carcinoid tumour from a patient who had Cushing's syndrome and abnormal pigmentation.

Following from the observations of Oates et al. (1964) that bradykinin appeared in the hepatic vein associated with flushing, it became necessary to be able to demonstrate that the change in BK levels which occurs in the hepatic vein extends also into the peripheral circulation, if these central events were to be causally related with the changes occurring peripherally. During the years 1964 - 1966 three patients with the carcinoid syndrome became available for study and this section describes the elevation in peripheral arterial blood BK levels which occurred in these patients during flushing.

Recently others have reported changes in peripheral blood kinin levels during carcinoid flushes. Mason and Melmon (1966)

have found elevations of peripheral arterial blood concentrations of bradykinin peptide and kallikrein during flushes. Zeitlin and Smith (1966) studied kinin levels in forearm venous blood in four patients with the carcinoid syndrome,in one of these they were able to show a rise in kinin level occurring during a flush induced by 10 μg of I.V. adrenaline and associated with this they detected falling levels of kinin precursor; in another subject no change could be detected in a lower kinin level. In a further two patients free kinin levels in venous blood are stated to have been elevated above (unspecified) normal levels during adrenaline provocation. The fall in kininogen level observed in the one patient during flushing is interesting, but a closer inspection of their data reveals that there is not the expected correlation between severity of disease, free kinin levels and kinin precursor, unless one postulates a greatly accelerated kininogen production in the severely flushed patient, for indeed this patient shows the highest plasma kininogen level. Full appreciation of their findings is hampered by lack of detail of their procedures of blood collection, the method of extraction and assay, particulars about blood BK levels in normal subjects as found by their method, and the effects on these levels from I.V. adrenaline. Mason and Melmon (1966) do not comment on the nature of BK levels in arterial blood in normal subjects either, but state that such levels are not significantly altered

by adrenaline administration.

The data on carcinoid syndrome patients presented here is of interest because the kinin levels can be evaluated in terms of normal arterial levels and the rise produced by adrenaline provocation is contrasted with the marked fall that occurs in normal subjects (Part 4). It is shown that I.V. BK can mimic the changes produced by I.V. adrenaline and in one patient data is also presented showing the effect of I.V. and oral methysergide on the intestinal and vascular manifestations of the syndrome. Methysergide is one of the most potent antiserotonin substances known (Doepfner & Cerletti, 1958) and its value in the management of the intestinal symptoms of the carcinoid syndrome well documented (Mengel, 1965, Melmon, Sjoerdsma, Oates & Laster, 1965).

(B) Arterial blood BK levels were examined in three patients hospitalised for advanced cirrhosis of the liver. This study arose from Dr. M.L. Mashford's interest in the cause of the hyperdynamic circulatory state that sometimes forms part of the picture of advanced cirrhosis, where a high-output state may persist into terminal hypotension and oliguria (Hecker & Sherlock, 1956; Mashford, Mahon & Chalmers, 1962).

The overall hyperdynamic cardiovascular manifestations in

cirrhosis have been described by Kowalski and Abelman (1953) and include warm extremities cutaneous vascular spiders, wide pulse pressure and capillary pulsations in the nail beds; the resting cardiac output may be elevated and associated with a large stroke volume and low peripheral vascular resistance. These workers raised the possibility of a circulating vasodilator material (VDM) involved in the mechanism producing peripheral vasodilatation. More recently Kontos, Shapiro, Mauck and Patterson (1964) have found that cirrhotic patients who have an increased cardiac index also have abnormal vasodilatation in the upper limbs either confined to skin or muscle or present in both vascular beds. They raise the possibility that bradykinin or a related vasodilating polypeptide could be a possible candidate for such a mechanism.

(C) The association of bradykinin with flushing in the carcinoid syndrome suggested the possibility that many other, more common flushing states may possibly be mediated this way. In menopause, the most prominent symptom is the hot flush, which is more prone to occur during exitement, emotional disturbances, eating, exercise, in warm weather and at night. Flushes may last from a few seconds to several minutes and are characterised by a gradually increasing redness of the skin, particularly over the

face and neck, which is followed by sweating. The cause is unknown, but the symptoms have been described as being due to vasomotor instability and treated with oestrogen replacement, sedatives and tranquilisers with moderate success (Hazan & Conneely, 1964). No specific stimulus that will induce a menopausal flush is known, but Robertson et al. (1962) have remarked that intravenous injections of adrenaline and noradrenaline, in doses that provoke a carcinoid flush, will not precipitate a flush in these women. Three women were selected for study because of the frequency of their flushing attacks --- several every day, but only one exhibited a typical spontaneous flush under laboratory conditions. Arterial blood BK levels were estimated in all women and in one comparison between levels during control and flushing states was made.

b. SPECIAL METHODS AND SUBJECTS

(a) METHODS & MATERIALS

Methods of blood collection and BK assay have been described (Part 2). Method for blood pressure recording -- see Part 3.

Skin temperature on the forehead was registered by a copper-constantan thermocouple and Elektrolaboratoriet galvanometer; temperatures were recorded manually at intervals of ½ or 1 min.

Cardiac output (CO) was estimated by single - injection dye dilution method (Hamilton, 1962), with tricarbocyanine green into a forearm vein. Arterial blood was sampled from the contralateral brachial artery by a mechanically driven withdrawal pump and output curves were obtained using a cuvette densitometer (Waters XC - 50 B) and an Offner Dynagraph (Model RS). Output curves were corrected for recirculation using the method of Gorten and Hughes (1964).

Total peripheral resistance (TPR), was calculated from the formula

$$TPR\ (\text{dyne} - \text{sec} - \text{cm}^{-5}) = 80 \times MAP\ \text{mm Hg} / CO\ (\text{l per min})$$

MAP = Mean arterial pressure = diastolic blood pressure plus 1/3 pulse pressure.

Methysergide dimaleate (Deseril, Sandoz).

Tricarbocyanine green (Cardio Green, Hynson, Westcott & Dunning Inc. Baltimore Md. U.S.A.)

5-Hydroxytryptamine (Serotonin-Kreatininsulfat SR 134, Sandoz).

(b) SUBJECTS

(A) Carcinoid syndrome

Patient 1. (E.B.) --- female aet. 69, presented with diarrhoea, weight loss and flushing attacks. Urinary 5-H.I.A.A. excretions were 165 - 600 mg in 24 hrs (N 10 mg/24 hrs). She deteriorated rapidly and died 12 months after she was first seen.. Autopsy revealed a small bowel carcinoid, with hepatic metastases.

Patient 2. (L.C.) --- female aet. 71, had undergone excision of an ileal tumour in 1953, which was histologically identified as a carcinoid. In 1962 she began to have diarrhoea and flushing attacks. In 1965 5-H.I.A.A. excretion was found to be 60 - 106 mg in 24 hrs. She has continued to progress slowly downhill.

Patient 3 (L.H.)--- male aet. 35, presented with weight loss, mental depression and continuous flushing and was found to have

tricuspid stenosis and incompetence. Urinary 5-H.I.A.A. excretion was 320 mg per 24 hrs. His deterioration was rapid and autopsy revealed widespread metastases from a small bowel carcinoid tumour. He was continuously flushed but noted a hot feeling in his face and trunk when his liver was compressed by twisting or bending movements.

Adrenaline infusions were effective in provoking a flush in E.B. and L.C. and arterial blood was taken before infusion and during the flush. L.H. was not given adrenaline, but blood samples were taken at rest in bed and while pressure was applied over the liver causing a subjective feeling of heat but no obvious change in his flushed appearance.

(B) Cirrhosis

Three patients who were hospitalised because of the severity of their disease were studied. One of these (I) had macroglobulinaemia but no clinical evidence of hyperdynamic circulation; another (II) was in an obvious hyperdynamic circulatory state and consequent hepato-renal failure, cirrhosis had been confirmed by liver biopsy. The third patient (III) was in a terminal state exhibiting jaundice, bounding pulses, warm extremities, flushed "liver" palms, was hypotensive and oliguric. The

diagnosis of cirrhosis of the liver was confirmed at necropsy.

(C) Menopausal

Three women were studied in four experiments. All had developed menopausal flushing in the last twelve months. Menopause was of natural onset in two D.R. aet. 47 and K.C. aet. 51. In the third H.M. aet. 45, menopause was a consequence of hysterectomy and bilateral oophorectomy for large fibroids. All three had sought medical attention for relief of hot flushes which recurred several times daily.

On the day of the experiment, the subject was made comfortable on a couch with an indwelling intra-arterial catheter in the brachial artery. Control arterial blood was collected and the subject was observed for up to 4 hrs with readiness for recording changes of blood pressure and forehead skin temperature. During this period endeavours were made to provoke flushing by heating the room and employing other non specific stimuli which from the individual personal history seemed likely precipitants. These latter included hot drinks, cigarette smoking, sucking or eating particular foods and interrogation touching on seemingly sensitive aspects of personal or family history.

All the subjects were aware of the purpose of the experiments and the attempted provocation. In four such experiments, spontaneous flushing occurred twice in succession in one subject. Blood pressure, skin temperature over the forehead area and arterial blood BK levels were measured before, during and after the event.

c. RESULTS

(A) Carcinoid

In L.C. the resting BK level of 0.15 ng/ml in arterial blood was very close to the average normal for the method used, but in the other two subjects resting concentrations were elevated. In L.H., who flushed continuously, the arterial level was 1.1 ng/ml which is higher than has been found in any normal subject. However, the level of 10 ng/ml in E.B. was by far the highest that has been found with this method, despite the absence of flushing at the time of sampling.

Both patients (L.C. and E.B.) who received an adrenaline infusion began to flush within two minutes and samples were taken at the height of the response. L.C. received adrenaline infusion at two dose levels, 1 μg/min and 2 μg/min. Both produced a flush, fall in B.P. and tachycardia. The higher dose of adrenaline caused more marked cardiovascular changes. The smaller dose caused a doubling of the arterial blood BK level from 0.15 to 0.31 ng/ml; the BK level with the higher dose, however, was lower (0.27 ng/ml) than would have been expected. The BK level

measured during pressure over the liver, ten minutes after the adrenaline infusion was back to 0.16 ng/ml. These events in subject L.C. are recorded in Fig. 32. The facial flush produced in this patient by a dose of 2 μg/min I.V. adrenaline is shown in Fig. 33.

In E.B. there was a 50% increase in arterial BK from 10.0 to 15.0 ng/ml during the adrenaline induced flush (dose 0.5 μg/min). The fall in B.P. and tachycardia from such a dose are shown in Fig. 34. In this patient it was further possible to demonstrate the failure of I.V. methysergide pretreatment to modify the adrenaline induced flush or B.P. response (Fig. 35).

Although methysergide was effective in blocking the abdominal symptoms which occurred during serotonin infusion (Fig. 36), serotonin in the dose used caused no flushing or change in B.P. In this patient it was also shown that an I.V. infusion of BK in a dose of μ16 g/min (Fig. 37) could produce a fall in blood pressure, a rise in heart rate and a flush which was subjectively and objectively identical to the one produced by I.V. adrenaline.

The effect of oral methysergide up to 18 mg daily over the hospitalisation period was to produce a marked reduction in bowel frequency from 12 - 15 per day to 3 - 5 per day. Con-

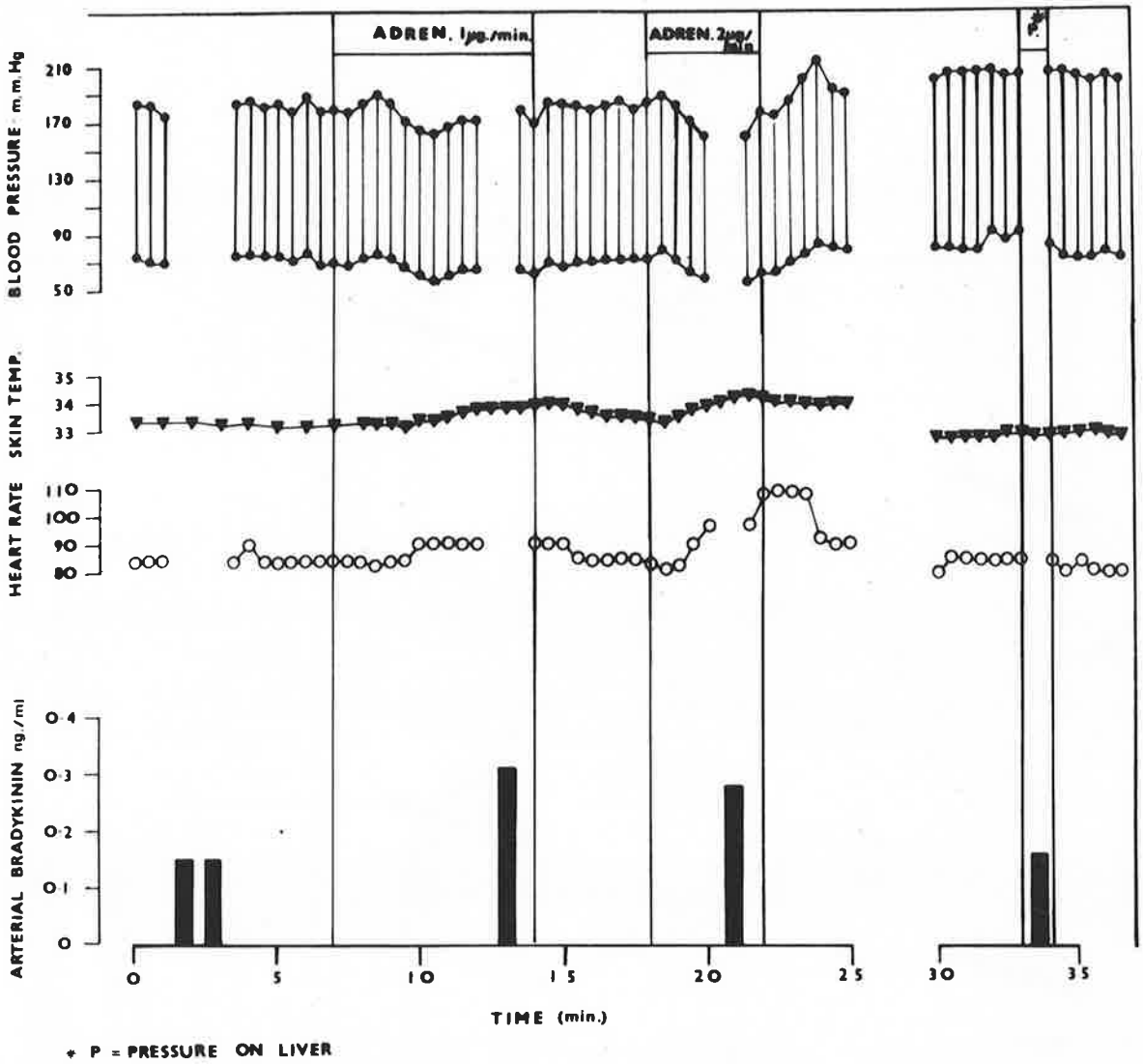


Fig. 32. CARCINOID SYNDROME --- subj. L.C.

Changes in BP, skin temperature, heart rate and arterial blood BK levels occurring during two adrenaline induced flushes are shown. Adrenaline in doses of 1 µg/min and 2 µg/min was infused into an antecubital vein, arterial blood was sampled from the contralateral brachial artery at the peak of the flush. Duplicate estimations of control arterial BK levels were 0.15, 0.15 ng/ml. A rise to 0.31 ng/ml occurred with the smaller dose of adrenaline and a level of 0.27 ng/ml was obtained with the larger dose (see DISCUSSION). Manual pressure over the liver produced no cardiovascular changes and the BK level was 0.16 ng/ml

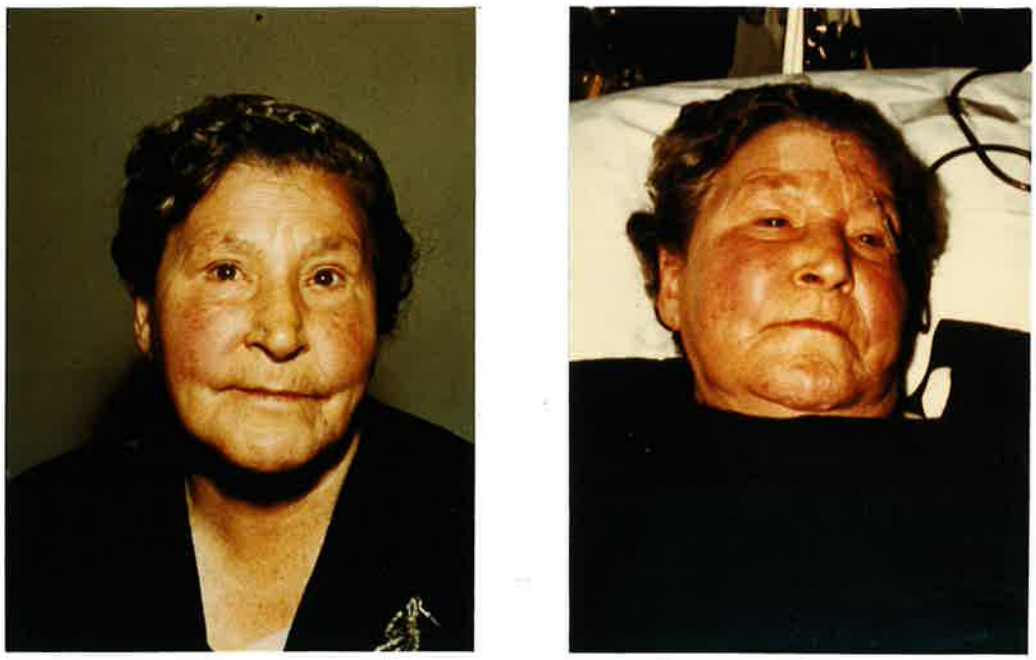


Fig. 33. CARCINOID SYNDROME --- subj. L.C.

The flushing state was produced by an I.V. infusion of adrenaline at the rate of 2 μg/min. A typical deep red facial flush and conjunctival suffusion are evident. (Patient was not icteric).

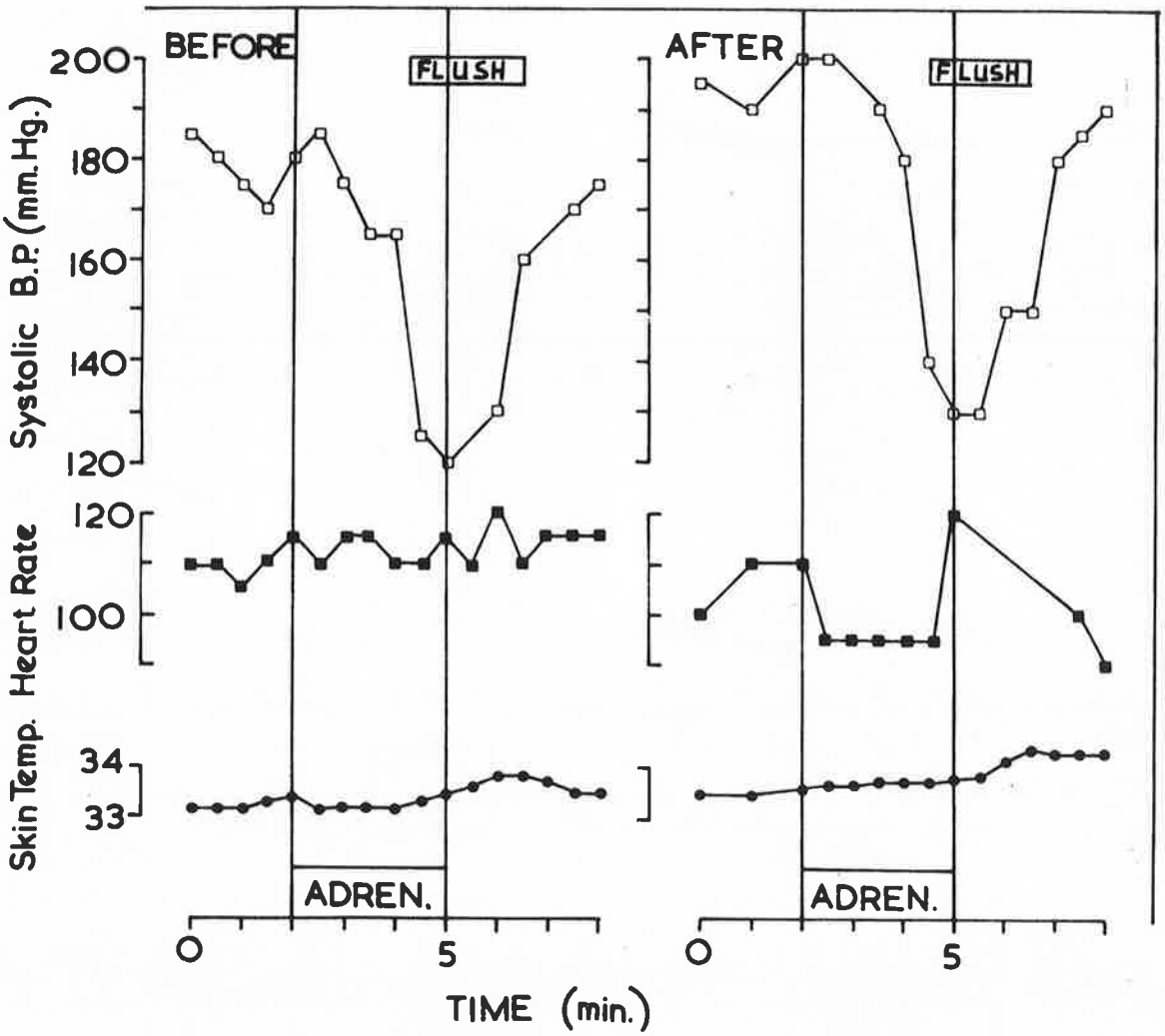


Fig. 35 CARCINOID SYNDROME --- subj. E.B.

A facial flush, fall in BP and a slight rise in heart rate were produced by I.V. adrenaline (0.5 μg/ml) (BEFORE) The same response could be elicited after Deseril pre-treatment (1 mg I.V.) (1 mg in 50 ml saline infused at 4 ml/min) (AFTER)

This dose of Deseril was effective in blocking the intestinal symptoms of I.V. serotonin (see Fig. 36).

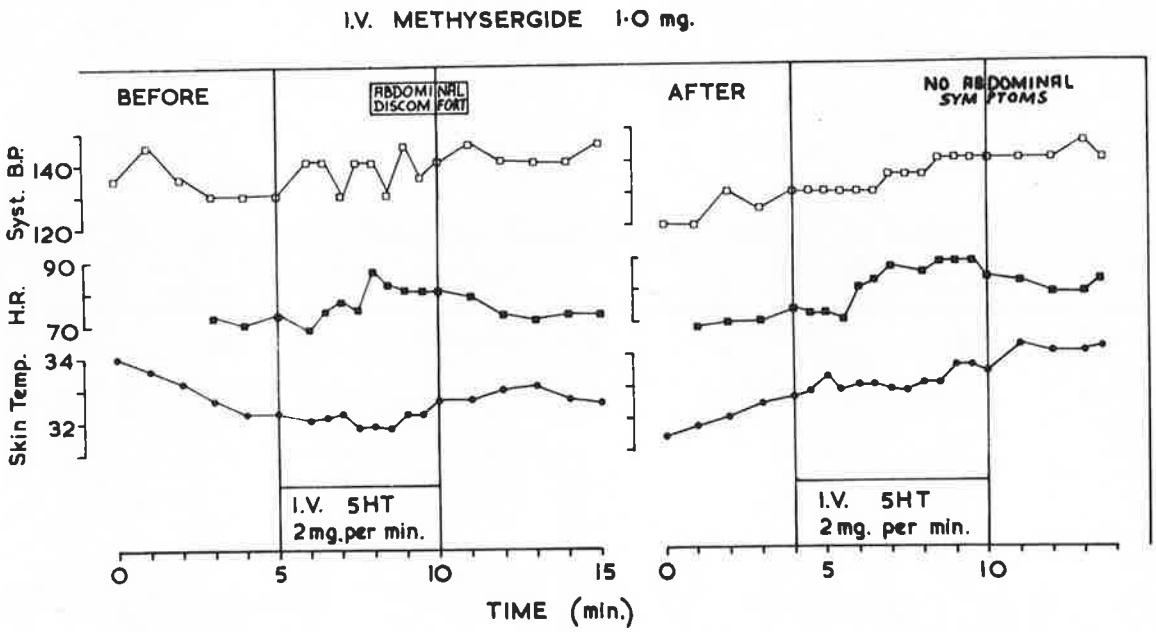


Fig. 36. CARCINOID SYNDROME --- subj. E.B.

Intravenous serotonin produced mild to moderate abdominal pain and a moderate increase in heart rate, but no flush. (BEFORE) Pretreatment with I.V. Deseril (methysergide) (see legend Fig. 35) effectively blocked the abdominal symptoms (tachycardia not blocked) (AFTER)

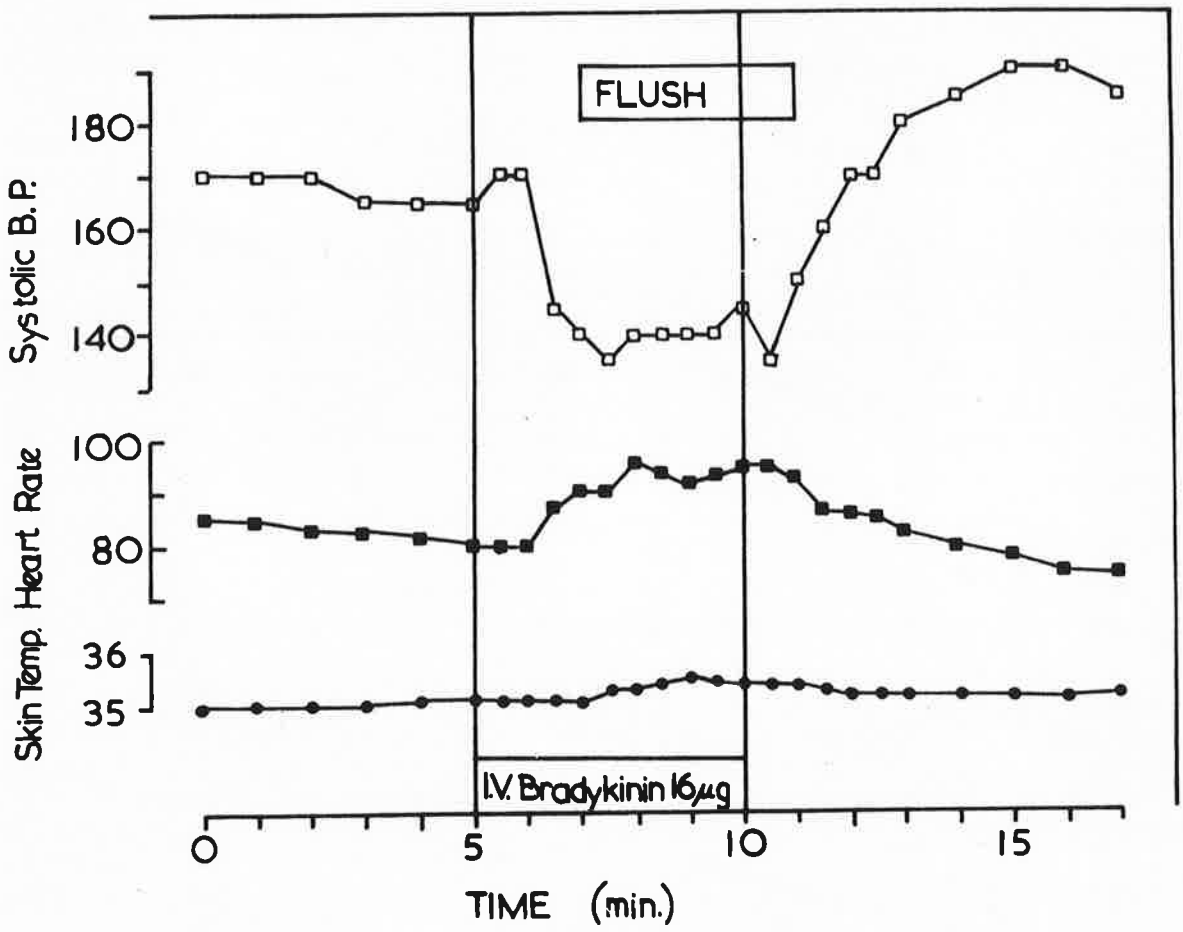


Fig. 37. CARCINOID SYNDROME --- subj. E.B.

Intravenous BK 16 µg/min produced a flush with a fall in BP and tachycardia, but NO ABDOMINAL SYMPTOMS. Flushing described by patient as identical to spontaneous flush.

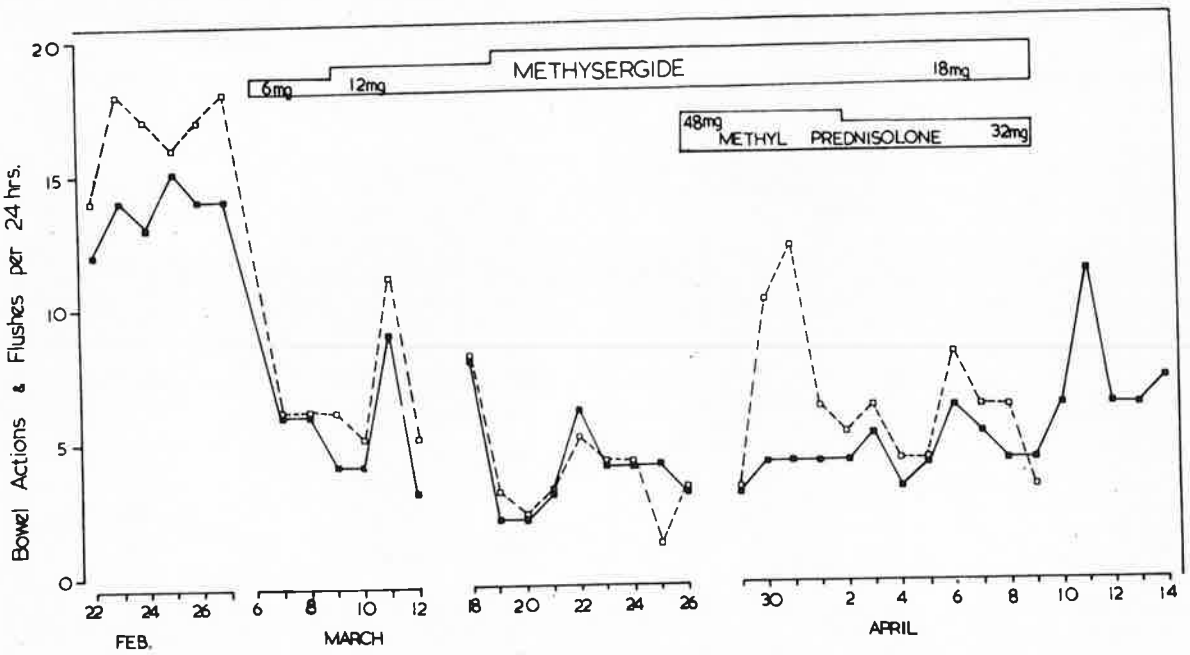


Fig. 38. CARCINOID SYNDROME --- subj. E.B.

Oral therapy with methysergide decreased the frequency of bowel actions _____ from 12 - 15 per day to 3 - 5 per day. Flushing attacks ------ which had occurred usually concommitantly with bowel actions, were reduced to 3 - 5/day. Additional prednisolone therapy had no beneficial effect on the symptoms.

PATIENT	ARTERIAL BLOOD BRADYKININ* LEVEL (ng./ml.)			URINARY 5 H.I.A.A. EXCRETION (mg./24 hrs)	CLINICAL STATUS
	Resting	Flushing	Provocation		
L.C. ♀ 71 yrs.	0·15	0·31	I.V. ADREN.	60–106	Ambulant.
L.H. ♂ 35 yrs.	✸ 1·1	1·3	PRESSURE on LIVER	320	Incapacitated; Right heart disease.
E.B. ♀ 69 yrs.	10	15	I.V. ADREN.	165–687	Rapid deterioration & death.

* NORMAL ARTERIAL BK LEVEL = 0·25 ng/ml (SD 0·23 ng/ml)

✸ PERMANENTLY FLUSHED

Table 10. CARCINOID SYNDROME

A summary of clinical status, urinary 5-H.I.A.A. output and changes in arterial blood BK levels during flushing in three patients with the carcinoid syndrome

commitant with this, there occurred a parallel decrease in the frequency of flushing (Fig. 38).

The patient L.H. had a 20% increase in arterial blood BK associated with pressure over the liver which caued an increased sensation of heat in the face. The changes in BK levels in the three patients are summarised in Table 10.

(B) Cirrhosis

The arterial blood BK level in the three patients studied were within the normal range. Results are summarised below.

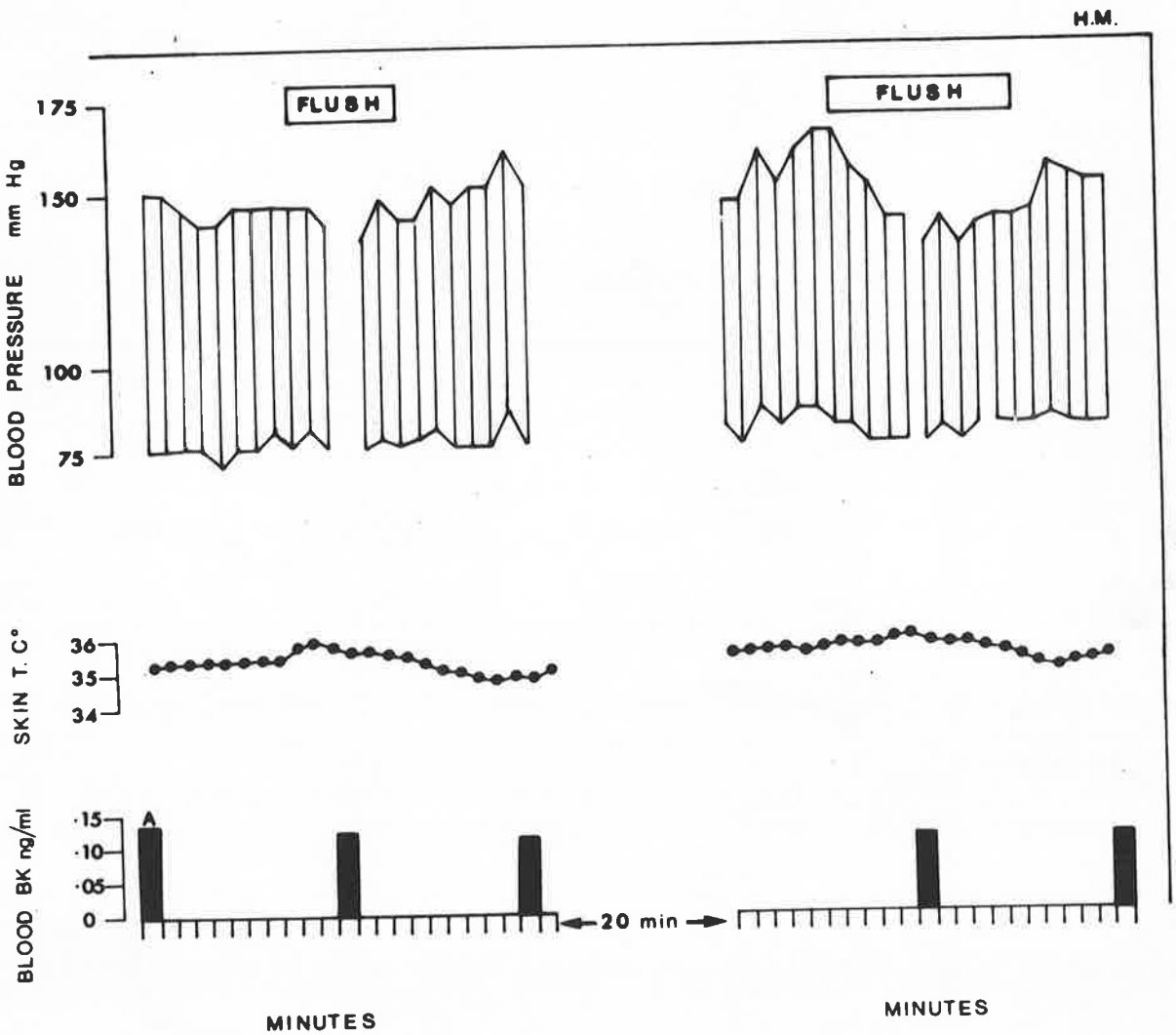
Patient	B.P.	C O T P R	Art. BK	Clinical status
I	$\frac{280}{160}$	4.5 2841	0.5 ng/ml	Macroglobulinaemia Abnormal L F T's Spider naevi
II	$\frac{90}{50}$	not estimated	0.27 ng/ml	Hyperdynamic circulation. Hepato-renal failure
III	$\frac{60}{20}$	5.53 477*	0.35 ng/ml	Hyperdynamic circulation Hepato-renal failure

Abbreviations:

C O	Cardiac output (l/min)
T P R	Total peripheral resistance, (dyne sec cm^{-5})
	* (N 600 - 2000)
L F T	Liver function test

(C) Menopause

Patient H.M. was studied on two occasions, on the first occasion, she experienced two spontaneous flushes. The slight fall in blood pressure and the rise in forehead skin temperature are shown in Fig. 39. There was no increase in arterial blood BK levels associated with the flush and the levels altered very little over the entire course of the experiment. On a subsequent occasion, no spontaneous flushing occurred and arterial blood BK levels were undetectable (<0.015 ng/ml). No flushing occurred in the other two women under laboratory conditions and BK levels in the arterial blood were too low to be detected with the sensitivity of the present method.



BK ng/ml 0.13 0.12 0.11 0.11 0.11

Fig. 39. MENOPAUSAL SUBJECT -- H.M.

Two spontaneous "hot" flushes which were accompanied by sweating and hyperpnoea occurred in this subject. A slight rise in forehead skin temperature (0.5 ^{o} C) and a fall in systolic BP (Maximal fall in mean SBP of 20 mm Hg) were recorded.

Expt	Subj.	Control arterial BK ng/ml	Arterial blood BK ng/ml during flushing
1	D.R.	<0.015	
2	K.C.	<0.015	
3	H.M.	0.13, 0.11, 0.11	0.12 0.11
4	H.M.	<0.015	

Arterial blood BK levels in three menopausal women. In three experiments levels were too low to be detected with the present method of assay.

d. DISCUSSION

Although the studies on the menopausal women gave negative results, a role for BK cannot be excluded. It is interesting to note that in three of four experiments blood BK levels could not be detected. This is similar to blood drawn for assay from a normal subject who is receiving an adrenaline infusion and blood levels of BK in such a situation are at the threshold of detectability. The "vasomotor instability" in these people is probably due to a labile sympatho-adrenal system but it is not unlikely that in spite of the low BK levels in the peripheral blood, there could be widely fluctuating BK levels in the subcutaneous tissues. Accumulation of BK would promote hyperaemia; a sympathetic discharge or increased levels of circulating adrenaline would lead to accelerated destruction of the peptide. The fluctuations in peripheral sites need not be reflected in systemic arterial blood. More information about BK changes during flushing could be obtained by studying the venous blood draining the skin, particularly from the flush area. This however, poses difficulties with the present method where large amounts of blood are needed for an individual assay and the cannulation of a vein draining the relevant area of skin in such

subjects is difficult.

Of the three patients with cirrhosis, II and III fit into the category of " hypotensive oliguric cirrhotics" (Mashford et al.,1962). Although the BK levels are within the normal range, information is inadequate to dismiss BK from a role in contributing to the hyperdynamic circulation. It is again probable that study of peripheral extracellular fluid and venous BK levels may give more information. Mashford et al. (1962) observed that "hypotensive, oliguric, cirrhotic patients" showed a decreased response to tyramine and argued that impaired tissue noradrenaline stores may play a part in the pathogenesis of the hypotension by withdrawal of the normal tonic action of the sympathetic nervous system. One can speculate further on their findings by suggesting that low tissue concentrations of noradrenaline would promote the survival of BK resulting in increased tissue concentrations of this peptide.

The findings in the carcinoid subjects add to the accumulating evidence that the flushing attacks which characterise the carcinoid syndrome are associated with and presumably due to elevated levels of a bradykinin-like peptide in the blood. This does not bear on the cause of the other components of the syndrome since bradykinin has variable effects on gut motility in vivo (Winkler, Bauer & Gmeiner, 1965; Bauer, Gmeiner & Winkler, 1966).

In one of the patients studied here (E.B.) it was possible to reproduce the bowel symptoms by infusion of the serotonin without any noticable vascular effect (Fig. 36) and on the other hand to reproduce the flush without abdominal discomfort by infusing BK (Fig. 37). The use of methysergide did not interfere in any way with the adrenaline - induced flushes; it did, however, abolish the effect of infused serotonin and had its usual beneficial effect in reducing the frequency of bowel actions (Fig. 38).

BK is bronchoconstrictor (Bhoola, Collier, Schachter & Shorley, 1962) in the guinea pig, but the evidence for this action in man is tenuous (Herxheimer & Stresemann, 1963) and asthma did not accompany the flushes or bradykinin infusion in either patient.

The blood levels of BK reported here are lower than values which have been described by others (Zeitlin & Smith, 1966; Mason & Melmon, 1966). However, the good recoveries of internal standards (Part 2) and the rise in blood level found during infusion of BK (Part 3) suggest that this method is giving an accurate reflection of true levels. The destruction of BK by blood is very rapid (Part 4) and approximately only 10% of BK infused into the brachial artery survives to appear in the venous blood on that side (Part 3). Under these circumstances

it is suprising that Zeitlin and Smith (1966) found that venous blood levels rose in the manner described.

One unexplained feature is that in patient E.B., with a level of BK in blood larger by an order of magnitude than any other subject studied, there was no permanent vasodilatation. This implies a reduced vascular sensitivity to the peptide. Such exhibition of tachyphylaxis has parallels in other situations where there are high circulating levels of other vasoactive substances such as noradrenaline in phaeochromocytoma and perhaps angiotensin in some cases with renal hypertension (Scroop, 1967). There is also some indication that the vasodilatation in the calf caused by infusion of BK is not well maintained (Coffman & Javett, 1963) and a similarly transient response has been found in the hand with brachial artery infusions (personal observations).

The rise in arterial blood BK levels caused by adrenaline infusions in the carcinoid syndrome is particularly striking in view of the fall seen in normal subjects (Part 4). This fall is due to activation of the bradykinin-destroying enzyme, kininase in the blood; evidence of this action is probably also to be seen in the carcinoid patient L.C. (Fig. 32) where the larger dose of adrenaline was associated with a slightly smaller rise in BK level. Release of kallikrein by catecholamines may

also occur in normal subjects, as suggested by the studies of Hilton and Lewis (1956), but it seems that the overriding effect of carboxypeptidase activation does not allow BK levels in the blood to rise. In the carcinoid syndrome, with an excess of kallikrein - containing tumour tissue, sufficiently large amounts of kallikrein are liberated into the circulation to cause a rise in blood BK levels.

The opposing effects of adrenaline to be expected on BK metabolism in the carcinoid syndrome has interesting implications in the possible use of adrenergic blocking agents e.g. phenoxybenzamine in the clinical management of these patients. It has been shown that oral phenoxybenzamine can be effective in reducing the frequency of spontaneously occurring flushes (Levine & Sjoerdsma, 1963), but it is interesting to note that this drug is very poorly tolerated in these patients. Therapy in a dose of only 10 mg/24 hrs may have to be discontinued because of severe drowsiness, confusion and visual blurring. Phenoxybenzamine is much better tolerated in other situations e.g. in therapy of peripheral vascular disease (Moser, Prandoni, Orbison & Mattingly, 1953) where doses of 30 - 240 mg/24 hrs (even up to 400 mg daily) have been used with only mild transient side effects. In chronic medical therapy for phaeochromocytoma, Engelman and Sjoerdsma (1964) have used

doses of 30 - 80 mg/24 hrs, the only side effect being an initial mild sedative effect.

Since phenoxybenzamine is expected to prolong the survival of BK once the latter has been released, the severe side effects found in some of the patients studied by Levine and Sjoerdsma (1963) may be due to rising levels of circulating BK on the central nervous system. Sicuteri et al. (1963) have shown that intravenously administered BK in healthy humans may produce mild stupor, drowsiness and lightning scotomata -- symptoms which are similar to those produced in some of the patients of Levine and Sjoerdsma (1963) receiving phenoxybenzamine therapy.

e. SUMMARY

1. Three cases are briefly discussed in which the carcinoid syndrome can confidently be diagnosed. Two of them had elevated resting levels of arterial blood BK and in all three the level rose associated with increase in vascular symptoms. In two of the patients adrenaline infusion was used to produce flushing and this resulted in an elevation of arterial BK levels above resting concentrations. This contrasts with findings in six subjects not suffering from the carcinoid syndrome in whom adrenaline caused a fall in arterial blood BK (Part 4).

2. Three patients with advanced cirrhosis of the liver were examined, two clearly had hyperdynamic circulation and hepatorenal failure. In all three, arterial blood BK levels were within normal limits.

3. Three menopausal women suffering from frequent "hot" flushes were studied. In three experiments no spontaneous flushing occurred under laboratory conditions and arterial blood BK levels were too low (<0.015 ng/ml) to be detected by the present method of assay. When spontaneous flushing occurred no changes in arterial blood BK levels were detected. The significance of the low levels is discussed.

APPENDICES TO METHODS in PART 2

APPENDIX 1. SOLUTIONS

APPENDIX 2. CHROMATOGRAPHY

APPENDIX 3. BIOLOGICAL ASSAY

APPENDIX 4. STATISTICAL ANALYSIS

APPENDIX 1.

SOLUTIONS

1. 0·1 M Ammonium Acetate buffer (pH 6·0)

This buffer solution is prepared as 0·1 M strength with regard to NH_4^+. A 0·1 M solution of ammonium acetate is prepared by dissolving reagent grade ammonium acetate crystals in deionised water (Elgastat) and the pH is carefully adjusted to 6·0 with glacial acetic acid using a direct reading Beckman pH meter. This buffer solution is stored at room temperature. At this temperature it is likely to become contaminated with fungal growth and fresh solutions have to be regularly made. Preservatives such as thymol have not been used. Storage of buffer at low temperatures is inadvisable, as gases which dissolve in the solution at low temperature are liberated over a long period of time when the buffer is returned to room temperature. Evolution of even minute amounts of gases during chromatography with I.R.C. 50 is one of the most common reasons for failure of the resin.

2. Ammonium hydroxide solutions 1 N and 0·1 N.

Stock concentrated ammonium hydroxide solution S.G. 0·90 17 N is used for the preparation of the weaker solutions. The stock is kept in a refrigerated room in a tightly sealed bottle. 58 ml stock diluted with deionised water to 1 litre will give a 1 N solution. This solution is maintained at room temperature for the same reasons as the ammonium acetate buffer. Since at this temperature the strength of the solution may rapidly change, it is avisable to prepare fresh solution every day. The 0.1 N solution is conveniently prepared from the 1 N solution by dilution whenever required.

3. Casein solution

Light white soluble casein (B.D.H.) is used. Fresh solution of 0·25% casein in deionised water is prepared whenever required.

4. de Jalons solution

de Jalons solution is the superfusate used in the bioassay and also for the preparation of the final extract. It is eminently suitable for the rat's uterus because of its low calcium content which keeps the tissue quiescent until stimulated.

(Gaddum, Peart & Vogt, 1949). The solution used in the present experiments, however, contains a little more calcium than that of Gaddum et al.; de Jalons solution of the following composition was used:

	gm/l
NaCl	9
$CaCl_2$	0.08
KCL	0.42
$NaHCO_3$	0.5
Glucose	0.5

A convenient way to prepare de Jalons is to first prepare two stock solutions. Stock A contains the chlorides and stock B the bicarbonate and glucose to avoid precipitation of insoluble $CaCO_3$. The stock solutions are prepared from reagent grade solids except for $CaCl_2$ which has been previously prepared as a standardised 10% solution.

Stock A

NaCl	360 gm
$CaCl_2$	3.2 gm
KCL	16.8 gm

made up to 2 litres with deionised water.

Stock B

$NaHCO_3$	20 gm
Glucose	20 gm

made up to 1 litre with deionised water.

The stock solutions are kept in the refrigerator and are usually prepared weekly. The final solution is prepared from 100 ml Stock A and 50 ml Stock B made up to 2 litres in such a way that Stock A is first adequately diluted before Stock B is added. Stock A should be checked for signs of Ca precipitation, if this is not occurring, stocks may be kept for longer periods of time.

APPENDIX 2

AMBERLITE I.R.C. 50 RESIN

a. Preparation of resin.

b. Packing of columns.

c. Chromatography.

d. Regeneration of resin.

PREPARATION OF RESIN

Resin of mesh size 100 - 200 is used. Although chromatographic grade resin is purchased, it is necessary to further purify it according to the method of Hirs, Moore & Stein, (1953). This involves the removal of "fines" by frequent washing with deionised water and decantation. The washing of the resin with acetone can be conveniently done after the water wash. The hydrogen form (as purchased) is cycled through the sodium form, back to the hydrogen form and finally converted to the ammonium form by allowing it to stand in a large volume of 2 N NH_4OH overnight.

The final ammonium form has to be equilibrated with 0·1 M

ammonium acetate buffer. A time saving, short cut to this is first to wash the excess ammonium hydroxide out with Elgastat and then titrate the suspension (stirred with a magnetic stirrer) with glacial acetic acid, to pH 6.0. A stable pH of 6.0 is reached only slowly and titration may have to be carried on for about an hour. When a stable pH 6.0 has been reached, the resin is allowed to settle and all the supernatant is decanted. The resin is now resuspended in 0.1 M ammonium acetate buffer (pH 6.0) and the suspension is then titrated again with glacial acetic acid to pH 6.0. The buffer is completely replaced again every time a stable pH 6.0 is reached. Replacement of buffer and titration are continued until the resuspension of the resin in fresh buffer gives a pH of 6.0 without any additional glacial acetic acid. The resin is now ready for use. The pH of this resin has a tendency to drift slightly to the alkaline side of 6.0 over a period of days and periodic checks of pH have to be made and the pH adjusted accordingly. Resin prepared in this way remains free of microbial contaminants for a long period of time amd preservatives have not been necessary. The batch of resin in use is stored at room temperature.

PACKING OF COLUMNS

Because the resin is equilibrated in a weak salt solution

and is at a slightly acid pH, satisfactory packing of the column requires patience and care. Quickfit columns are first rinsed through with the buffer solution and checked for satisfactory flow through the sinter and the tap, and then half filled with the buffer solution. Resin slurry is conveniently transferred to the column with a teat pipette; with the tap closed, the resin is allowed to settle, addition of resin is continued until the resin level is 0.5 to 1.0 cm above the 5 cm mark in the column. Gentle tapping of the column (heavy wooden ruler) is necessary now, with intermittent opening of the tap. The resin continues to pack for a period of 10 min. with this procedure. A column is considered to be adequately packed when upon opening the tap and running through the buffer solution no distortion of the packing is evident. Excess resin is now pipetted off the top of the column and a filter paper disc (Whatman No 2) is allowed to settle on top of the resin. The filter paper disc should be exactly the same size as the top surface of the column and prevents stirring up of the resin surface when the column is loaded.

To check on the packing of the column, the adequacy of flow through it and the compatibility of the buffer with the resin preparation, 35 ml of the buffer solution is allowed to wash

through the resin and the effluent is discarded. The column is visually checked again, if the buffer solution has been unsuitable in some way e.g. excessive gaseous content, or the original packing of the resin inadequate, the packing in the column will now show distortion, air bubbles, lamination etc. Such a column is unsuitable and has to be repacked with the appropriate adjustment.

Inadequate packing of the column or disruption of the resin bed by use of buffer at unsuitable temperature or pH are the commonest causes for the failure of the column to function properly.

CHROMATOGRAPHY

Flow rate through the column is not critical and the maximal flow rate (atmospheric pressure) gives satisfactory results. The flow rate is not interfered with during the buffer wash of the loaded column nor during the eluting with 1 N NH_4OH. However, with 0.1 N NH_4OH the flow can be safely accelerated and time saved. This is conveniently done by applying positive pressure to the top of the column. This can be done manually with the aid of a rubber tube from a sphygmomanometer squeeze-bulb connected to the top of the column.

REGENERATION OF THE RESIN

Resin used once is pooled until sufficient has been accumulated for another batch. This resin is in the ammonium form and has a significant amount of proteinaceous impurities. The impurities can be removed by a complete recycling of the resin (including the Na^+ form and the acetone wash). If complete recycling is not done and the resin merely cycled through the hydrogen form and the ammonium form, the resultant preparation contains significant organic impurities and fungal growth is evident in a few days.

APPENDIX 3

BIOLOGICAL ASSAY

a. Rats -- stilboestrol priming, -- temperature in the assay.

b. Significance of Ca^{++} in the superfusate.

c. Recording of changes in muscle tone.

d. Use of chymotrypsin

RATS

The virgin rats should be at least 150 gm in weight to obtain a suitable uterus preparation. The best duodenum, however, is more frequently obtained from small rats. Stilboestrol priming (Honvan, Charles McDonald) is performed with a subcutaneous injection in the scalp between the ears.

The ambient temperature in the tissue chamber is of critical importance in determining the sensitivity of the tissues and the uterus in particular. Since maximum sensitivity is necessary in most BK assays, the contribution from temperature must be borne in mind. Increase of temperature is accompanied with in-

creasing sensitivity until the point of onset of spontaneous activity. There is no one optimal temperature for all uterus preparations and it is often profitable to try and achieve the maximum temperature at which the uterus will still be stable. Temperature range from 25° C to 35° C can be explored.

SIGNIFICANCE OF Ca^{++} IN THE SUPERFUSATE

de Jalons containing 0·08 gm/l $CaCl_2$ (7.3×10^{-4} M) is the optimal solution. Lower concentrations have not been tested in cases where the uterus has to be discarded because of persistent spontaneous activity. In some cases, however, an initially insensitive uterus can be improved by increasing the Ca^{++} concentration in the superfusate by up to 50%.

RECORDING OF CHANGES IN THE MUSCLE TONE

The fine points on the recording levers are likely to encounter much friction on the smoked kymograph paper. The traditional "pharmacological tap" is the best way to prevent the tips from sticking in the soot. This applies particularly to the uterus. A metal bar connecting the Braun pump with the

writing lever scaffolding can be used to provide constant mechanical vibration.

USE OF CHYMOTRYPSIN

The use of chymotrypsin (α-chymotrypsin 3 x crystallised, Worthington) to increase the sensitivity of the uterus to BK has been of immense value. With the aid of chymotrypsin, the ratio of preparations suitable for assay has been increased from about 1/2 to 4/5 which results in great economics of time and animals. The method of priming with chymotrypsin is as follows. Superfusion is turned off 1 min. prior to the application of standard or unknown solution. Three drops of chymotrypsin solution are used to bathe the surface of the uterus 30 sec before the application of BK. The uterus is primed prior to every application of BK. The most suitable chymotrypsin concentration for the superfused uterus has been found to be 400μg/ml. By repeated dosing with a standard BK solution the development of increased sensitivity can be followed and the maximum sensitivity is achieved in about 20 min. The increase in sensitivity is accompanied by a continous decrease

in tone of the preparation (Fig. 12). Chymotrypsin seems to have little or no effect on the duodenum when used in the manner described for the uterus. Fresh chymotrypsin solutions are prepared daily and during use are kept at room temperature.

APPENDIX 4

STATISTICAL ANALYSIS

A. Analysis of variance of individual assay.

B. 1. Paired t test on the differences between uterus and duodenum results obtained in recovery experiments.

2. Paired t test on the differences between uterus and duodenum results obtained in endogenous BK estimations.

C. Paired t test on differences between the slopes of the log dose-response curves of standard and unknown, individually for uterus and duodenum in recovery and endogenous BK assays.

D. Regression analysis of bias of duodenum estimations to uterus estimations of endogenous BK levels.

A Analysis of variance of results from a four-point assay

The calculation of the relative potency of the unknown extract depends on parallelism between dose-response lines for extract and standard. The significance of deviation from parallelism may be tested by an analysis of variance. In the calculations shown below such an analysis of variance is applied to a set of data from one assay. The assay results analysed are from the duodenum* assay of P.A. (Table 2).

Duodenum

S_1	S_2	U_1	U_2				Tot_1	Tot_2	Tot_s	Tot_u
12	20	20	20	72	(R_1)	i	32	40	32	40
17	17	18	20	74	(R_2)	ii	35	39	34	40
15	21	19	22	77	(R_3)	iii	34	43	36	41
20	19	19	21	79	(R_4)	iv	39	40	39	40
64	77	76	85	302	T. Tots.		140	162	141	161

Totals

* Similar analysis has been applied to uterus results.

Total Sums of Squares $= \sum x^2 - \frac{(\sum x)^2}{16}$

$= 5804 - \left| \begin{matrix} 5700.25 \\ \text{Corr.)} \end{matrix} \right| = 103.75$

Corr. = Correction Factor

Sources of variation. (SS)

1. Standard v Unknown. (S)

$$\frac{(\sum S)^2 + (\sum U)^2}{8} - \text{Corr}$$

$$= \frac{(141)^2 + (161)^2}{8} - \text{Corr}$$

$$= 5725.25 - 5700.25$$

$$= 25$$

2. Dosage (D)

$$\frac{(\text{T. Tot}_1)^2 + (\text{T. Tot}_2)^2}{8} - \text{Corr}$$

$$= \frac{19600 + 26244}{8} - \text{Corr}$$

$$= 5730.5 - 5700.25$$

$$= 30.25$$

3. Replicates (R)

$$\frac{(\sum R_1)^2 + (\sum R_2)^2 + (\sum R_3)^2 + (\sum R_4)^2}{4} - \text{Corr}$$

$$= \frac{5184 + 5476 + 5929 + 6241}{4} - \text{Corr}$$

$$= 5707.5 - 5700.25$$

$$= 7.25$$

Interactions

4. S x D

$$\frac{(\sum s_1)^2 + (\sum s_2)^2 + (\sum u_1)^2 + (\sum u_2)^2}{4} - \text{Corr} - S - D$$

$$= \frac{64^2 + 77^2 + 76^2 + 85^2}{4} - \text{Corr} - S - D$$

$$= 5756.5 - 57000.25 - 25 - 30.25$$

$$= 1$$

5. S x R

$$\frac{Tot_{si}^2 + Tot_{sii}^2 + Tot_{siii}^2 + Tot_{siv}^2 + Tot_{ui}^2 + Tot_{uii}^2 + Tot_{uiii}^2 + Tot_{uiv}^2}{2} - \text{Corr} - S - R$$

$$= \frac{32^2 + 34^2 + 36^2 + 39^2 + 40^2 + 40^2 + 41^2 + 40^2}{2} - Corr - S - R$$

$$= \frac{11478}{2} - Corr - S - R$$

$$= 6.50$$

6.

D x R

$$\frac{Tot_{1i}^2 + Tot_{1ii}^2 + Tot_{1iii}^2 + Tot_{1iv}^2 + Tot_{2i}^2 + Tot_{2ii}^2 + Tot_{2iii}^2 + Tot_{2iv}^2}{2} - Corr - D - R$$

$$= \frac{32^2 + 35^2 + 34^2 + 39^2 + 40^2 + 39^2 + 43^2 + 40^2}{2} - Corr - D - R$$

$$= 5748.0 - 5700.25 - 30.25 - 7.25$$

$$= \underline{10.75}$$

Analysis of Variance

	SS		Mean square (Variance estimate)		DF	Variance Ratio
S	25.00		25		1	5.6* $0.01 < p < 0.05$
D	30.25		30.25		1	6.8* $0.01 < p < 0.05$
R	7.25		2.4		3	1.9 (N.S.) $p > .2$
S x D	1.00		1.0		1	4.4 (N.S.) $p > .2$
S x R	6.50)		2.2)		3)	
D x R	10.75)	40.25	3.8)	4.47	3)	9
Res.	23.00)		7.7)		3)	
Total	103.75				15	

The interaction S x D represents the contribution to the total SS of the divergence from parallelism. The SS of interactions S x R and D x R are combined with the residual SS, resulting in an error variance of 4.47 and increasing the DF of error variance to 9.

The divergence from parallelism of the dose response lines of standard and extract is not significant ($p > .2$) and it is valid to use the method of Gaddum (1959) for estimation of relative potency of the unknown. Such a statistical test was applied to all assay results where doubt existed about parallelism. All data in Table 2, showing endogenous BK estimations by the two tissues, has been validated in this respect.

B. Table App. i shows recovery estimations by uterus and duodenum assays. Twenty consecutive estimations of recovery of 10 /ml of BK from blood have been examined.

Table App. i

Per Cent Recovery

	Uterus	Duodenum	(Ut - Duod.) X
1.	102	90	+ 12
2.	89	90	- 1
3.	65	115	- 50
4.	119	91	+ 28
5.	90	100	- 10
6.	75	97	- 22
7.	105	112	- 7
8.	100	98	+ 2
9.	98	85	+ 13
10.	94	115	- 21
11.	110	104	+ 6
12.	101	82	+ 19
13.	79	104	- 25
14.	107	91	+ 16
15.	71	123	- 52
16.	78	105	- 27
17.	81	100	- 19
18.	91	91	0
19.	80	112	- 32
20.	83	112	- 29

$\Sigma x = -192$

$\bar{x} = \frac{-192}{20} = -9.6$

$$\sum (x_n - \bar{x})^2 = 9635.3$$

Variance of mean $(V\bar{x}) = \dfrac{\sum (x_n - \bar{x})^2}{n(n-1)} = \dfrac{9635}{380}$

$$t_{19} = \frac{\text{Error of mean}}{\text{S.E. of mean}}$$

$$= \frac{+\ 9.6}{\sqrt{\dfrac{\sum (x_n - \bar{x})^2}{n(n-1)}}}$$

$$= \frac{+\ 9.6}{5.03} = +\ 1.9$$

N.S. at 5%

When the differences between uterus and duodenum assay results for endogenous BK (from Table 2) are examined in a similar manner, the values are found to be significantly different at the 5% level.

C. Test of significance of differences in parallelism of the slopes of log-dose response curves for standard and unknown from uterus and duodenum for: a. Recovery estimations

b. Endogenous BK estimations

On the null hypothesis, the slope calculated for responses to standard

$$\frac{(s_2 - s_1)}{\log 2}$$

should equal the slope calculated for the unknown:

$$\frac{(u_2 - u_1)}{\log 2}$$

(log 2, since the ratio of high dose to low dose is 2:1)

$$\text{then} \quad \frac{s_2 - s_1}{0{\cdot}3010} = \frac{u_2 - u_1}{0{\cdot}3010}$$

$$\text{and } s_2 - s_1 - u_2 + u_1 = 0 \quad \text{(from null hypothesis)}$$

The actual values for the differences are presented in the table below (Table App ii) and the significante of their deviation from zero calculated.

Table App. ii

	Value of $u_2 - u_1 - s_2 + s_1 = x$			
	Recovery		Endogenous	
	Uterus (A)	Duodenum (B)	Uterus (C)	Duodenum (D)
1.	+ 17	- 25	+ 40	- 20
2.	- 54	+ 2	- 40	- 33
3.	+ 76	+ 7	- 5	- 4
4.	- 39	+ 3	- 60	- 10
5.	+ 256	+ 6	+ 46	- 11
6.	+ 70	- 3	- 64	- 10
7.	+ 263	+ 21	- 156	+ 7
8.	+ 6	+ 17	+ 49	+ 1
9.	+ 26	0	- 28	- 7
10.	+ 73	- 12	- 36	- 5
11.	+ 130	- 2	- 26	- 24
12.	+ 29	+ 18	+ 144	+ 12
13.	+ 22	+ 5	- 6	+ 12
14.	- 48	- 4	- 208	+ 2
15.	- 21	- 19	- 211	- 8
16.	- 30	- 15	- 36	- 1
17.	- 6	+ 18	- 20	- 7
18.	- 61	- 21	- 182	- 32
19.	+ 112	+ 12	- 1	- 5
20.	+ 112	- 17	+ 1	- 27
21.			+ 34	+ 1
22.			+ 37	+ 17
23.			- 79	0
24.			- 44	- 11
25.			+ 93	- 10

Analysis of above data:

Set of values	A	B	C	D
$\sum x$	+ 671	- 9	- 758	- 173
Number of values (n)	20	20	25	25
Mean $\bar{x}$	33.5	- 0.45	- 30.32	- 6.92
$\sum(x_n - \bar{x})^2$	200,467	3,755	182,277	5221
Degrees of freedom	19	19	24	24
S.E. of mean $\sqrt{\frac{\sum(x_n - \bar{x})^2}{n\,(N-1)}}$	22·96	3·14	17·45	2.94
$t = \frac{\text{Error in mean}}{\text{S.E. of mean}}$	± 1·46	± 0·14	± 1.74	± 2.67*
t (tables) (5%)	2.1	2.1	2.06	2.06
p	10%<p<20%	80%<p<90%	5%<p<10%	1%<p<2% *

These results show that the nature of the extract of endogenous blood BK is such that it gives falsely high results on the duodenum*, and it does so by changing the log dose-response line away from parallelism to the standard log dose-response line. In the recovery experiment, where the initial level of BK is 10 ng/ml and greater dilution of the extract can be made for assay, the impurity through greater dilution does not significantly effect the duodenum.

D. REGRESSION ANALYSIS & ANALYSIS OF VARIANCE

Null hypothesis: No significant trend in duodenum assay bias from uterus results over the range of normal BK levels.

Data from 25 parallel assays of endogenous BK levels from 18 subjects (Table 2).

In table below,

t_i = uterus assay value

x_i = % difference in corresponding duodenum assay.

* see table previous page.

t_i	0.02	0.07	0.07	0.09	0.10	0.09	0.11	0.11	0.14	0.13
x_i	150	100	157	122	120	122	127	136	100	131

t_i	0.14	0.17	0.12	0.14	0.16	0.17	0.21	0.23	0.19	0.37
x_i	114	88	150	129	125	106	138	143	158	97

t_i	0.52	0.50	0.60	0.70	0.70
x_i	125	154	150	128	172

Total sum of squares $= \sum x_i^2 - \frac{1}{25} \sum^2 x_i$

$= 431,520 - 418,350$

$= 13,170$

Regression SS $= \frac{\left\{\sum x_i t_i - \frac{1}{n} \sum x_i \sum t_i\right\}^2}{\sum t_i^2 - \frac{1}{n} \sum^2 t_i}$

$= 1021$

Deviation from regression $= 13170 - 1021$

$= 12149$

Analysis of variance

Source of variance	Sum of squares	D of F	Variance	Ratio
Regression	1021	1	1021	F 1, 23=1.93
Dev^n. from regression	12149	23	528	$.1 < p < .2$
Total	13170	24		

$F_{1,23}$ = 1.93 which is not significant at 5% level.

i.e. in endogenous BK estimations over a range encountered in normal subjects, the bias from the duodenum does not vary significantly over this range.

The bias of the duodenum is

$$\frac{1}{n}\sum x_i = 129.68\%$$

Confidence limits of bias (a)

Variance (a) = $\frac{1}{25}$. Variance of deviation from regression

$$= \frac{528}{25} = 21.12$$

∴ S.E. of (a) = 4.595

∴ Bias of duodenum = + 29.68% ± 4.595 x t_{32}

= + 29.68% ± 4.595 x 2.07

= + 29.68% ± 9.47% (with 95% confidence).

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