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LA THÈSE A ÉTÉ  
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THE ISOLATION AND CHARACTERIZATION OF HUMAN SALIVARY KALLIKREIN.  
FROM NORMAL SUBJECTS AND CYSTIC FIBROSIS PATIENTS

by  
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Submitted in Partial Fulfillment of the Requirements for the  
degree of Doctor of Philosophy at Dalhousie University

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Dedicated to the memories of my Grandmother (Ms. Emily Mighty)  
and my Brother (Phillip Arthur Hare).

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# ABSTRACT

A protein with arginine esterase activity was purified from whole saliva. The protein lowers the blood pressure in rabbits and produces kinins in acid treated dog plasma. The enzyme was inhibited by Kunitz's bovine pancreatic trypsin inhibitor and diisopropylfluorophosphate. However, it was inhibited by soybean trypsin inhibitor and it did not bind  $\alpha_2$ -macroglobulin. Therefore the protein is a glandular or tissue kallikrein.

The purified kallikrein had a pI of 4.07 and a molecular weight of 40,000 D as determined by sodium dodecylsulfate gel electrophoresis and by sedimentation equilibrium centrifugation. The enzymatic activity was inhibited at high benzoyl arginine ethyl ester concentrations.

The amino acid composition showed a large number of polar residues, especially acid residues. Fluorescence spectra indicated the presence of tryptophan. Since the fluorescence was quenched by iodide at least part of the aromatic residues are located on the protein surface. The  $\text{NH}_2$ -terminal amino acid is mostly serine.

Circular dichroism spectra exhibit one minimum at 197 nm. Computer analysis indicated that the enzyme was devoid of  $\alpha$ -helix but may contain a relatively large amount of pleated sheet structure. The structure is stable and dodecylsulfate does not have much effect on it.

Saliva of CF patients showed normal levels of kallikrein. Comparison of the purified salivary kallikrein from CF (Cystic Fibrosis) patients and control subjects showed no significant difference in enzymatic activity, polyacrylamide gel electrophoresis, chromatographic behaviour, amino acid composition or spectroscopic behaviour. Therefore it was concluded that the salivary kallikrein in CF patients is similar to the salivary kallikrein in non-CF subjects and thus not involved in the pathogenesis of CF, contrary to some previous speculations.

# SYMBOLS AND ABBREVIATIONS

## Symbols

$dc/dr$	distance between the schlieren patterns produced by two sectors of the cell at the distance $r$ from the axis of rotation.
$r$	distance in cm from a position in the cell to the axis of rotation.
$v$	partial specific volume
$\lambda$	wavelength
%T	concentration of polyacrylamide gel monomer
%C	concentration of cross linker relative to the total monomer.
$k_{cat}$	maximum number of substrate molecules converted to products per active site per unit time or the number of times the enzyme turns over per unit time ( $= V_{max}/E_0$ , where $E_0$ is total enzyme concentration).
$K_m$	concentration of substrate giving half maximal activity (the Michaelis constant)
$v$	velocity of reaction
$V_{max}$	velocity of an enzyme reaction when saturated with substrate.
pH	$-\log_{10}H$ where $H$ is the hydrogen ion concentration
pI	the isoelectric point (the pH at which the net charge on the protein is zero)
pK	$-\log_{10}K$ where $K$ is the equilibrium constant

[ $\theta$ ] mean residue ellipticity

Abbreviations

BAEE	$\alpha$ -N-benzoyl-L-arginine ethyl ester.HCl
BAPNA	$\alpha$ -N-benzoyl-DL-arginine-p-nitroanilide.HCl
BPTI	bovine pancreatic trypsin inhibitor
CF	cystic fibrosis
D	daltons
CD	circular dichroism
DEAE	diethyl aminoethyl
DFP	diisopropyl fluorophosphate
ES	enzyme-substrate complex
HPLC	high performance liquid chromatography
LBTI	lima bean trypsin inhibitor
Mw	molecular weight
nm	nanometer ( $10^{-9}$ meter)
PAGE	polyacrylamide gel electrophoresis
rpm	revolutions per minute
SBTI	soybean trypsin inhibitor
SDS	sodium dodecylsulfate
Tris	tris (hydroxymethyl) aminomethane
v/v	volume per volume
w/v	weight per volume

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## CHAPTER I

### GENERAL INTRODUCTION

#### A. INTRODUCTION

Cystic fibrosis (CF) is an inborn error of metabolism which has escaped precise definition but which involves all the exocrine glands and probably other tissues as well (di Sant'Agnese and Talamo, 1967). The majority of the clinically significant abnormalities in CF are caused by obstruction of the organ ducts by abnormal secretions. Normally the secretions from the digestive or respiratory systems are fluid and mobile, delivering needed chemicals or aiding in 'clearing' open passages. But in CF something abnormal happens to the chemistry of the body fluids and the secretions become very thick and viscous, so they obstruct passages especially in the lungs and in the pancreatic and bile ducts.

#### Genetics

CF is inherited from both parents, each of whom must be a carrier (obligatory heterozygote). The abnormal gene has a frequency of approximately five percent in the population, which means that one person in twenty is a carrier of the gene. The carriers of the disease are asymptomatic and unfortunately there is, at present, no reliable method to detect them. The incidence of the disease is approximately one in two thousand live births (Nadler et al., 1978). If two carriers (one in approximately four hundred marriages is estimated to involve two carriers) produce



children, there is a twenty-five percent chance with each pregnancy that the child will inherit the abnormal genes from both parents and thus have CF. Once having such a child, the parents now know that they are carriers. If such a couple has further children, there is no accurate method, at present, to determine before birth, whether or not the infant is affected.

#### B. MAIN AREAS OF CF RESEARCH

The basic biochemical defect in CF is not yet known. At the present time the following four main hypotheses are being investigated :

##### 1. Abnormal Secretion

Abnormal secretions have been recognized for some time as one of the basic abnormalities in children with CF. Various chemical abnormalities have been detected in these secretions (Mangos et al., 1967a) but these have not as yet led to the underlying biochemical defect. In general the secretions have an increased viscosity which is responsible for many of the signs and symptoms of CF.

##### 2. Autonomic Dysfunction

The autonomic nervous system has been subjected to a great deal of studies in relation to CF, because it is the system which controls the secretory activity of the exocrine glands (Barbero et al., 1966).

Structural and functional studies of the autonomic nervous system have thus far failed to produce an explanation for the disease.

### 3. Humoral Abnormality

The possibility of a humoral abnormality has received a great deal of attention. Spock et al. (1967) described a serum factor in children with CF, and in their parents, which causes the disorganization of the ciliary rhythm in explants of respiratory epithelium from rabbits. The mechanism by which this serum factor produces disorganization of the ciliary beat is not as yet understood.

Mangos and McSherry, in 1967b also detected a factor in CF sweat and saliva which inhibited sodium reabsorption in rat parotid and human sweat glands. It was proposed by these investigators that a circulating inhibitor to sodium reabsorption was responsible for the abnormally high concentrations of sodium and chloride in CF sweat. The relationship of the factor or factors to the pathogenesis of CF is presently unknown, but its presence in CF genotypes appears to be related to the basic defect.

### 4. Ion Transport Defect.

Ion transport dysfunction has been demonstrated in CF and this is the basis for the major diagnostic test; the 'Sweat Test' (di Sant'Agnese et al., 1953). The sweat of virtually all CF patients shows elevated sodium and chloride levels (sweat chloride >60 mEq per liter in CF compared to <50 mEq per liter in normal).

It should be noted that the 'Sweat Test' is not completely accurate as diagnostic for CF and that it should be used in conjunction with family history, chronic pulmonary disease and malabsorption. The 'Sweat Test' is also not reliable during the prenatal period.

In summary there are several hypotheses as to the pathogenesis of CF none of which has been proven and it may well be that several of them are interrelated and controlled by more than one genetic factor (Wood et al., 1976). As stated previously, CF is characterized by thick viscous secretions. Therefore it seems likely that something has been added to the secretion, or that something is absent that should be present.

The fact that the cause of CF is not known has forced investigators to search widely for its cause. But unfortunately CF research is plagued by irreproducible results which hampers progress.

#### C. KALLIKREINS AND KININS

Kallikreins are serine proteases with specific and limited proteolytic actions (Schachter, 1980) and which also show arginine esterase activity. These enzymes are present in different body fluids such as saliva, urine, plasma and also in organs of different species such as the pancreas and salivary glands of man. The major known action of these enzymes is to liberate kinins from kininogens present in the plasma, lymph and other body fluids.

The formation of kinins from kininogens by kallikreins is shown in Fig. 1. The kinins which are liberated are vasoactive peptides known to lower blood pressure and contract smooth muscles. Kinins are also known to be involved in vascular permeability, shock, inflammation and pain production.

The physiological importance of kinins.

a) Release of Catecholamines

Kinins indirectly affect airway function through their ability to release catecholamines, either from the adrenals or by stimulation of sympathetic ganglia. In the cat, only 50 ng of bradykinin injected into the abdominal aorta, releases catecholamines from the adrenal gland (Feldberg and Lewis, 1964). It was estimated that one molecule of bradykinin released approximately fifty molecules of catecholamines. Staszewska-Barczak and Vane (1967) have studied this effect further using the blood-bath organ technique, in which blood flowing in an extracorporeal loop of the circulation, is superfused over test tissues with different sensitivities to adrenaline and noradrenaline. Their experiments showed that the only catecholamine released was adrenaline. Feldberg and Lewis (1964), found that bradykinin was slightly more effective than kallidin, but the reverse order of potency was reported by Staszewska-Barczak and Vane (1967) although both sets of experiments were done with cats. Experiments done by Collier (1970) on cats also demonstrated that kinins stimulate the release of

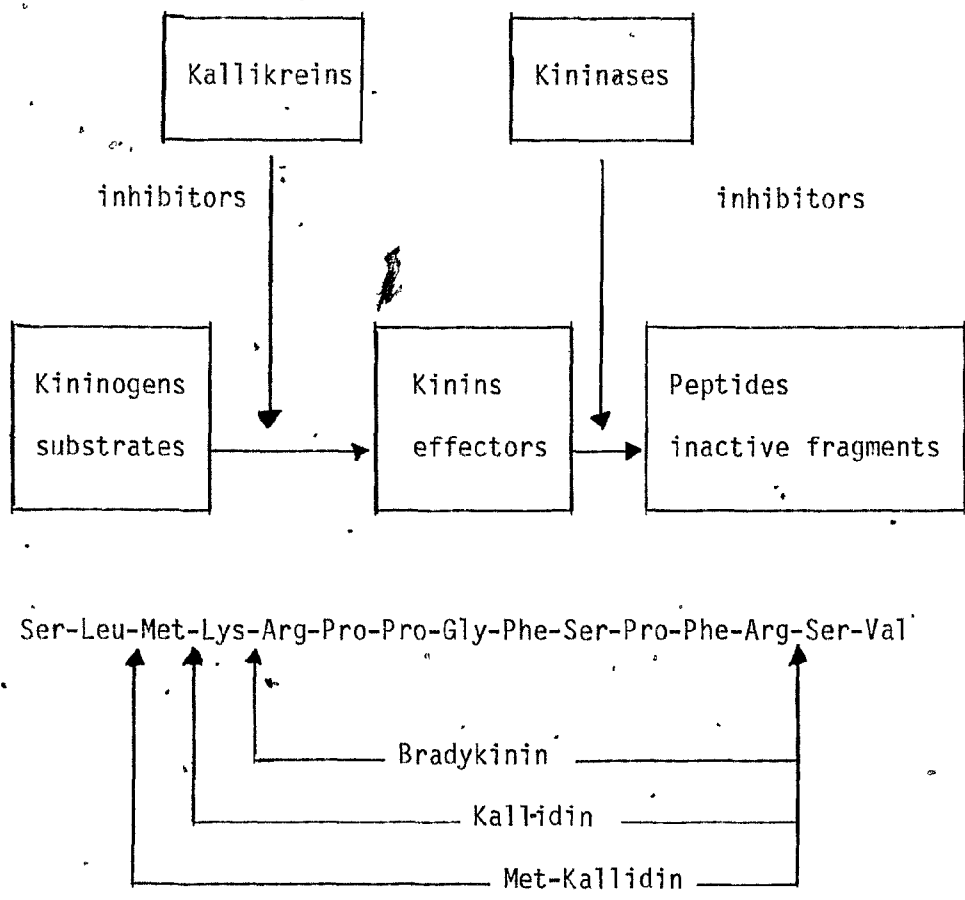


Fig. 1 : The kallikrein-kinin system: Diagrammatic representation of the major constituents of the kinin-forming pathway (Fritz , 1983).

catecholamine from the superior cervical ganglia.

Bradykinin is not always the most effective compound for releasing adrenaline. On a weight for weight basis, when used via the intra-arterial route in the cat angiotensin was most effective in releasing adrenaline followed by histamine which was also more effective than bradykinin. However, the opposite was the case in the dog, where bradykinin was more effective than angiotensin or histamine.

b) Pain

The pain-producing actions of the plasma kinins and specifically bradykinin were first studied by Armstrong et al. (1952). Blisters can be raised by applying cantharidin (the anhydride of cantharidic acid) plaster to the forearm of human subjects. The raised epidermis is cut off and test solutions are poured onto the denuded base. The pain-producing actions of applied kinins are recorded on a pain chart. Bradykinin and 5-hydroxytryptamine produce pain that is burning in character and is delayed in onset for about 20 - 25 seconds, and the pain reactions are slow in reaching a peak. When bradykinin and structurally related agents are applied to the exposed cantharidin blister base (generally referred to as blister base) they also show a characteristic induction of 'after pain'. This 'after pain' is often intense, and is felt during and especially after the removal, of the agents by gentle washing of the area (Armstrong, 1970). This characteristic

action would help a subject or an observer to guess at the nature of the agent that had been applied, because 5-hydroxytryptamine does not cause this 'after pain'. The same 'after-pain' phenomenon can occur also after subthreshold application for pain production.

Tissue injuries liberate 5-hydroxytryptamine and bradykinin-like substances in addition to histamine (Armstrong, 1970). The pain producing actions of histamine are qualitatively and quantitatively different from 5-hydroxytryptamine and bradykinin. In the case of histamine the effective concentrations are very high ( $5 \times 10^{-3}$  to  $5 \times 10^{-4}$ M). Because of this high effective concentration, Keelè and Armstrong (1964) suggested that the direct pain producing actions of histamine are probably not of great physiological significance. The 5-hydroxytryptamine and bradykinin-like agents, on the other hand, are several thousand-fold more effective in inducing pain on the human blister base than is histamine and thus may well be of physiopathological importance. The effective concentrations of 5-hydroxytryptamine for moderate pain productions are low (about  $10^{-6}$ M), bradykinin is at least ten times more active, and its threshold concentrations for pain production range from about  $10^{-8}$  to  $10^{-7}$  M.

#### c) Inflammation

The relation of kinin products to the early stages of inflammation has been explored by many scientists. Since kinins have potent effects on vascular permeability, and since a multiplicity of factors and kallikreins, stimulate their production,

they are prime candidates for inflammatory mediators.

Bradykinin may be a mediator of carrageenin edema, which is a model for testing anti-inflammatory drugs. This type of edema is not readily blocked by anti-histaminic or antiserotonergic drugs (Leme et al., 1973).

d) Prostaglandins

The first evidence that bradykinin could release other vasoactive substances from tissues came from Piper and Vane (1969b). They used lungs isolated from guinea pig and perfused with Krebs solution and measured the release of active substances from the lungs by a cascade bioassay system (Vane, 1964, 1969). When lungs from sensitized guinea pigs were challenged, these researchers detected release of histamine, SRS-A (slow reacting substance of anaphylaxis),  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  (prostaglandins  $\text{E}_2$  and  $\text{F}_{2\alpha}$ ), and a previously unknown substance that contracted the rabbit aorta (Piper and Vane, 1969a, 1969b). This "rabbit aorta contracting substance" (RCS) disappeared very rapidly from the Krebs solution and had a half-life of 1 to 2 minutes. Gryglewski and Vane (1972) suggested that the "rabbit aorta contracting substance" was a prostaglandin intermediate, possibly the unstable cyclic endoperoxide. This suggestion was supported by the fact that arachidonic acid, the prostaglandin precursor, causes a continuous generation of "rabbit aorta contracting substance" from guinea pig lungs (Vargaftig and Dao, 1972). Furthermore, aspirin or indomethacin



which interfere with prostaglandin biosynthesis by substrate competition at the active site of the enzyme (Lands et al, 1974), also prevent the generation of "rabbit aorta contracting substance" (Piper and Vane, 1969a, 1969b). In the 1970's several groups have shown that the cyclic ~~endoperoxide~~ intermediates in prostaglandin biosynthesis, which Hamberg and Samuelsson (1974) have named  $\text{PGG}_2$  and  $\text{PGH}_2$ , also contracted the rabbit aorta. RCS is now known to contain thromboxane  $\text{A}_2$  ( $\text{TXA}_2$ ) which is a prostaglandin (Hamberg et al., 1975).

Bradykinin releases prostaglandins from isolated organs such as guinea pig lung, dog kidney and dog spleen (Vane and Ferreira, 1974). Substances such as indomethacin and meclofenamate have a selective inhibitory action on prostaglandin synthetase at low concentrations, and antagonism by these agents on organ or tissue responses to bradykinin can be assumed to be due to the abolition of prostaglandin synthesis and release. Antagonism by aspirin has been described for bradykinin induced bronchoconstriction or hypotension in guinea pigs, hypotension in the rabbit and renal vasodilation in the dog (Vane and Ferreira, 1974). The mechanism of prostaglandin release by bradykinin is independent of the vascular effects by bradykinin and may reflect activation of phospholipase  $\text{A}_2$  (Vargaftig and Dao Hai, 1972). It has since been demonstrated by McGiff (1980) that bradykinin increases the activity of phospholipase  $\text{A}_2$  in dog kidney cells and this resulted in an enhanced down flow of the arachidonic acid cascade.

Prostaglandins also contribute to the action of bradykinin by potentiating the latter's intrinsic effects. The main contribution of prostaglandins to the pain of inflammation is the sensitization of pain receptors to mechanical or chemical stimulation (Vane and Ferriera, 1974). Vane and Ferriera (1974) did a set of experiments in which prostaglandins have been shown to sensitize nerve ending to the pain-producing activity of bradykinin in the skin of man and in the spleen, knee joints and heart surface of dogs. Prostaglandin in low concentrations has also been reported to potentiate the increased vascular permeability induced by bradykinin (Thomas and West, 1973). Thus, endogenous bradykinin and prostaglandins interact in many biological systems, and aspirin-like drugs modify or abolish the contribution of endogenous prostaglandin generation of these interactions.

In summary, the kinins have an impressive array of effects on various physiological functions. They can affect organ systems directly or indirectly through their interactions with neural pathways and with other mediators. The recognized effects of kinins on prostaglandin synthesis and metabolism emphasize the potentially broad-range effects these potent peptides may have. However, kinins presently have no generally accepted role in normal physiology.

## Kallikreins

Kallikreins were discovered, over fifty years ago by Frey (1926), but even today the name kallikrein still applies to two totally different groups of enzymes. The glandular, organ or tissue kallikreins on the one hand and the plasma or serum kallikreins on the other. The relationship between the two groups of kallikrein appears to be no closer than for example, those between trypsin and thrombin. Plasma kallikreins liberate the nonapeptide bradykinin, (Arg-Prp-Pro-Gly-Phe-Ser-Pro-Phe-Arg) from kininogens whereas the salivary kallikrein and other glandular kallikreins generate the decapeptide kallidin, lysylbradykinin (Webster et al., 1970).

### 1. Glandular Kallikreins in Man

Glandular kallikreins occur mainly in the pancreas, pancreatic juice, saliva, salivary glands, urine, kidney, sweat and sweat glands. They are not inhibited by soybean trypsin inhibitors (SBTI) as opposed to plasma kallikreins which are inhibited by SBTI. Also as mentioned before, the plasma kallikreins release bradykinin from kininogens whereas the glandular kallikreins release kallidin. Glandular kallikreins are present in exocrine secretions in their active form whereas plasma kallikreins are present in the plasma as pre-kallikreins, that is, in an inactive form. The plasma pre-kallikrein is activated enzymatically by a process of limited proteolysis, by the Hageman factor (factor XII) and by trypsin (Wuepper and Cochrane, 1972). In addition, the

plasma kallikreins release kinins mainly from high molecular weight kininogens and may be inactivated by different inhibitor(s) in plasma which do not inactivate the glandular kallikreins (Webster et al., 1970). It has become clear in recent years that in some instances the physiologically significant proteolytic actions of these enzymes are not always related to the release of kinins. Glandular kallikreins are now believed to be involved in the regulation of secretion, sperm motility, cell proliferation, blood flow capillary permeability, and electrolyte concentration (Bhoola et al., 1979). A synopsis of the functional aspects of the glandular kallikrein-kinin system is shown in Fig. 2.

## 2. Cellular localization of Glandular Kallikrein.

There have been numerous studies describing the localization of kallikrein in pancreatic tissues. In every paper except one kallikrein was shown to be present in the apical granule containing part of the pancreatic acinar cells. The only discrepancy was, reported for human pancreatic kallikrein, which was found to be present only in the  $\beta$ -cells of the exocrine gland (Ole Moi Yoi et al., 1979). Amouric et al. (1982) have investigated the immunolocalization of human pancreatic kallikrein at the cellular level, using an indirect immunofluorescence and immunoperoxidase method, and at the subcellular level using the protein A-gold technique (Roth et al., 1978). All their results demonstrated that human pancreatic kallikrein was unambiguously located within the zymogen

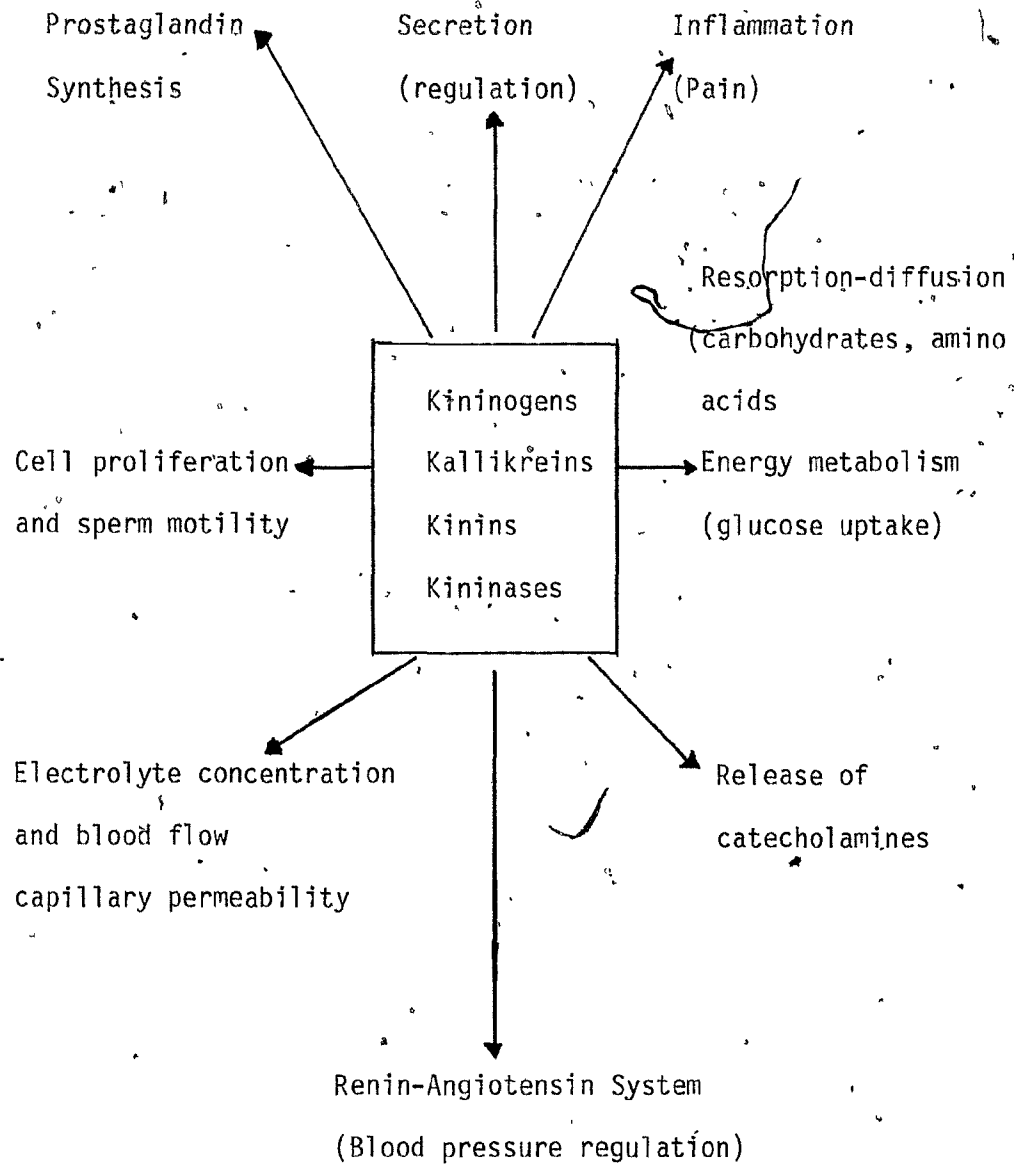


Fig. 2 : Synopsis of functional aspects of components of the glandular kallikrein-kinin system ( Fritz, 1983)

granule of the pancreatic acinar cells and that the enzyme seems to follow the same classic pathway as that described for the other secretory enzymes (Palade, 1975).

However, the cellular localization of salivary kallikrein has only recently been clarified. Earlier studies (Emmelin and Henrikson, 1953) correlated the light microscope appearance of parasympathetically denervated submandibular glands in cat with the kallikrein activity of saliva. They concluded that the demilune cells were the sources of kallikrein. On the other hand, studies done by Bhoola et al. (1966) and by others, for example, Erdos et al. (1968) and Gautvik et al. (1974a), demonstrated that kallikrein is present in secretory granules of many mammalian salivary glands. Bhoola and his co-workers (1973) suggested that kallikrein was located in the secretory granules in the acinar cells guinea-pig submandibular gland.

Most recent studies, however, by Uddin et al. (1981) and Barton et al. (1975) have indicated that salivary kallikrein is located in the striated duct cells and not in the acinar demilune cells.

### 3. Salivary Kallikrein(s)

In 1939, Werle and von Roden discovered kallikreins in the saliva and salivary glands of man.

The salivary glands are innervated by adrenergic and cholinergic nerve fibres which activate the corresponding receptors

(Garrett, 1967). Activation of the cholinergic and  $\alpha$ -adrenergic receptors raises the intracellular calcium ion concentration and this causes a rapid transport of ions and water. At the same time the activation of the  $\alpha$ -adrenergic receptors stimulates the secretion of kallikrein into the saliva in the ducts. As discussed before kallikrein is located in the duct cells of the salivary glands. Since the duct cells are surrounded by a capillary plexus (Burgen et al., 1958) it is possible that a small amount of the salivary kallikrein may also reach the capillary plexus where it could regulate local blood pressure and blood flow. This latter kallikrein function seems possible because:

(i) the vasodilation which occurs in the capillaries of the submandibular glands of the cat after stimulation of the  $\alpha$ -adrenergic receptors is sustained by bradykinin (Gautvik et al., 1974b) and

(ii) the presence of an antigen which is immunologically identical to submandibular kallikrein has been demonstrated in rat plasma (Nustad et al., 1977).

Activation of the  $\beta$ -adrenergic receptors results in increased levels of cyclic AMP, because of an induction of adenylate cyclase which transforms ATP into cyclic AMP. The increased levels of cyclic AMP cause extrusion of enzymes, glycoproteins and other macromolecules (Bhoola et al., 1979). Activation of cholinergic muscarinic receptors and  $\alpha$ -adrenergic receptors stimulates guanylate cyclase to form cyclic GMP from GTP. Perhaps the cyclic

nucleotides so formed combine with specific protein kinases to either phosphorylate or dephosphorylate key membrane proteins and hence alter cation permeability. This could result in an intracellular rise in free calcium and initiate the exocytotic release of kallikrein into the secretory lumen (Albano et al., 1976). It is also likely that the cyclic nucleotides are involved in mediating the effect of kinins on the cellular levels (Albaño et al., 1977). Thus increased levels of calcium ions and cyclic nucleotides; the latter as a result of the activity of kinins on the adrenergic receptors; could modify the activities of intracellular enzymes like glycosyltransferases. In fact Baker et al. (1977) have demonstrated that canine tracheal explants acquire enhanced galactosyltransferase activity in the presence of kallidin (lysyl-bradykinin) which is produced by the salivary kallikrein. It has been demonstrated by Catanzaro et al. (1978), that the salivary kallikrein levels in rats are related to the control of the secretion of sodium ions, potassium ions and water. More recently it has been shown by Martinez et al. (1981), that kallidin inhibits  $\text{Na}^+$ -reabsorption in the rat parotid gland. Perhaps these latter kallidin effects should be attributed to the production of prostaglandin  $\text{E}_1$ , which is stimulated by kinin (Terragno, 1979), and which has also been implicated in the inhibition of  $\text{Na}^+$ -reabsorption.



#### 4. Some Views on the Functional Role of Kallikrein in the Exocrine Glands

The experiments of Hilton and Lewis (1955, 1956) and subsequently those of Gautvik (1970) established the general view that kallikrein regulates vasodilation in all glandular organs during secretory activity, in particular in salivary glands and sweat glands and the pancreas. The generalization and the specific involvement of glandular or tissue kallikrein in regulating functional vasodilation has been questioned by Schachter (1969, 1970) and by Bhoola (1968, 1970). The physiological experiments by Schachter have provided evidence which indicates that kallikrein is not implicated in the vasodilation of the salivary glands produced on stimulation of the chorda tympani nerve.

Kallikrein is secreted primarily into the exocrine secretion of the glands and therefore may have enzymatic function in the gastro-intestinal tract (Bhoola et al., 1979). Furthermore, because of its location in the striated duct cells of all the species examined so far, and its physiological activity in the kidney (Mills and Ward, 1975), it may modulate ion transport across the striated duct cells of the exocrine glands. Although this is an attractive proposition, no evidence exists for such an action in the submandibular glands or the pancreas. Furthermore, the detailed mechanism and sequential steps involved in such an effect is not known. The question whether kallikrein may act directly to alter cell membrane permeability or whether this action depends

entirely on the release of kallidin remains to be answered. Another interesting aspect, of the effect of the kallikrein-kinin system, is the regulation of sperm motility and migration (Palm et al., 1975). The precise mode of action and role of kallikrein in the male and female reproductive tracts remains to be elucidated. Clearly the different glandular kallikreins may have different but specific physiological roles. But if we are to seek some significant function of the glandular kallikrein which is to apply in every case where it is found, then it must surely be some aspect of the secretory process itself that we should look to, because of the ubiquity of the kallikrein in secretory structure.

#### 5. Assay Methods

The method of assay for kallikreins is an important problem as the components involved in the kallikrein-kinin system are rapidly metabolized and their contents in tissues and body fluids are low, extensively variable and dependent upon the physical conditions of the animal and its species.

The hypotensive action of kallikreins has been of great use in determining their activity, but measurement of blood flow has also been used because of its increased sensitivity and other advantages (Moriya et al., 1965). Isolated smooth muscle tissues, such as guinea pig ileum or rat uterus are contracted by the kinins, and this response has also been applied as an assay method. Webster et al. (1961) demonstrated that kallikreins hydrolyze N-substituted

Red

arginine esters and that this esterolytic activity was nearly proportional to the vasodilator activity, especially in urine. This provided a method for the assay of kallikrein by a simple enzymatic procedure.

The bioassay is generally performed by recording the hypotensive activity of the kallikrein on the dog's blood pressure and measuring the ability of a kallikrein to release kinin from a suitable substrate, e.g., acid treated dog plasma. The amount of kinin released is then measured with an isolated muscle preparation such as the guinea-pig ileum or rat uterus. The advantage of bioassays over chemical tests is that they measure directly a specific biological property of the kallikrein. However, in addition to being imprecise, bioassays have pitfalls in so far as the potency of the enzyme with respect to the release of kinin depends on the specific mammalian kininogen on which it is acting. For example, cat salivary kallikrein does not release kinin from horse plasma but horse salivary kallikrein does so readily (Bhoola et al., (1965).

All glandular kallikreins hydrolyze synthetic arginine esters such as N-benzoyl-L-arginine ethyl ester (BAEE) and N-toluenesulfonyl-L-arginine methyl ester (TAME). Other synthetic substrates for trypsin such as benzoyl-arginine-naphthylamide (BANA) and benzoyl-arginine-p-nitroanilide (BAPNA) are not hydrolyzed by salivary or urinary kallikreins (Schachter, 1980). Also, unlike trypsin, kallikrein does not have proteolytic activity towards casein.

Synthetic chromogenic peptide substrates have been introduced recently to measure kallikreins and other proteases. These substrates are p-nitroanilides of tri- and tetrapeptides with various groups substituted at the terminal amino group. The chromogenic substrate itself is colorless but the p-nitroaniline liberated on proteolysis is a colored compound that is readily measured with a spectrophotometer (Cleason et al., 1978). A synthetic peptide substrate, with fluorogenic substituent has also been introduced and is said to have greater sensitivity than the chromogenic substrates (Morita et al., 1977). The chromogenic and fluorogenic substrate methods, like those with the synthetic ester substrates have problems of sensitivity and specificity, and in each case, methods must be developed in order to deal with interfering factors.

Finally radioimmunoassay procedures have been developed (Mann et al., 1977). These are alleged to have the property of remarkable sensitivity, detecting concentrations of kallikreins in picograms per ml. However, there are problems with the complexity of the method and also with its limited specificity. The latter resulting, for example, from the immunological cross reactivity of salivary, pancreatic and urinary kallikreins in the same species (Proud et al., 1977) e.g. in the case of the rat.

D. GLANDULAR KALLIKREIN(S) : IMPLICATIONS IN CF

In searching for a valid biochemical difference between normal subjects and CF patients one has to focus on a definite area. Elevation of the sodium chloride concentrations in sweat and in secretions from the minor salivary glands is a consistent finding in CF (Weisman et al., 1972). This electrolyte abnormality in eccrine sweat of CF patients, was first described by di Sant'Agnese et al. in 1953. It was postulated that, the elevated concentrations of sweat electrolytes reflect a disturbance in cation transport which is generalized among exocrine glands and restricted to them (di Sant'Agnese et al., 1968). The same exocrine glands also produce kallikreins.

When the salivary glands are stimulated they secrete an isotonic solution of electrolytes and macromolecules. During the passage of this solution through the glandular ducts, ions such as sodium are reabsorbed and the final solution leaving the ducts is hypotonic. This reabsorption is believed to be an active process and therefore must be initiated by a component in the secretion (Sutcliffe et al., 1968). Dann and Blau (1978) suggested that the reabsorption of ions is induced by a kinin. According to their hypothesis this kinin (which they called kinin E) is produced by the proteolytic action of a kallikrein (an arginine esterase) on a precursor in the acinar cells. The kallikrein itself is activated by what is suggested to be another exocrine arginine esterase. Their proposal calls for a deficiency in either of these two

enzymes, the kallikrein or the arginine esterase, to be the cause for abnormal secretion in CF. This hypothesis has become less attractive since it has been shown that kallidin inhibits sodium reabsorption (Martinez et al., 1981) and one would therefore anticipate a decreased level of sodium in saliva, if the hypothesis of Dann and Blau was true.

In 1981 Hallinan expanded the hypothesis of Dann and Blau to include a defect in an intracellular enzyme which carries out some functionally important post-translational modification to the arginine esterase, for example a glycosylation or phosphorylation. The result of such a defective enzyme could be an abnormal exocrine secretion process and the particular manifestation of the disease then depends on the nature of the exocrine glands.

The sequence of significant events in CF, in relation to Hallinan's hypothesis is outlined in Figure 3. The primary mutation can be in the structural gene for either of the proteins, numbered 1 to 3. The proposed defects will cause altered interactions between the component molecules of the processing system, before the discharge of the secretion. The result is a defective exocrine secretion processing which could manifest itself differently in the various glands. Perhaps the most direct manifestation is that observed in the sweat glands where a defect in ion reabsorption factor metabolism, results in altered ion concentrations in the sweat. In mucus glands a number of interacting events may account for the clinical problems. On the one

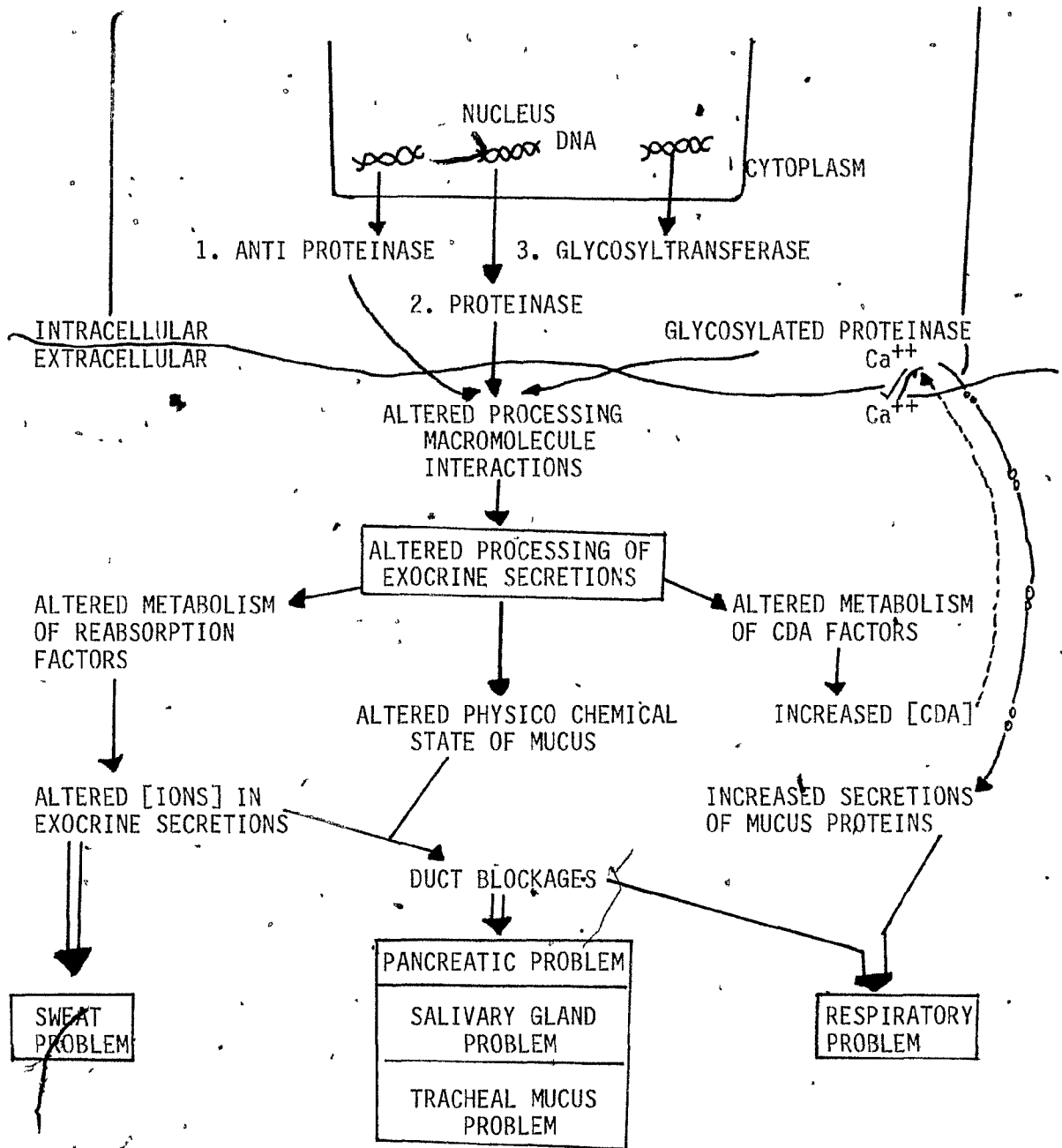


Fig. 3 : Altered regulation of a proteinase mediated primary secretion processing system as the basic defect in CF (Hallinan, 1981).

hand, a combination of altered ion concentrations and/or altered processing of mucus proteins results in an altered physicochemical state of mucus, eventually causing blockages of the duct passages. In the lung on the other hand, increased levels of the CDA (ciliary dyskinesia) factors cause increased secretion of mucus (Conover and Conod, 1978) which, in combination with an altered physicochemical state of mucus, inhibits the ciliary activity thereby initiating the respiratory problems. In Hallinan's hypothesis the CDA factors and other such CF factors, are considered to be normal products, in the primary secretions of the cells. During processing in the glands they are normally broken down but not so in the case of CF where the processing enzyme is believed to be defective.

The altered  $\text{Ca}^{++}$  metabolism in CF (Feigal and Shapiro, 1979) is considered to be the result of the  $\text{Ca}^{++}$  ionophore-like effect (Conover and Conod, 1978) of the CDA factors. If the defect is in the glycosyltransferase which is modulated by  $\text{Ca}^{++}$  concentrations (Bhoola, 1979) it will introduce a further level of complexity to the interpretation of many experimental observations on CF. Aldaheff (1978) has also speculated that the CF defect is an altered glycosylation because of several reports on altered glycosylations of CF proteins. However, as discussed before kallidin itself might also be able to regulate the glycosyltransferases in one way or another, and it is therefore not clear to what extent defects in the primary secretion processing are involved.



The central role of the secretion process implies that it may be more valuable to examine the protein components of an exocrine secretion, such as saliva, in search for the basic defect rather than the serum or plasma, despite the convenience of obtaining the latter.

A glandular kallikrein, specifically salivary kallikrein was chosen for this study. This enzyme was chosen for several reasons:

1. CF is an inherited disease characterized by a generalized dysfunction of the exocrine glands. These glands produce and secrete glandular kallikreins as are found in the pancreas, pancreatic juice, salivary glands, saliva, sweat glands, sweat, kidney and urine.

2. Although the physiological functions of kallikreins in exocrine glands are incompletely understood, it has nevertheless been demonstrated by Catanzaro et al. (1978), that the glandular kallikreins have various physiological functions. Apart from their ability to liberate kinins from kininogens, kallikreins also regulate electrolyte concentrations, in particular sodium and potassium. In vivo experiments with the salivary glands of rats have shown that the amounts of sodium and potassium that are secreted are correlated with a parallel change in kallikrein esterase activity (Catanzaro et al., 1978). The latter results led to the hypothesis that the salivary kallikrein-kinin system is involved in salivary control of sodium, potassium and water secretions.

Since there is an increased level of sodium ions in the saliva of CF patients (Weismann et al., 1970) one might speculate on the relationship between the salivary kallikrein-kinin system and the disease. It should be noted that there is also a lack of water in CF secretion (Wood et al., 1976) which is partly responsible for the increased viscosity and elevated levels of ions such as sodium. The paucity of water in mucous secretions may reflect decreased water secretion, increased secretion of solids without appropriately increased secretions of water, or increased reabsorption of water from the primary secretory fluid. It should be noted that at present there is no evidence to support any of these mechanism.

3. It has been shown that kinins are directly involved in the regulation of secretion. For instance Manning et al. (1982) demonstrated that kallidin and bradykinin inhibit the absorption of electrolytes in the guinea pig ileum.

Thus in summary the fact that CF is characterized by abnormal secretions and ion transport defects makes glandular kallikreins an interesting prospect.

#### E. SCOPE OF THESIS

During the past years, a number of macromolecular "factors" presumably unique to CF have been described, first by Spock et al., (1967), then by Mangos et al. (1967a, 1967b) and later by others.

Rao and Nadler (1972a) have postulated that various "CF factors" are present because of a deficiency of a proteolytic enzyme which would normally hydrolyze and inactivate them. Since the "factors" have not been purified to homogeneity, an empirical approach using synthetic substrates has been taken. Because polylysine can mimic the effect of the "factor" on reabsorption of sodium in rat parotid gland (Mangos et al., 1968), and because polylysine and other cationic polypeptides can be hydrolyzed by enzymes with a specificity similar to that of trypsin, the activity of the 'trypsin-like' enzymes has been assayed in saliva using  $\alpha$ -N-benzoyl-L-arginine ethyl ester as substrate. 'Trypsin-like' activity was defined as the fraction of activity inhibited by an excess of soybean trypsin inhibitor. This activity had an optimum around pH 8.

The mixed saliva of CF patients had about one-eighth the 'trypsin-like' activity of controls, whether the controls were age-matched or adults. Obligate heterozygotes had activities higher than those observed in patients but less than those in controls. Studies on individual families suggested that in most cases, the parents had levels of 'trypsin-like' activities intermediate between those observed in controls and those in the affected children. In other instances, sibs had levels similar to those of their parents. Though this suggested a genetic component of the activity, the individual obligate heterozygote could not be identified with certainty in each case. This deficiency of

'trypsin-like' activity was the first quantitative enzymatic difference between controls and patients with CF (Rao and Nadler, 1972a). Attempts to demonstrate hydrolysis of an amide or peptide bond, which is a more precise measurement of proteolytic activity, were unsuccessful. However, hydrolysis of a typical trypsin substrate and inhibition of the activity by soybean trypsin inhibitor supported the concept of a deficiency of proteolytic activity.

A number of studies have been carried out to determine whether a deficiency of proteolytic activity might account for the CF serum factors (Rao and Nadler, 1974 and 1975). Since many proteolytic enzymes in blood plasma are present as zymogens, a variety of conditions of activation, such as kaolin, acid pH, ellagic acid, and exposure to glass surfaces (Colman et al., 1971), were tested on the plasma of patients with CF and controls. The optimal difference between activity, as measured by the hydrolysis of a typical trypsin or kallikrein substrate,  $\alpha$ -N-toluenesulfonyl-L-arginine methyl ester (TAME), has been observed when activated by 4,4',5,5',6,6'-hexahydroxy-diphenic acid 2,6,2',6'-dilactone which is called ellagic acid (Rao et al., 1972b). This activity was termed plasma kallikrein by virtue of its ability to hydrolyze only esters (but not amides or peptides) of L-arginine and because of its inhibition by soybean trypsin inhibitor. Assays of this activity in samples of plasma of CF patients, their parents and controls showed that the

"kallikrein" activity was about one-half the activity observed when the fractions of activity inhibited by soyabean trypsin inhibitor were compared. Thus, the deficiency observed in mixed saliva was confirmed in plasma.

It has been postulated that the partial deficiency of arginine esterase activity might represent a total deficiency of one of several arginine esterases (Nadler et al., 1978).

Plasma fractionated on 5% polyacrylamide gels by isoelectric focusing at pH 5-8 revealed six bands of arginine esterase activity in control samples, but only five bands in samples from CF patients (Rao and Nadler, 1974). This deficiency of a plasma arginine esterase in CF has been questioned by Leberman (1974) but was confirmed by Coburn et al., (1974). However, Goldsmith et al. (1977) demonstrated normal levels of prekallikrein in CF by coagulant and esterase assays as well as by immunochemical determinations. More recently Bury and Barrett (1982) have demonstrated normal levels of plasma kallikreins in both CF and normal subjects. These studies, however, do not rule out the deficiency of glandular kallikrein in CF because plasma kallikrein and glandular kallikrein are two entirely different enzymes (Webster et al., 1970). Also studies have demonstrated that glandular kallikrein may be transported, somehow, into the plasma (Nustad et al., 1977). Therefore considering the previously mentioned evidence and the involvement of the exocrine glands in CF it seems that it would be essential to study glandular kallikrein

in an effort to find the underlying biochemical defect in CF. Thus the work presented in this thesis was carried out on the premise that the salivary kallikrein, and, in a broader sense the glandular kallikreins, in CF is defective.

The human saliva is an available source of glandular kallikrein. However, the enzyme had to be purified before comparative studies could be carried out. The isolation of human salivary kallikrein has been reported by Fujimoto et al. (1973) and Modeer (1977), but the degree of purification was questionable in each case. Hojima et al. (1975) reported that the protein purified by Fujimoto was not homogeneous when isoelectric focused, while Modeer (1977) did not report on the purity of the protein at all. Hofmann et al. (1983) isolated salivary kallikrein which they obtained by selective catheterization of the glandular duct. Kallikrein was also obtained from the human parotid saliva and purified by Wong et al. (1983), however they did not do a detailed study of the enzyme. The latter group obtained a molecular weight of 38,900 D using SDS-Page which was similar to that found by Modeer (1977) using gel filtration. However this value differed markedly from that of Fujimoto et al. (1973) which was 23,000 D by ultracentrifugation and 28,000 D by gel filtration. The Mw obtained by Wong et al. (1983) also differed from that obtained by Hoffman et al. (1983), who found a molecular weight of 32,000 D. The results from two isoelectric focusing experiments reported, were not similar. Also one of the three amino acid compositions reported

was very different. Therefore, the first step was to purify normal salivary kallikrein and characterize it. After which the CF salivary kallikrein was similarly purified and characterized. The normal and CF salivary kallikreins were compared in terms of their physical and chemical properties.

## CHAPTER 2

### EXPERIMENTAL

#### A. Materials

All reagents used were analytical grade unless otherwise specified. Organic solvents and water were glass distilled prior to use.

Whole saliva stimulated by chewing parafilm wax (Sybron/Kerr) was obtained from both male and female volunteers (5 CF patients between the ages of 19 -24 years, each contributing about 100 ml of saliva per isolation).

Chromatographic media (Sephacryl S-200 and DEAE-Sephacel); standard protein mixture containing  $\alpha$ -Lactalbumin ( $M_w = 14.4$  KD) soybean trypsin inhibitor ( $M_w = 20.1$  KD), carbonic anhydrase ( $M_w = 30$  KD), ovalbumin ( $M_w = 43$  KD), bovine serum albumin ( $M_w = 67$  KD) and phosphorylase b ( $M_w = 94$  KD); and Pharmalyte (pH 3-10 and pH 2.5-5.0) were obtained from Pharmacia.

The Spherogel-TSK 3000 SW column (7.5 x 300mm), 4000SW column (7.5 x 300mm) and a Ultrasphere-ODs column (4.6 x 250mm) were purchased from Beckman Instruments.

Most of the synthetic substrates were obtained commercially from Sigma Chemical Co. Sources of other materials were :  
bovine serum albumin - Miles Laboratories; soybean trypsin inhibitor, lima bean trypsin inhibitor and trypsin - Worthington Biochemical Corp.; diisopropyl fluorophosphate - Aldrich Chemical



Company Inc.

All other unspecified chemicals were highly purified commercial products.

## B. METHODS

### 1. Column Chromatography

Columns of DEAE-Sephacel and Sephacryl were prepared according to the manufacturer's instructions.

An affinity column of bovine trypsin inhibitor (BPTI) bound covalently to Sepharose 4B was prepared locally; CNBr activated Sepharose was purchased from Pharmacia and 1 g of this gel was washed with 200 ml of 1 mM HCl on a glass filter. After which the gel was washed with 25 ml of 0.25 M  $\text{NaHCO}_3$  buffer, pH 8.8, containing 0.5 M NaCl and then suspended in 5 ml of the same bicarbonate-sodium chloride buffer containing 10mg BPTI (Sigma). The suspension was gently shaken overnight at  $2^\circ\text{C}$  after which the gel was washed with 0.25 M  $\text{NaHCO}_3$  pH 8.8 and then resuspended in 10 ml of 0.2 M glycine buffer, pH 8.5. After gentle shaking for 2 h at room temperature, the gel was washed once more with at least 100 ml of 0.1M sodium acetate buffer, pH 4.0. The gel was poured into a column of 0.9 cm diameter and carefully equilibrated with 0.05 M sodium phosphate buffer, pH 8.0. The amount of BPTI coupled to 1 g of gel was sufficient to bind the kallikrein present in at least 500 ml of saliva.

HPLC was performed with a system consisting of a Beckman Model 110A pump and a Hitachi model 100-40 spectrophotometer which was set at 275 nm. The sensitivity range of the spectrophotometer output was set at 0.2. In each chromatographic experiment the Spherogel-TSK 3000 SW column was loaded with 100  $\mu$ l of the protein solution previously purified by affinity chromatography and this solution was pumped through the column with a flow rate of 0.5 ml per min at room temperature.

## 2. Enzyme Assays

The arginine esterase activity, was used to monitor the purification of salivary kallikrein. The activity was determined with the method described by Schwert and Takenaka (1955). The assay was carried out using the artificial substrate BAEE and the increase in absorbance at 253 nm was followed on a Zeiss PMQ II or Cary Model 14 spectrophotometer.

Kallikrein activity was also measured by the release of kinins from acid treated dog plasma (Horton, 1959). The kinins released were extracted and assayed by the contractions of guinea pig ileum (Stewart et al., 1970) or rat uterus (Trautsohold, 1970) using a force transducer and a Sanborn Pen Recorder.

The effect of the salivary kallikrein on a rabbit's blood pressure was also measured (Horton, 1959). A rabbit weighing approximately 2.5 Kg was anaesthetized with sodium barbitol and then given a steady supply of oxygen, via an oxygen mask, thus

negating the need to cannulate the trachea. The blood pressure was recorded from a carotid artery using a Statham SP1400 monitor. An injection of about 10 µg kallikrein, in saline, into the femoral vein was used to lower the blood pressure.

### 3. Analytical Methods

#### (i) Protein determination

Protein was determined by the reaction with Folin-Ciocalteu reagents as described by Lowry et al. (1951) and with Coomassie Blue by the Bio-Rad method (Bradford, 1976). A protein standard (Bio-Rad) was included in each determination.

#### (ii) Gel Electrophoresis

##### (a) Gel electrophoresis at pH 9.5

Gel electrophoresis was done at 2°C with polyacrylamide slab gels (7.5% T, w/v) of 16 x 16cm. A continuous buffer of 0.37 M Tris-glycine, pH 9.5 was used (Gordon, 1969). The protein was dissolved in a 1:10 dilution of the buffer with 10% glycerol and approximately 20-100 µg was applied to a pre-equilibrated well of the slab gel (15 mA for 1 hr). Electrophoresis was conducted at approximately 30 mA until the marker dye (Bromophenol Blue) had moved to the bottom of the gel. The gel was stained with 0.125% Coomassie Blue in 10% trichloroacetic acid (Fazekas de St. Groth et al., 1963). The purity of the kallikrein was determined from a tracing with an automatic recording densitometer of pictures taken of the gel after destaining.

(b) Gel electrophoresis in the presence of dodecylsulfate.

Estimates of the  $M_w$  (molecular weight) values were obtained by gel electrophoresis on 10%T (w/v) polyacrylamide resolving gel and a 4%T stacking gel. The slab was run in a buffer of 0.025 M Tris, containing 0.192 M glycine, pH 8.3 plus 0.1% sodium dodecylsulfate (Laemmli, 1970). The slab gel, 16 x 16 cm, was calibrated with a standard mixture of proteins. The kallikrein samples were dissolved in 0.01 M Tris-glycine buffer along with at least 5-fold excess of dodecylsulfate with or without 1% (v/v)  $\beta$ -mercaptoethanol. The samples were heated at 100°C for 5 min and glycerol and tracking dye were added before about 20  $\mu$ g of protein was loaded on the pre-equilibrated gel. Electrophoresis was conducted at 30 mA until the tracking dye had moved to the bottom of the gel. The slab was treated with 10% (w/v) trichloroacetic acid in 25% isopropyl alcohol for about 30 min and then stained with Coomassie Blue in methanol/acetic acid/water (50/10/40, v/v). The slab was destained in a solution of 7% acetic acid, 5% methanol in water.

(c) Activity from Page-SDS

Gels, 8x0.5 cm, were prepared in 0.1 M sodium phosphate, pH 7.2 containing 0.1% SDS as described by Weber and Osborn (1975). The gels were prepared with 7.5% acrylamide with 3% crosslinking. The gels were electrophoresed for 1 h at a low current of 1 mA/gel and then loaded with an aliquot of 50  $\mu$ l of kallikrein solution, containing from 5 to 50  $\mu$ g enzyme. The gels were

electrophoresed at 8 mA/gel together with a gel which was loaded with a standard protein mixture (Pharmacia). When the tracking dye, bromophenol blue, reached a level close to the bottom of the gel, the electrophoresis was stopped. The gels were sliced with a device obtained from Miles laboratories and the slices were dispersed in 0.1 M Tris-HCl buffer with a Polytron (Kinematic GMBH, Switzerland). The activities of the suspensions were measured with BAEE.

(iii) Amino Acids

The purified enzyme (about 20 - 30  $\mu$ g) was hydrolyzed in evacuated sealed tubes in 6 M HCl at 110°C for 24 h. The solution was evaporated to dryness and the residue suspended in 100  $\mu$ l of 0.2 M citrate buffer, pH 2.2. Analyses were performed with a Beckman Model 6300 Automatic Amino Acid Analyser, with the system recommended by the Beckman Company. Separate samples were oxidized with performic acid at 20°C for 4 h and then analyzed for cysteic acid and methionine sulfone following acid hydrolysis (Hirs, 1967).

Tryptophan was determined by the method of Goodwin and Morton (1946).

From the absorption measurements at 294.4 and 280 nm one can calculate the tyrosine/tryptophan ratio with the equation:

$$\frac{\text{Tyrosine}}{\text{Tryptophan}} = \frac{0.592 A_{294.4} - 0.263 A_{280}}{0.263 A_{280} - 0.170 A_{294.4}}$$

The wavelength of 294.4 nm represents the isosbestic point for tyrosine and tryptophan at pH 13.0 (0.1 M NaOH) with a molar extinction coefficient  $A = 2,395 \text{ cm}^{-1}$ .

At 280 nm the molar extinction coefficients of tyrosine and tryptophan at pH 13.0 are respectively,  $A = 1,540$  and  $A = 5,340 \text{ cm}^{-1}$ . Since both wavelengths are close to the maximum absorption, the slopes of the absorption curves are not yet too steep and the method is fairly reliable when :

- a) Beer's law applies
- b) no other absorbing materials are present, and
- c) the molar extinction coefficients of tyrosine and tryptophan are independent of their incorporation into proteins.

To ensure that the extinction coefficients are independent of incorporation into the polypeptide chain the protein solutions at pH 13.0 were heated at  $70^{\circ}$  for 1 hour.

#### (iv) Carbohydrates

Carbohydrates were assayed by the method of Dubois et al. (1956) using a mixture of galactose and mannose (1:1) as standard. The method of Warren (1959) was used for sialic acid determinations after hydrolysis at pH 2.0 and  $80^{\circ}\text{C}$  for 1h.

The purified enzyme was also chromatographed on Concanavalin A- and wheat germ lectin- Sepharose (both purchased from Sigma).

(v) N-Terminal end group

The phenylthiohydantoin derivative was obtained by the Edman method (Edman and Begg, 1967). The phenylthiohydantoin residue was identified by reversed phase chromatography using an Ultrasphere-ODS (4.6 x 250 mm) column obtained from Beckman Instruments. HPLC was carried out with the ternary isocratic solvent system; consisting of 68.5% of 0.01 M sodium acetate (pH 5.2), 31.5% acetonitrile plus 0.5% dichloroethane as described by Lottspeich (1980). The HPLC was carried out at room temperature using a Beckman HPLC system a flow rate of 1.0 ml/min and a detection wavelength at 265 nm.

(vi) Preparation of  $\alpha_2$ -Macroglobulin.

Plasma was obtained from volunteers.  $\alpha_2$ -Macroglobulin was prepared according to the method of Richman and Verpoorte (1981) and assayed as described by Ganrot (1966).

The purity of the  $\alpha_2$ -macroglobulin preparation was determined by gel electrophoresis in the presence of dodecylsulfate and by HPLC on a Sphérogel TSK 4000SW (7.5 x 300 mm) column with 0.02 M phosphate buffer, pH 6.0 and a flow rate of 0.5 ml/min.

#### 4. Physical Methods

##### (i) Isoelectric Focusing

Isoelectric focusing was done using a LKB 8101 column (LKB, Sweden). The pH gradients were formed with Pharmalyte pH 3-10 or Pharmalyte pH 2.5-5.0 in sucrose density gradients. The anolyte (+) and catolyte (-) solutions were, respectively, 0.01 M sulfuric acid and 0.1% dimethylamine or 0.1 M NaOH.

##### (ii) Ultra Violet Absorption Spectra

Ultra violet absorption spectra were measured with a Cary Model 14 recording spectrophotometer. The rate of hydrolysis of BAEE by the salivary kallikrein was also followed on this spectrophotometer.

##### (iii) Sedimentation Equilibrium

The molecular weight,  $M_w$ , of salivary kallikrein from normal individuals was calculated from schlieren pictures taken at sedimentation equilibrium. The experiment was done with a Spinco Model E analytical ultracentrifuge at 20° using a double sector centerpiece.

A solution of pure kallikrein containing ca 150 µg enzyme was exhaustively dialysed against water and then freeze dried. The protein was redissolved in 100 µl of 0.1 M sodium acetate buffer, pH 5.0 and transferred to one sector of the double sector cell. In the other sector was placed 110 µl of acetate buffer.



An initial rotor speed of 15,000 rpm (revolutions per minute) was used for 3 h, after which the rotor speed was reduced to 11,000 rpm. The run was continued for another 16 h and pictures were taken using a bar angle of 40 degrees.

The molecular weight,  $M_w$  was calculated by the method of Lamm (1929), using the following equation:

$$M_w = \frac{2RT}{(1-v\rho)\omega^2} \frac{d[\ln(1/r \, dc/dr)]}{dr^2}$$

where R is the molecular gas constant (  $8.314 \times 10^7$  ergs per degree per mole)

T = temperature in  $^{\circ}\text{K}$

$\omega$  = angular velocity ( $2\pi \times$  revolutions per sec)

v = partial specific volume of kallikrein which was calculated from the amino acid composition as suggested by Cohn and Edsall (1943).

$\rho$  = the density of 0.1 M sodium acetate

r = the distance in cm from a position in the cell to the axis of rotation.

$dc/dr$  = the distance between the schlieren patterns produced by the two sectors of the cell at the same distance r from the axis of rotation.

It can be seen from the equation that a plot of  $\ln(1/r \, dc/dr)$

versus  $r^2$  should yield the molecular weight  $M_w$ .

Once the value of  $M_w$  is obtained one can estimate the ratio of the kallikrein concentrations at the cell bottom and the meniscus by the equation:

$$M_w = \frac{RT}{(1-v_p)\omega^2} \frac{dc/dr}{rc}$$

In this calculation it was assumed that the  $M_w$  by the latter equation equals the value of  $M_w$  as calculated by the Lamm equation.

So that:

$$\frac{|dc/dr|}{rc} \Big|_{\text{meniscus}} = \frac{|dc/dr|}{rc} \Big|_{\text{cell bottom}}$$

This equation can be used to calculate the concentration ratios at any two positions in the cell.

#### (iv) Fluorimetry

Fluorescence measurements were made with an Aminco-Bowman spectrofluorimeter at room temperature, using a 3 mm square cuvette which requires only 100  $\mu$ l of solution. The method of Melhuish (1962) was used to correct the spectra for variation in transmission, photomultiplier response and light source intensity with wavelength.

A horizontally oriented polarizer was used in the excitation beam to reduce scattering during measurements on the intrinsic fluorescence (Chen, 1966).

The intrinsic fluorescence spectra of proteins can be analysed to determine the number of independent contributing fluorophores, that is, fluorescing components (Weber, 1961). These analyses can be done by simple algebraic calculations. The fluorescence intensities at  $n$  different wavelengths must be measured with  $m$  different wavelengths of excitation. A rank analyses of the resulting  $m \times n$  matrix then will give the number of fluorophores, since the rank of a matrix is the order of the largest square submatrix whose determinant is different from zero. The determinant of a submatrix is considered zero as its value divided by the permanent, which is the sum of all the determinant products, falls within the experimental error of the fluorescence intensity measurements.

Since only three fluorophores, i.e., tryptophan, tyrosine and phenylalanine contribute to the intrinsic fluorescence spectra, a  $4 \times 4$  matrix normally suffices to determine whether all the groups contribute. Four different emission spectra were measured for kallikrein, each with a different excitation wavelength. The fluorescent intensities at four different wavelengths in each spectrum were corrected according to Melhuish (1962) and the corrected intensities were combined in a  $4 \times 4$  matrix and then analysed.

Reference spectra were measured with N-acetyl tyrosine ethyl ester and N-acetyl tryptophan ethyl ester, both purchased from Aldrich Chemical Co.

(v) Circular Dichroism.

The circular dichroism (CD) was measured below 250nm, using a Cary 6001 instrument. The data were converted to  $\text{deg.cm}^2.\text{dmol}^{-1}$  using a mean residue weight of 106 which was calculated from the amino acid composition. The experiments were conducted with protein concentrations of about 100  $\mu\text{g}$  per ml in a 1mm cuvette.

The mean residue ellipticity,  $[\theta]$ , at any wavelength,  $\lambda$ , can be expressed as:

$$[\theta] = f_H [\theta]_H^{\bar{n}} + f_\beta [\theta]_\beta + f_t [\theta]_t + f_R [\theta]_R$$

(Chang et al., 1978) where  $[\theta]_H$ ,  $[\theta]_\beta$ ,  $[\theta]_t$  and  $[\theta]_R$  are the reference ellipticity values of the  $\alpha$ -helix,  $\beta$ -form,  $\beta$ -turn and unordered form at the wavelength  $\lambda$ . The  $f_j$ 's are the corresponding fractions of the structural elements so that :

$$\sum f_j = 1 \text{ and } 1 \geq f_j \geq 0$$

The superscript  $n$  refers to the average number of amino acid residues per helical segment and was chosen to be  $\bar{n} = 10$ .

To account for the chain length dependence of the reference value of  $[\theta]_H$  one can use :  $[\theta]_H^{\bar{n}} = [\theta]_H^\infty (1-k/\bar{n})$  where  $[\theta]_H^\infty$  refers to a helix of infinite length and  $k$  is a wavelength dependent constant. Values for  $[\theta]_H$ ,  $k$ ,  $[\theta]_\beta$ ,  $[\theta]_t$  and  $[\theta]_R$  were obtained from Dr Yang (personal communication).

With the 4 reference spectra and the equation for the observed mean residue ellipticities the fractions of secondary structure of the salivary kallikrein were estimated by a least

squares curve fitting method, using ellipticity data between 240 and 195 nm.

## CHAPTER 3

### FRACTIONATION OF WHOLE SALIVA

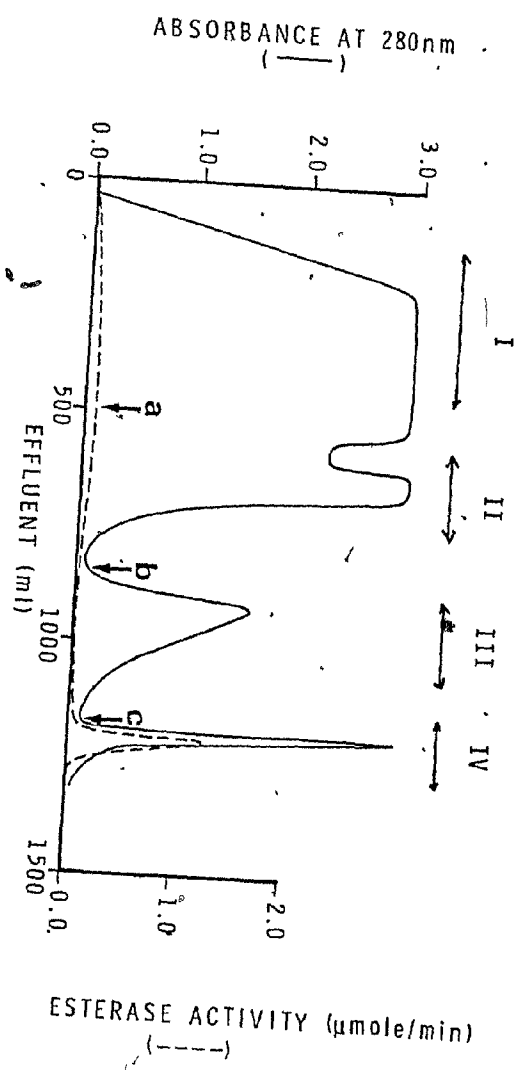
#### 1. Results

##### (i) Fractionation

Whole saliva was obtained after stimulation by chewing parafilm wax. The saliva was kept frozen until used. About 500 ml of saliva was thawed and centrifuged at 4000x g for 10 min. The supernatant was collected and filtered through a nylon screen with 37  $\mu$ m mesh opening to remove any other particulate materials. The clear solution was directly poured over a 10 x 3 cm DEAE-Sephacel column equilibrated with 0.01M sodium phosphate buffer, pH 8.0. The unbound material was collected and after all of the saliva supernatant had passed through, the column was washed with 0.01 M sodium phosphate buffer, pH 8.0. Washing with this phosphate buffer initially increased the absorbance of the effluent and was continued until the eluate had a very low absorbance at 280 nm. The column was then further washed with 0.1 M sodium acetate buffer, pH 5.5. The absorbance of the eluate at 280 nm, which was monitored, showed that this buffer removed more material from the column. When the absorbance was approximately zero the column was washed with 0.1 M sodium acetate, pH 5.5 containing 0.5 M NaCl to remove the remaining protein. The chromatogram obtained with saliva of normal individuals is shown in Fig 4. Each set of fractions were dialysed against distilled water and

Fig 4.: Fractionation of normal filtered whole saliva supernatant by DEAE-Sephacel chromatography. A DEAE-Sephacel column 10 x 3 cm, equilibrated with 0.01 M phosphate, pH 8.0 was loaded with 500 ml saliva supernatant. After all the saliva had passed through, the column was washed with buffers. Four fractions were obtained which are respectively; unbound material (I), material eluted with 0.01 M phosphate (II), with 0.1 M sodium acetate pH 5.5 (III) and with 0.1 M sodium acetate, pH 5.5 containing 0.5 M NaCl (IV). Only fraction IV showed arginine esterase activity which was assayed with BAEE. The activity is expressed in  $\mu$ mole arginine ester hydrolysed per min at 37°C and pH 8.0.

The arrows indicate changes in the eluant; to 0.01 M phosphate, pH 8.0 at a; to 0.1 M sodium acetate pH 5.5 at b; and to 0.1 M sodium acetate plus 0.5 M sodium chloride, pH 5.5 at c.





used for further experiments.

Separate experiments were conducted with normal whole saliva and the whole saliva from CF patients. The amounts of non-dialysable materials in the 4 fractions were weighed after drying and the results are summarised in Table 1.

#### (ii) Gel Electrophoresis

The results of SDS-PAGE of the individual DEAE-Sephacel fractions at pH 8.3 are shown in Fig. 5. The gels were stained with Coomassie Blue as shown in Fig. 5A, or with Schiff's reagent after periodic acid oxidation, see Fig. 5B. The latter staining method gives broader bands but also demonstrates the presence of several carbohydrate containing components of fairly high Mw values.

The major components which stained with Coomassie Blue do appear to contain little or no carbohydrate. No difference has been detected between the various comparable fractions obtained from normal or CF saliva, at least not in the predominant protein and carbohydrate compounds. There appears a difference in fraction IV obtained by eluting the DEAE-Sephacel column with 0.1 M sodium acetate buffer plus 0.5 M NaCl. This fraction from normal saliva contains a band which stains with Coomassie Blue and which has a Mw value of about 30,000. However, when the same gels after staining with Coomassie Blue were treated with the silver stain this band disappeared, see Fig. 6. With silver stain one

Table 1

DEAE-Sephacel fractionation of filtered saliva supernatant.

Fraction	CF mg*	Normal mg*
Non-bound	12.4	8.5
0.01M Phosphate, pH 8.0	5.1	2.7
0.1M Sodium acetate, pH 5.5	3.3	3.0
0.1M sodium acetate/0.5M NaCl	7.6	4.7

\*Average of samples taken from 3 individuals, original volume 10 ml.

Fig. 5 : SDS-PAGE at pH 8.3 of fractions from DEAE-Sephacel chromatography. The gels were stained with Coomassie Blue (A) or with Schiff's reagent after periodic acid oxidation (B). CF saliva fractions I to IV in lanes 1 to 4, normal saliva fractions I to IV in lanes 5 to 8. Each lane was loaded with 100  $\mu$ g material. The arrow indicates the position of the 30,000 Mw polypeptide which appears to be absent in CF.



A



B



1  
2  
3  
4  
5  
6  
7  
8

1  
2  
3  
4  
5  
6  
7  
8

Fig. 6 : The gel shown in Fig. 5A was washed free of Coomassie Blue and stained by the silver staining procedure. The DEAE-Sephacel fractions I to IV from CF saliva in lanes 1 to 4, and fractions I to IV from normal saliva in lanes 5 to 8.



1 2 3 4 5 6 7 8

observes more bands than with Coomassie Blue, however, the patterns obtained with normal and CF fractions are still qualitatively the same.

### (iii) Esterase Activity in Whole Saliva

The esterase in whole saliva supernatant is very stable. The supernatant can be stored at 25<sup>0</sup> for at least 72 h without any loss in activity. At least 70% of the esterase activity in whole saliva supernatant can be found in fraction IV from DEAE-Sephacel obtained with 0.1 M sodium acetate/0.5 M NaCl, pH 5.5.

The esterase is degraded rapidly by trypsin, but this reaction is blocked by soybean trypsin inhibitor (STBI). The arginine esterase in saliva supernatant does not appear to be active against BAPNA (benzoyl arginine-p-nitroanilide), and it is not inhibited by STBI although it is inhibited by bovine pancreatic trypsin inhibitor (BPTI). Inhibitors of the esterase will be discussed in detail in the next chapter.

## 2. Discussion

Whole saliva was obtained from normal individuals and CF patients while they were chewing dental wax. The saliva was mostly collected during the afternoon or early at night, thus at comparable stages during the daily cycles of the salivary glands (Dawes, 1974). The CF patients were young adults who had been known to have the disease for about 15 years or longer. Because of their ages the age matching of saliva controls was no problem.

The initial fractionation of centrifuged whole saliva by DEAE-Sephacel chromatography resulted in four fractions. A surprisingly large amount of macromolecular materials, mostly protein, did not bind to the DEAE-Sephacel at the pH of the saliva which is normally close to pH 7.8. Furthermore some compounds, again mostly protein that bound very weakly could be eluted with 0.01 M sodium phosphate buffer, pH 8.0 (fraction II). We suspect that these fractions of macromolecules form large aggregates in solution which either did not bind or bound very weakly to the DEAE-Sephacel. The effect of the phosphate ions on this binding, however, at this moment is not clear.

The amounts of non-dialysable materials, that is, the four fractions obtained by chromatography were more or less the same for normal and CF saliva. Although, the CF saliva showed consistently larger amounts of macromolecular compounds in the non-bound fraction I.



Gel electrophoresis of all fractions from DEAE-Sephacel chromatography at pH 8.9 was attempted. The results were disappointing in that the non-bound material (fraction I) did not move into the gel at all whereas most of the materials in fraction II also refused to move into the gel.

Gel electrophoresis in the presence of SDS was more interesting and demonstrated the qualitative similarity in the macromolecular compositions of centrifuged control and CF saliva. Not only were the Coomassie Blue stained gels very much alike, attesting to the similar protein compositions, the gels stained with Schiff's reagent also showed that the carbohydrate compounds are similar. Only one band in the Coomassie Blue stained gels of fraction IV from DEAE-Sephacel chromatography with Mw of approximately 30,000 appeared absent in the CF samples. Although this band was noted on several occasions it was not consistent and we have no evidence to show whether or not this band is related to CF. Furthermore when the gels were stained with Coomassie Blue and then washed with 40% methanol/10% acetic acid (v/v) followed by 10% ethanol/5% acetic acid (v/v) this band disappeared and was not found by staining with the silver stain reagent. (Merrill et al., 1981). It may therefore be argued that this band represents a protein which is rather poorly fixed in the gel and that it could be lost during staining. Thus it seems possible that this component may also be present in the CF saliva. The procedure for staining for phosphate (Cutting et al., 1973) has

also been applied. This method gives much less sharply defined bands. The gels obtained by SDS-PAGE showed very broad zones of phosphate which indicated that the 3 major protein components, with Mw about 60,000, and also the carbohydrate components contained phosphorous. Because of this observation it is suggested that the major protein components are phosphoproteins, probably proteins A and C of Bennick (Bennick, 1977) and that they show abnormal electrophoretic mobility during SDS-PAGE.

About 75% of the arginine esterase activity in the centrifuged whole saliva could be found in the most strongly bound fraction on DEAE-Sephacel. Although CF saliva at first appeared to have slightly less activity than normal saliva, a statistical analysis of the data indicated no significant difference. This material served as a starting material for the further purification of salivary kallikrein.

CHAPTER 4  
PURIFICATION AND CHARACTERIZATION OF KALLIKREIN FROM 'NORMAL'  
INDIVIDUALS

1. Results

A. Purification

(i) DEAE-Sephacel column chromatography

Whole saliva was centrifuged, filtered and the clear supernatant was chromatographed on a DEAE-Sephacel column as described in the previous chapter.

The arginine esterase activity was eluted with 0.1 M sodium acetate, pH 5.5 containing 0.5 M NaCl (see chapter 3), Fig. 4, fraction IV. The combined fractions with arginine esterase activity were dialysed against saturated ammonium sulfate which precipitated the enzyme.

(ii) Sephacryl S-200 column chromatography

The material precipitated with ammonium sulphate was extracted 2x with a small amount of 40% saturated ammonium sulfate. The combined extracts were pooled and contained 100% of the enzymatic activity. This solution was again dialysed against saturated ammonium sulfate and the precipitate was collected. The precipitate was dissolved in a small volume of water and then chromatographed on a 60 x 1.5 cm column of Sephacryl S-200 equilibrated with 0.02 M sodium acetate pH 5.5. The column was

eluted with the same acetate buffer and the results are shown in Fig 7.

(iii) Affinity Chromatography on BPTI-Sepharose 4B

The most active fractions after chromatography on the Sephacryl S-200 column were dialysed briefly against distilled water and adjusted to pH 8.0 with phosphate buffer to a final concentration of 0.05 M. Soybean trypsin inhibitor (SBTI), 0.01 volume of a 0.2% solution, was added and the solution was chromatographed on a BPTI-Sepharose 4B column. When the solution had completely run into the column the elution was started with 0.05 M phosphate buffer until the absorbance of the effluent at 280 nm was essentially zero. Further washing of the column with phosphate buffer was done while the absorbance of the effluent was monitored at 230 nm, until this absorbance was also virtually zero. The enzyme bound strongly to the column at pH 8.0 but could be eluted by washing with 0.05 M sodium acetate, pH 3.3, see Fig.

8:

(iv) High performance liquid chromatography (HPLC)

The active fractions from the BPTI affinity column chromatography were dialysed against water and concentrated by freeze drying to a volume of less than 0.5 ml. The pH was adjusted by addition of sodium phosphate buffer pH 6.0 to a final concentration of 0.02 M. The solution was chromatographed on a

Fig. 7 : Chromatography of partially purified salivary kallikrein on Sephacryl S-200, with 0.02 M sodium acetate, pH 5.5. The 60 X 1.5 cm column was eluted with a flow rate of 15 ml per hour at 2°C. The absorption of the fractions was measured at 280 nm and the arginine esterase activity was monitored with the substrate BAEE.

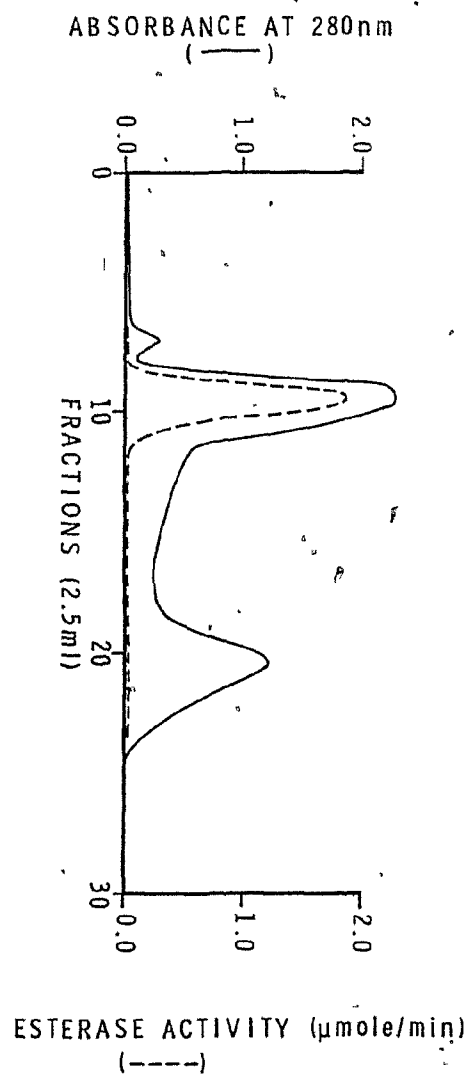
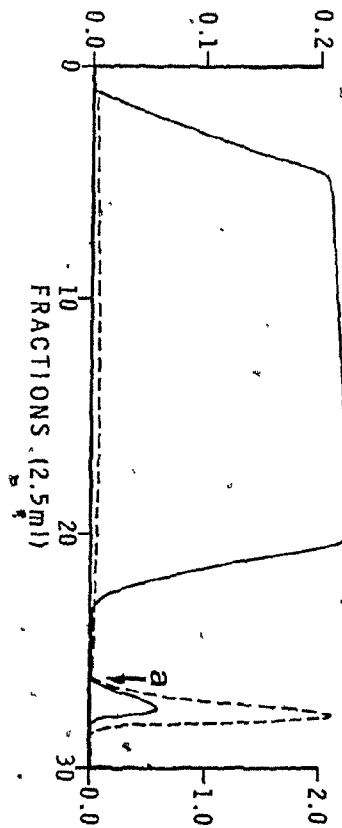


Fig. 8 : The affinity chromatography of partially purified kallikrein on a BPTI-Sepharose 4B column. The column was washed first with 0.05 M phosphate buffer, pH 8.0 which, at arrow a, was changed to 0.05 M acetate buffer pH 3.3.

The absorbance was measured at 280 nm as well as 230 nm before the change in buffer. Activity was followed using BAEE for substrate.

ABSORBANCE AT 280nm  
(—)



ESTERASE ACTIVITY ( $\mu\text{mole/min}$ ).  
(---)



0.75 x 30 cm Spherogel TSK 3000 SW column. The column was loaded with 0.1 ml of the protein solution and a flow rate of 0.5 ml per min was maintained. As shown in Fig 9, HPLC resulted in three fractions but more than 80% of the arginine esterase activity was found in the first peak. While the last peak did not contain any arginine esterase activity.

The procedure for the purification of salivary kallikrein is summarised in Table II. No excessive loss of activity was observed during any of the purification steps. Although affinity chromatography on the BPTI-Sepharose column provides the largest purification factor of a single step in the procedure, the enzyme was still not pure after passing through the column. The last step in the purification procedure was done by HPLC at pH 6.0 and this removes the remaining inactive materials from the enzyme.

#### B. Purity of the Kallikrein

The solution containing the arginine esterase activity obtained by HPLC was dialysed against water, concentrated and rechromatographed on the HPLC column. A single peak was obtained (see Fig. 10). In order to assess the homogeneity further the solution from the first HPLC peak was subjected to isoelectric focusing in the range of pH 3.0 to 10.0 and also in the range of pH 2.5 to 5.0. A typical pattern obtained with the protein is shown in Fig. 11. A single esterase activity peak,  $pI = 4.07$  was

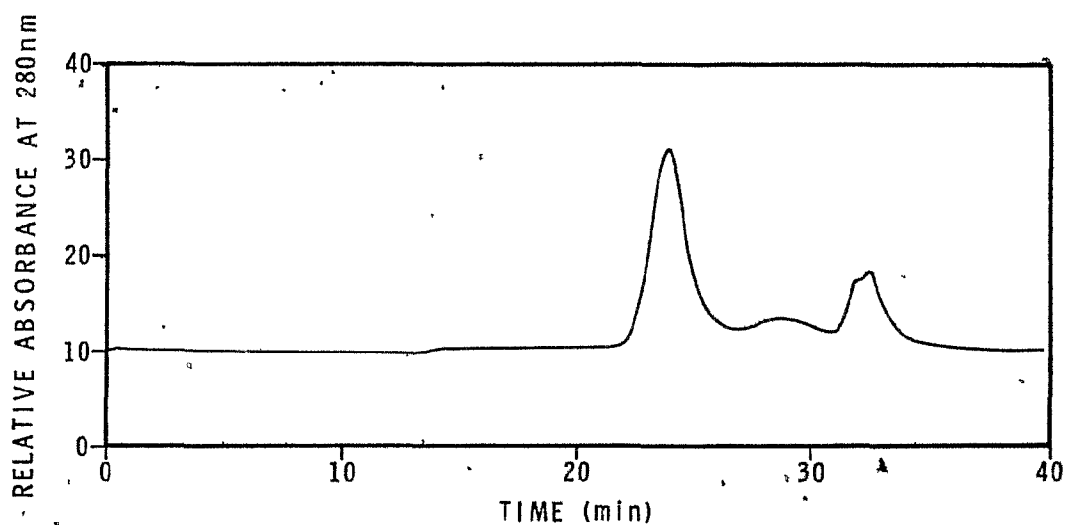


Fig. 9 : HPLC of partially purified salivary kallikrein on a Spherogel TSK 3000 SW column with 0.02 M sodium phosphate pH 6.0 and a flow rate of 0.5 ml per min. About 80% of the arginine esterase activity emerged from the column between 23 and 25.5 min. The fractions which emerged between 26 and 31 min showed a small amount of activity but the peak which was eluted between 31 and 34 min did not have any activity towards BAEE.

Table II

Purification of Normal Human Salivary Kallikrein

Preparation	Volume	Protein <sup>a)</sup>	Total	Specific <sup>b)</sup>	Total	Yield	Purification
(steps)	(ml)	(mg/ml)	protein	activity	Units	(%)	(fold)
			(mg)	(nmol/min/ mg protein)	(nmol/min)		
1. Whole saliva centrifuged and filtered	500.0	1.21	505.0	1.78	1076.9	100.0	1.0
2. DEAE-Sephacel	74.0	0.80	59.2	15.96	944.8	87.7	9.0
3. Sephacryl S-200	50.0	0.240	12.0	60.50	726.0	67.4	34.0
4. BPTI-Affinity	4.5	0.1150	0.520	987.0	510.8	47.4	544.0
5. HPLC	7.5	0.0102	0.0765	4880.0	373.3	34.7	2742.0

a) Data based on the protein assay by the Bio-Rad method.

b) BAEE was the substrate used.

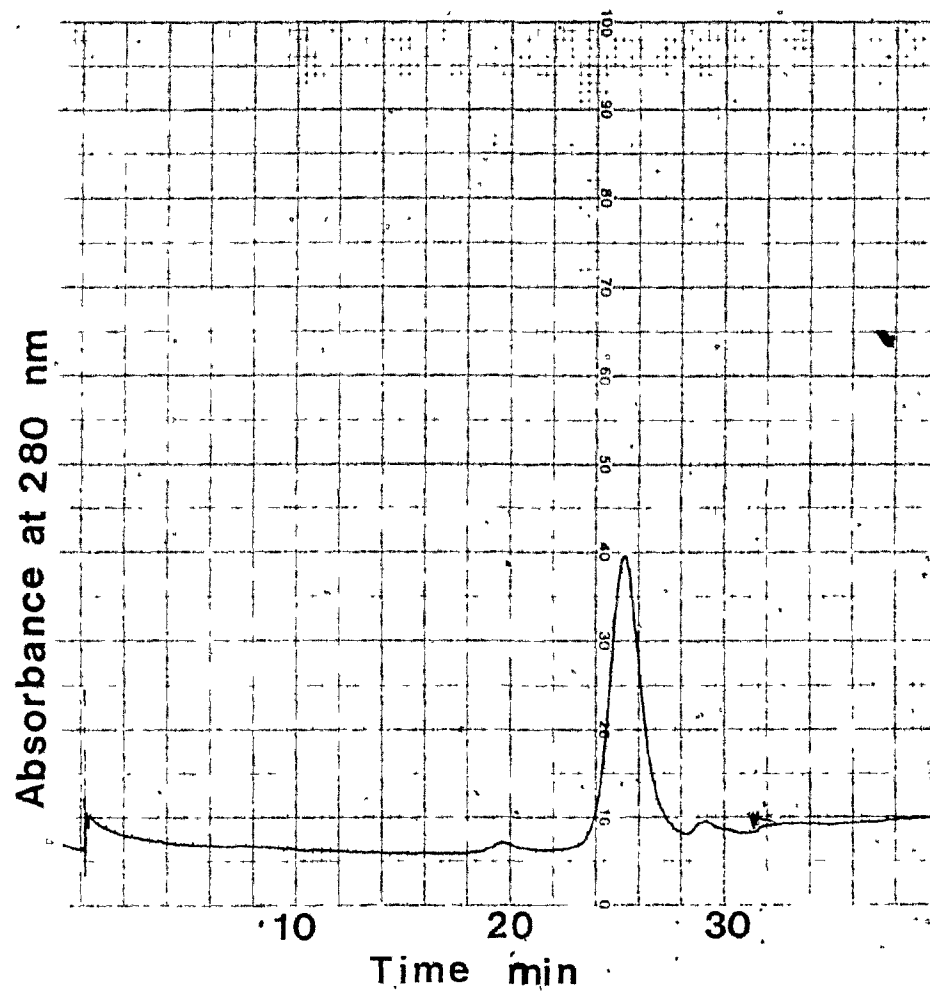
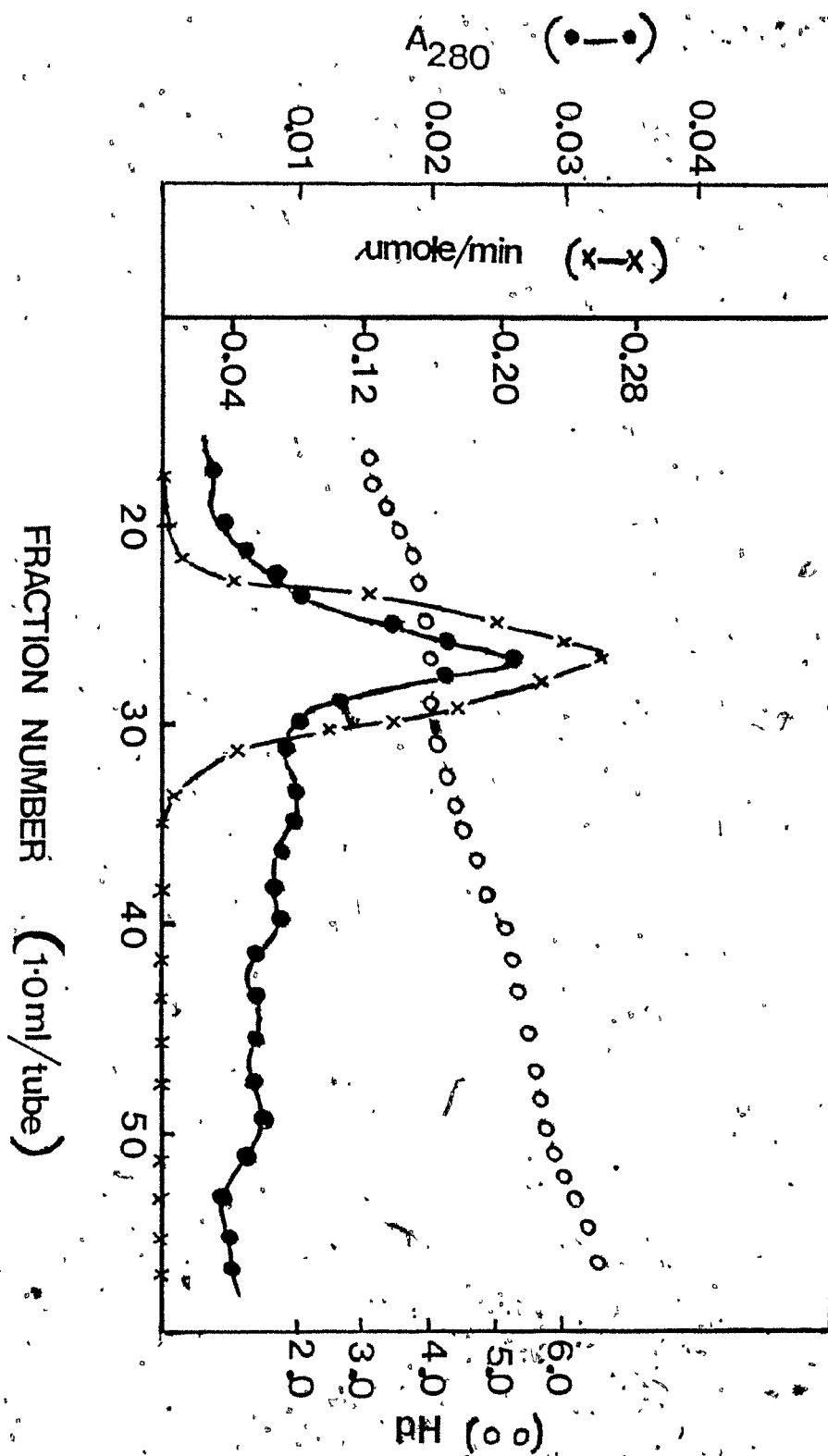


Fig. 10 : Rechromatography of the first peak from HPLC (shown in Fig. 9) on spherogel column. Absorbance from 0.0 to 0.1.

Fig. 11 : Isoelectric focusing of purified human salivary kallikrein with Pharmalyte pH 2.5 - 5.0. The experiment was conducted at 500V for 24h at low temperature in a sucrose density gradient.

The pH of each fraction was measured on a pH meter from Radiometer Copenhagen, and the activity was assayed with BAEE.



obtained, and no other protein could be detected by absorbance measurements at 280 nm.

### C. Gel Electrophoresis

#### (i) Gel electrophoresis at pH 9.5

The results of a typical polyacrylamide gel electrophoresis experiment at pH 9.5 is shown in Fig. 12A. The enzyme moves a fair distance into the gel and shows only a single band.

#### (ii) Gel electrophoresis in the presence of SDS

Polyacrylamide gel electrophoresis in the presence of SDS has been carried out with the active fraction obtained by HPLC and also with the inactive material that emerges from the column between 31 and 34 min (see Fig. 9 on page 67).

No impurities in the active fraction from HPLC could be detected, see Fig. 12B. From the mobility of the enzyme in the gel, a  $M_w$  of about  $40,000 \pm 1,800$  ( $n = 6$ ) has been calculated. The same result has been obtained when the electrophoresis was done at pH 7.2 in tubes. In the latter case a slice containing the protein was cut from a gel which was not stained. The gel was homogenized in 0.1 M Tris-HCl, pH 8.0 and the suspension was shaken gently overnight at  $20^\circ$ . About 50% of the arginine ester-ase activity that was loaded onto the gel was found in the slice extract.

Fig. 12 : Gel electrophoresis of purified kallikrein at pH 9.5 (A) and at pH 8.3 in the presence of SDS (B).

The gel electrophoresis of a standard mixture for molecular weight determination is shown in lane 1, purified kallikrein is shown in lane 2 while the last fraction from HPLC is shown in lane 3.



A

B

94 K



67 K



43 K



30 K



20.1 K



14.4 K

1

2

3

The Mw of the enzyme was not changed by boiling the sample, by treatment of the sample with 9 M urea or by addition of  $\beta$ -mercaptoethanol prior to electrophoresis.

#### D. Sedimentation Equilibrium

The molecular weight of kallikrein in 0.1 M sodium acetate buffer, pH 5.0 was calculated from a sedimentation equilibrium experiment. The results from an analysis of a schlieren picture taken at equilibrium are shown in the inset of Fig. 13. The Lamm plot (Fig. 13) was linear between the meniscus and a level in the cell close to the cell bottom where the protein concentration was 3.42 times the concentration at the meniscus. A molecular weight of 39,700 was calculated from the slope of the Lamm plot when a partial specific volume of  $v = 0.71$  was used.

#### E. Enzymatic Properties

##### (i) Demonstration of kallikrein activity

An aliquot (25  $\mu$ l) of a solution of pure arginine esterase after HPLC, was added to a solution of acid treated dog plasma, buffered at pH 7.3. After incubating at 37 $^{\circ}$  the solution was extracted with ethanol and the extract tested for kinins. The results of this assay using rat uterus is shown in Fig. 14.

Fig. 13 : Sedimentation equilibrium of purified human salivary kallikrein (1.7 mg/ml) in 0.1 M sodium acetate buffer, pH 5.0 at 11000 rpm and 16.7°C. The inset shows the schlieren picture from which the molecular weight was calculated.

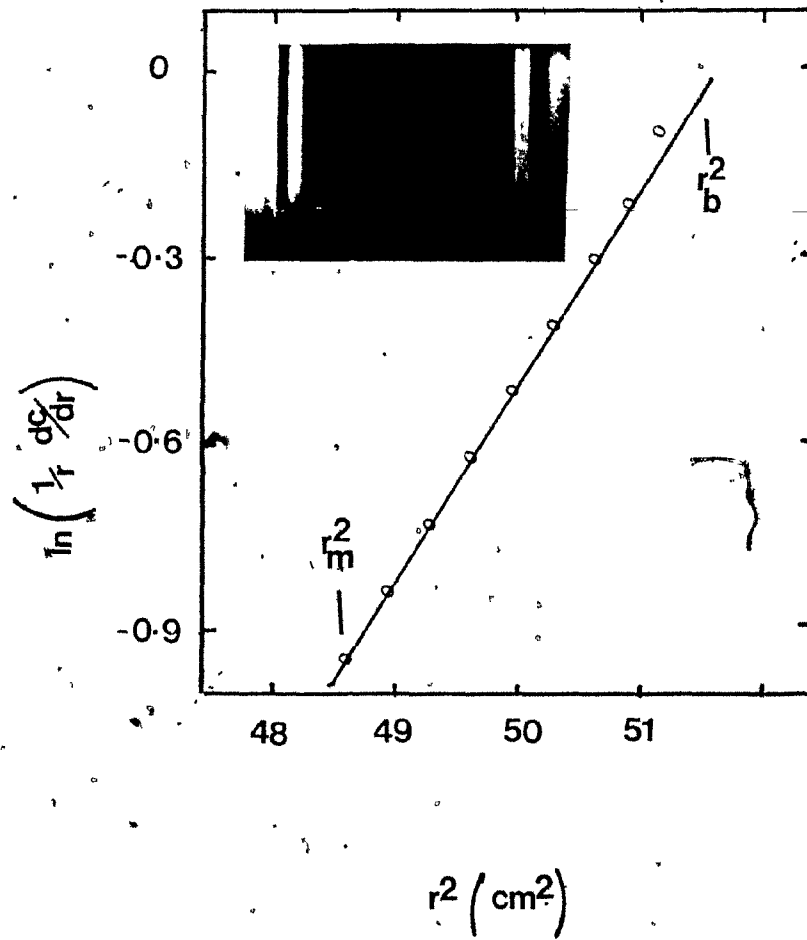
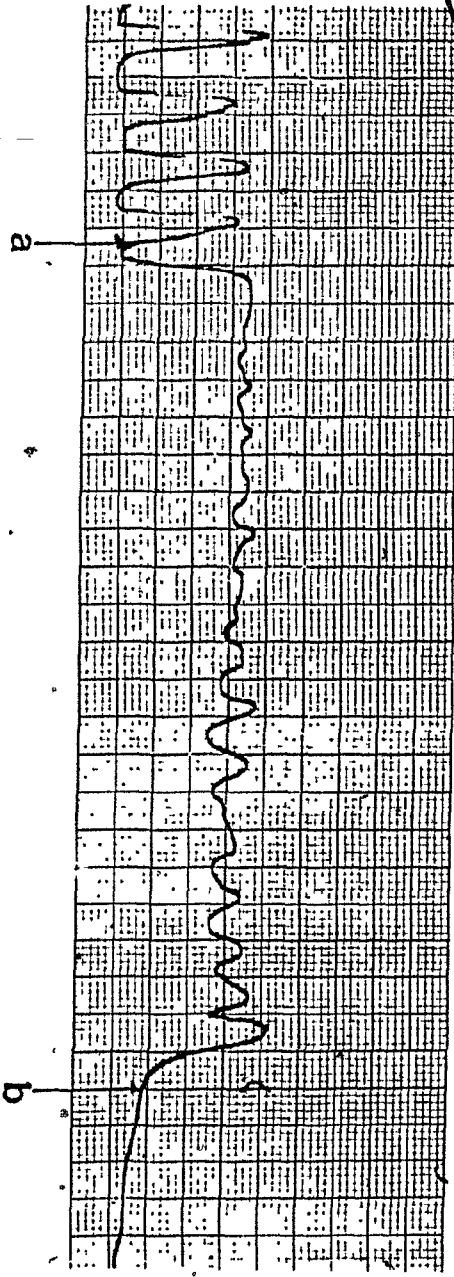


Fig. 14 : The contraction of isolated rat uterus by an extract of acid treated dog plasma after incubation with purified arginine esterase. The isolated rat uterus was suspended in a 6 ml bath containing de Jalon solution with  $10^{-6}$  M atropine and  $10^{-6}$  M pyrilamine sulfate.

Acid treated dog plasma (5 ml) was incubated with 0.5 ml pure salivary kallikrein, at pH 7.3 and at  $37^{\circ}\text{C}$ , for 2 h and the kinin precipitated with ethanol as described by Horton (1959). The kinin was redissolved in a small volume of de Jalon solution and aliquots were used to contract the uterus. At the arrow (a) 25  $\mu\text{l}$  of the kinin solution (the equivalent of 2.5  $\mu\text{g}$  kallikrein) was added to the bath. After about 6 min. the kinin effect decreases and the pattern returns to normal (b).



When the same purified arginine esterase was injected intravenously into a rabbit a dramatic lowering of the blood pressure was observed almost instantaneously. An amount of enzyme equivalent to about 5 ml of saliva reduced the blood pressure from 50 mm Hg to 30 mm Hg.

The purified salivary kallikrein did not cleave azocasein, nor N-benzoyl arginine-p-nitroanilide.

(ii) Variation in the reaction velocity with kallikrein concentration

The curve describing the variation in the rate of hydrolysis of BAEE at pH 8.2 with the enzyme concentration is shown in Fig. 15. It can be seen from this plot that the arginine esterase activity is linearly proportional to the enzyme concentration until a concentration of about 20  $\mu$ g per ml is reached. At higher concentrations of enzyme the rate of hydrolysis no longer increases.

(iii) pH optimum and stability

It was found that the enzyme has an optimum pH between 8.0 and 8.5 when BAEE was used as substrate. The activity was completely eliminated below pH 5.0, see Fig. 16.

The enzyme remained active when titrated to pH 2.5 with phosphoric acid and the activity determined after adding 10  $\mu$ l of the enzyme to 1 ml of substrate strongly buffered at pH 8.2,

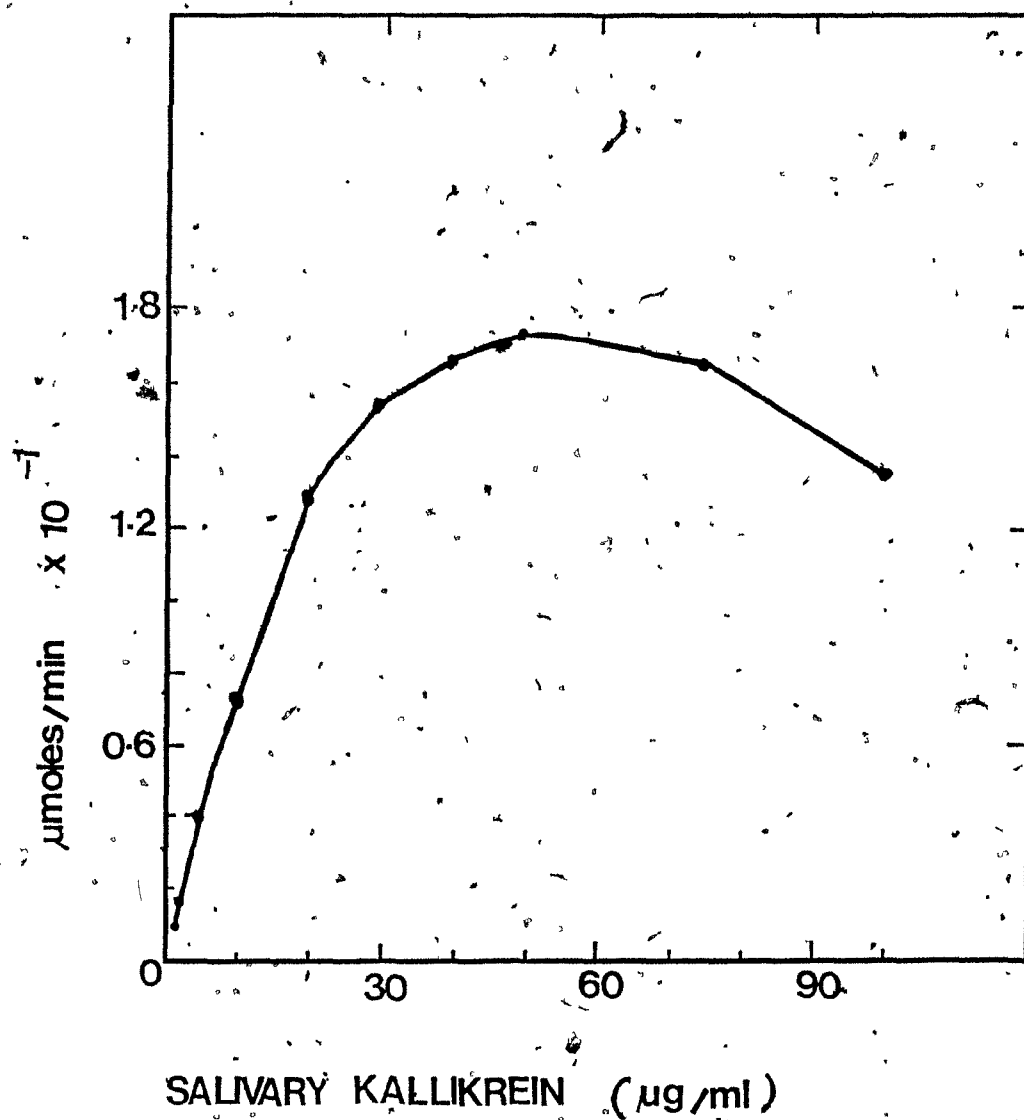


Fig. 15 : Variation of esterase activity with salivary kallikrein concentrations using 1.5 mM BAEE as substrate (at pH 8.2)



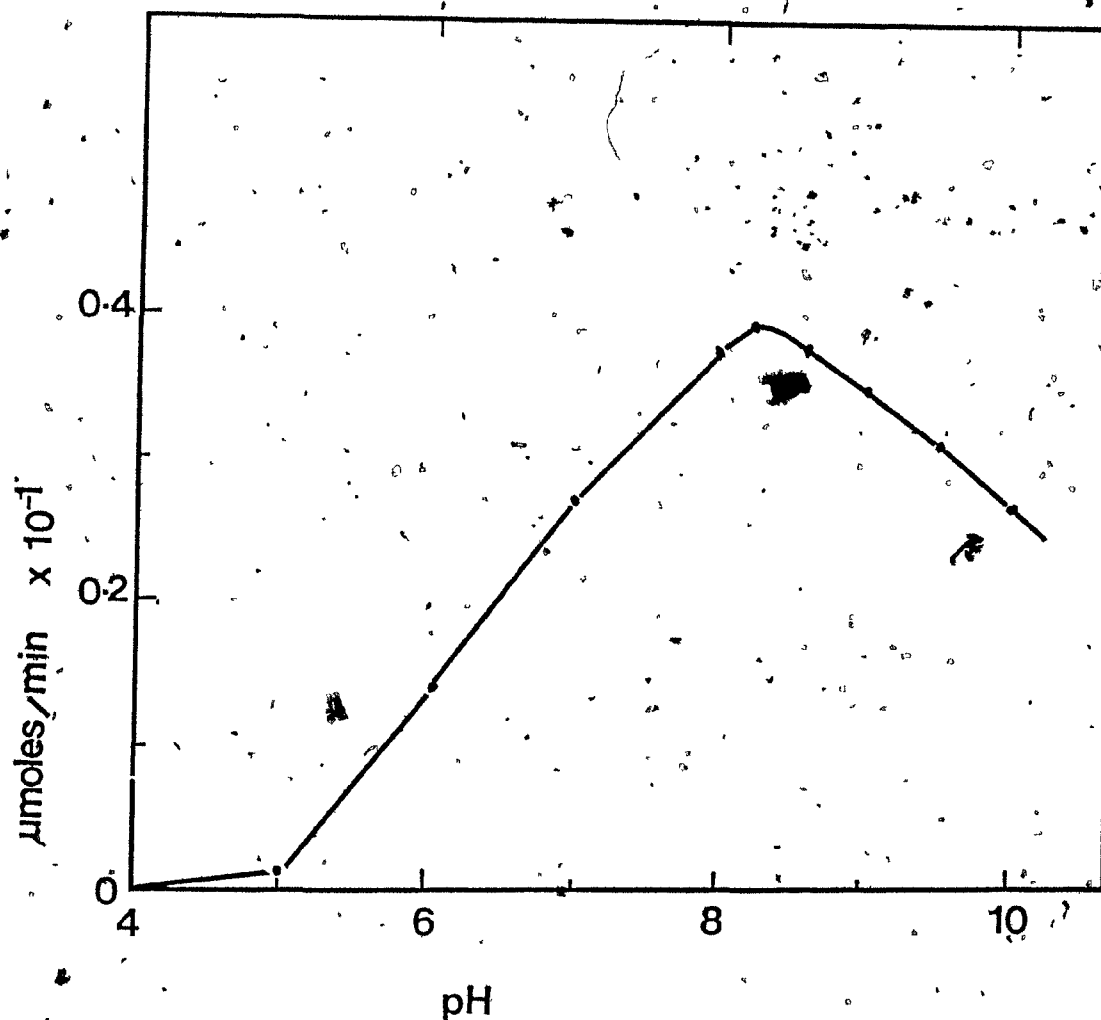


Fig. 16 : The arginine esterase activity of salivary kallikrein at various pH's. The activity was determined with 1.0 mM BAEE as substrate and a protein concentration of 4  $\mu\text{g}$  per ml.

The following buffers were used : 0.1 M  $\text{NaH}_2\text{PO}_4/\text{NaOH}$  (pH 4.0 - 7.0) and 0.1 M Tris-HCl (pH 7.0 -10.0).

But it lost its activity completely in 8 M urea. When heated at pH 5.0 and 100°C for 5 min the enzyme remained at least 50% active, even when 0.5% (w/v) SDS was added to the solution before heating.

(iv) The effect of substrate concentration on enzyme activity

The BAEE concentration was varied and the activity of the enzyme at pH 8.2 was measured using ca 5  $\mu$ g enzyme per assay. The data were plotted as shown in Fig. 17. At each substrate concentration the rate of hydrolysis was constant for at least 30 min. The rate of reaction increased linearly with substrate concentration to about 0.6 mM. At higher substrate concentrations there appeared to be a decrease in enzymatic activity.

The Michaelis-Menten constant  $K_m$  and also  $V_{max}$  were determined from Lineweaver-Burk plots. The substrate concentrations were kept below 1 mM of BAEE so that the plot was linear, see Fig. 18. A value of  $K_m = 0.4$  mM was found by extrapolation while  $V_{max}$  was found to be 20  $\mu$ mole/min/mg protein. From the value of  $V_{max}$  and a molecular weight of 40,000 the  $k_{cat}$  is found to be 13  $s^{-1}$  assuming that the enzyme has only one active-site.

(v) Inhibitor studies

The arginine esterase activity was not inhibited by common trypsin inhibitors from soybean and lima bean. On the other hand, aprotinin and bovine trypsin inhibitor did inhibit the

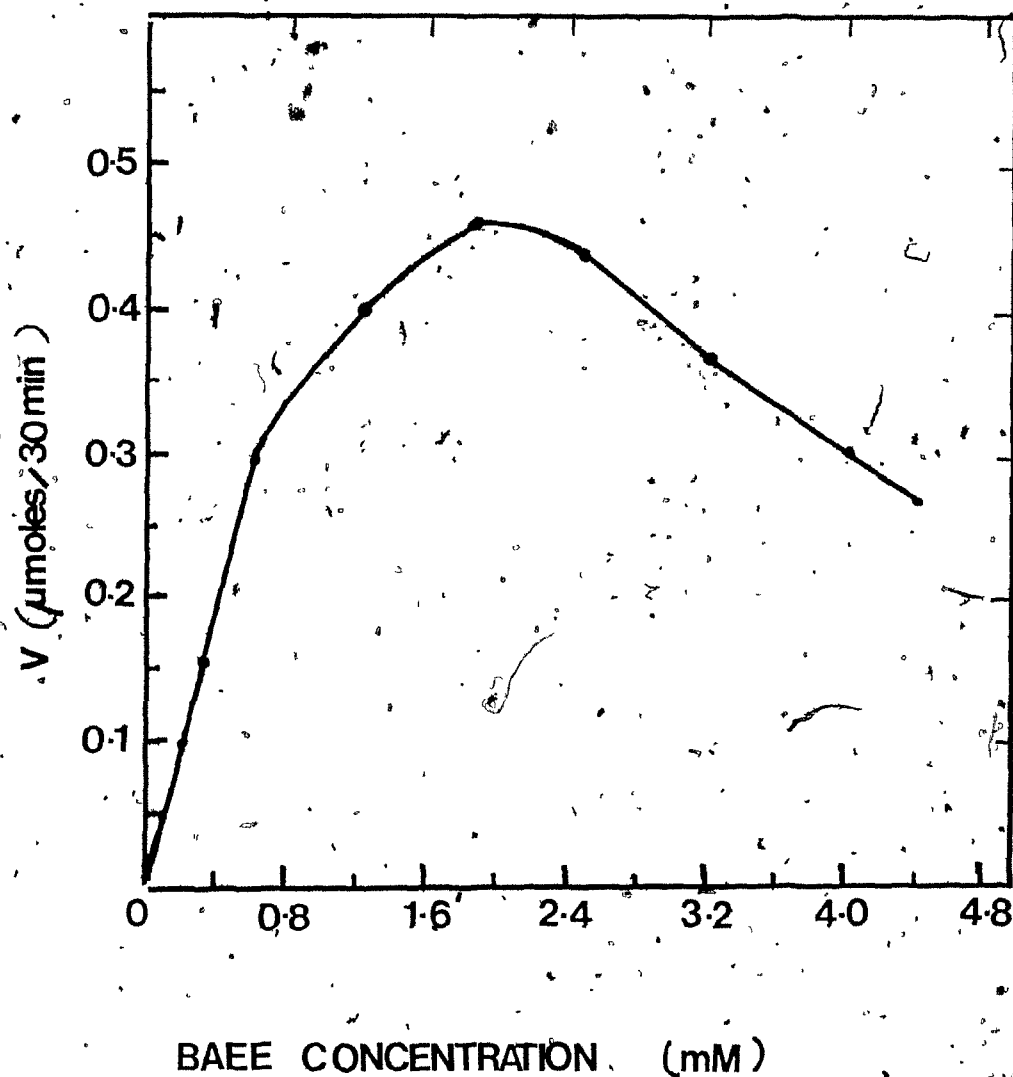


Fig. 17 : The change in the rate of hydrolysis of BAEE with increased substrate concentration. The reactions were carried out at pH 8.2 and  $37^{\circ}$  with 5  $\mu$ g of purified enzyme per ml.

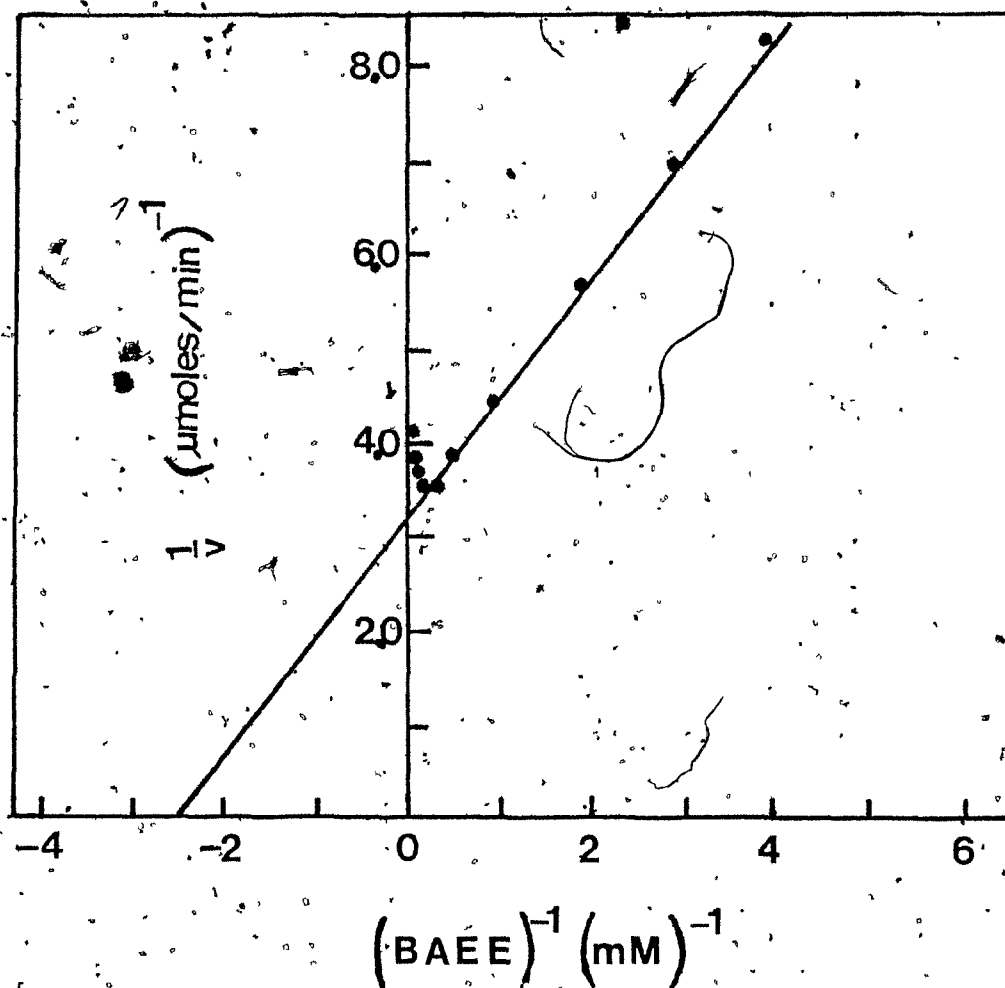


Fig. 13 : A Lineweaver-Burk plot for the hydrolysis of BAEE by salivary kallikrein. The plot was constructed using velocities that were constant for at least 30 min, at pH 8.2 and at 37°C. The kallikrein concentration was 13  $\mu\text{g}$  per ml.

esterase activity. The esterase activity of human salivary kallikrein was inhibited by diisopropyl fluorophosphate (DFP). However, a concentration of 3 mM DFP inhibited the esterolytic activity of trypsin to a larger extent than the activity of the salivary kallikrein (see Fig. 19). From the inhibition of the salivary kallikrein by DFP it was concluded that it belongs to the group of serine proteases. The DFP inhibition is reversible with time, and full recovery can be obtained by dialysis of the sample at pH 7.0 to 8.0.

(vi) The interaction of kallikrein with  $\alpha_2$ -macroglobulin

As is the case with trypsin,  $\alpha_2$ -macroglobulin does not inhibit the arginine esterase activity of the salivary kallikrein when BAEE is used for substrate.

The interaction of salivary kallikrein with  $\alpha_2$ -macroglobulin can therefore be studied by chromatography on Sephacryl S-300 and by HPLC using a Spherogel TSK 4000SW column, while monitoring the arginine esterase activity in the eluate.

No binding of kallikrein by  $\alpha_2$ -macroglobulin could be detected by the chromatographic methods, see Figures 20 and 21. A similar conclusion was drawn from gel electrophoresis experiments in the presence of dodecylsulfate, see Fig. 22. This latter experiment also showed that  $\alpha_2$ -macroglobulin was not cleaved by salivary kallikrein.

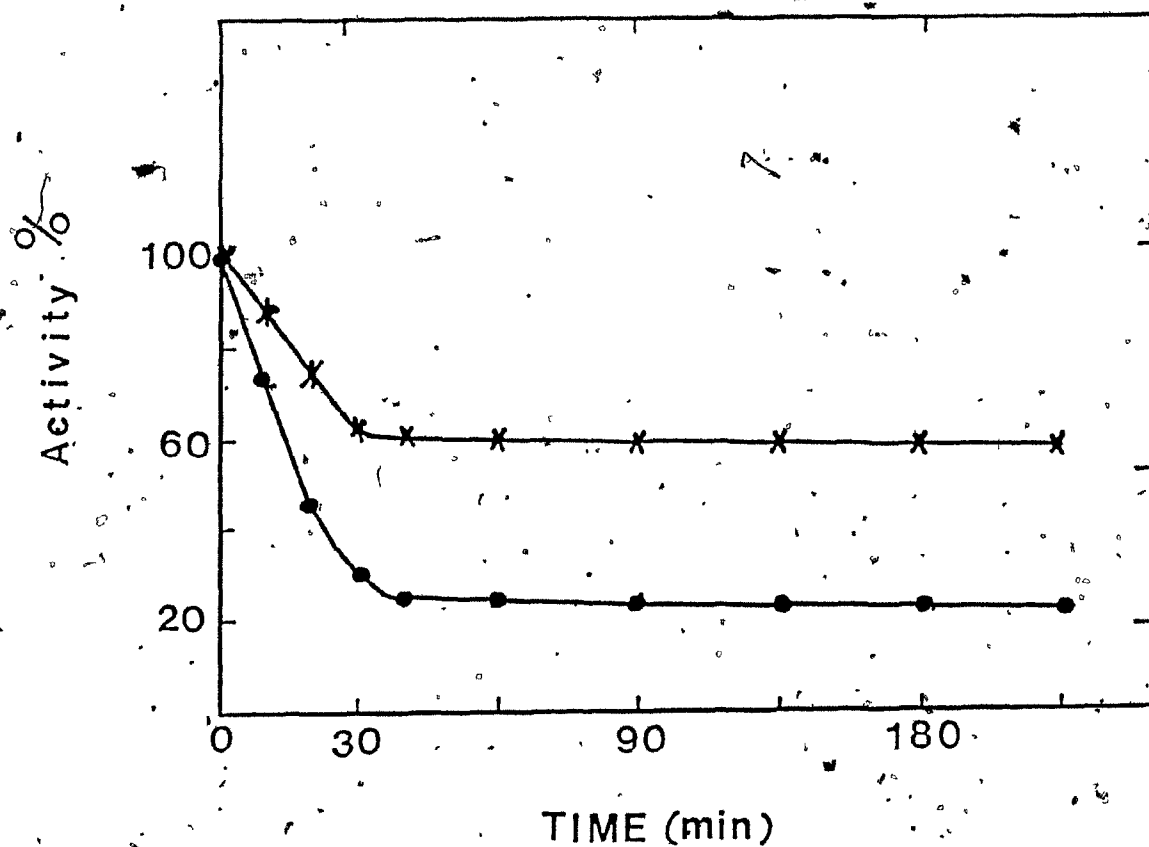


Fig. 19 : Inhibition of salivary kallikrein (x) and trypsin (●) by DFP. The enzymes were incubated with 3 mM DFP in 0.1 M Tris-HCl, pH 8.2 with 0.1 mM  $\text{CaCl}_2$ . The enzyme concentrations were about 10  $\mu\text{g}$  per ml.

Fig. 20 : Chromatography of a mixture of 50  $\mu$ g kallikrein and 800  $\mu$ g of  $\alpha_2$ -macroglobulin on a 60 x 1 cm column of Sephacryl S-300. The mixture was incubated for 1 h at pH 8.2 and at 37°C before being chromatographed. The absorbance at 280 nm was measured (—●—) and the arginine esterase activity was assayed with BAEE at pH 8.2 (x—x). Free  $\alpha_2$ -macroglobulin was determined by the method of Ganrot (1966) and is expressed as trypsin-esterase activity, (o—o).

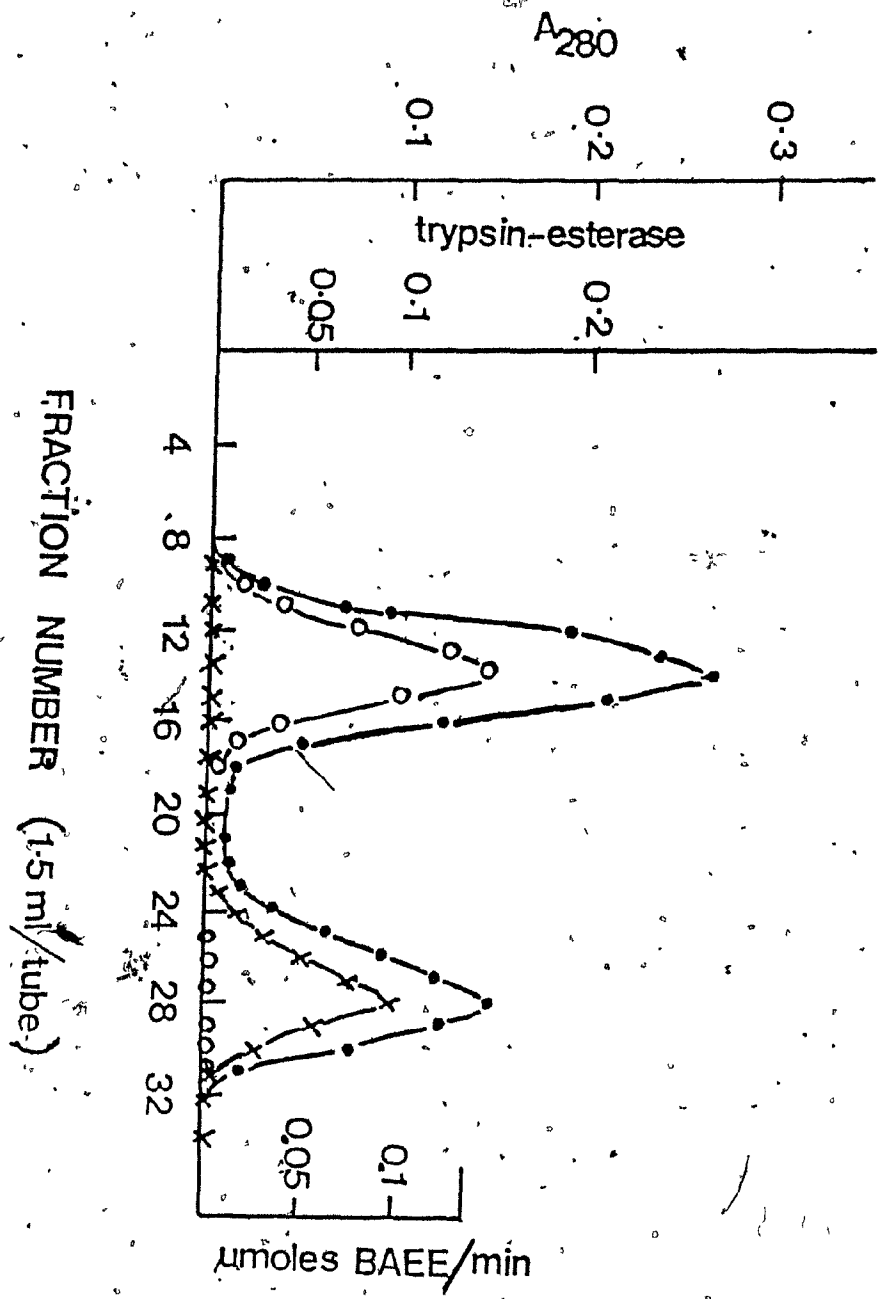




Fig. 21 : A. HPLC of  $\alpha_2$ -macroglobulin.

B. HPLC of a mixture of 400  $\mu$ g of  $\alpha_2$ -macroglobulin and 50  $\mu$ g of kallikrein after 6h incubation at 37<sup>0</sup>

C. HPLC of 50  $\mu$ g kallikrein

A spherogel 4000SW column was used with an eluting buffer of 0.02 M phosphate pH 7.2.

No activity could be detected in the fractions emerging from the column between 15 and 18 min.

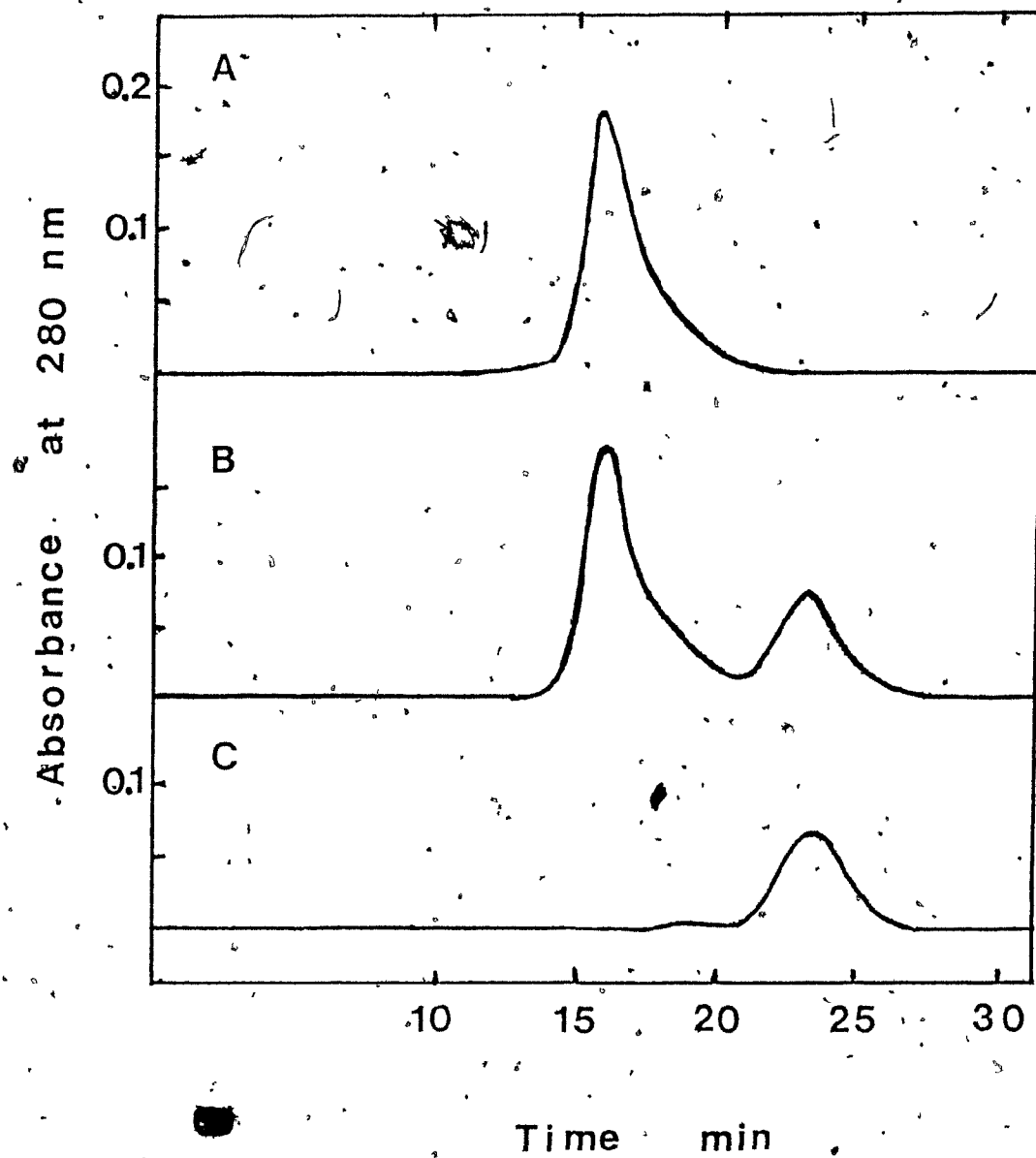


Fig. 22 : SDS-Page of a mixture of  $\alpha_2$ -macroglobulin and human salivary kallikrein.

A. is 100  $\mu$ g  $\alpha_2$ -macroglobulin

B. is 20  $\mu$ g kallikrein

C. is a mixture of 100  $\mu$ g  $\alpha_2$ -macroglobulin with 20  $\mu$ g kallikrein which was incubated at 37° before addition of SDS and electrophoresis.



A

B

C

Some data on the enzymatic activity of the human salivary kallikrein are summarized in Table III.

#### F. Carbohydrate Analyses

The enzyme did not contain a detectable amount of sialic acid, while the carbohydrate analysis showed less than 5% (w/w) was present. Preliminary results with Concanavalin A-Sepharose or Wheat germ lectin-Sepharose column chromatography did not indicate binding and thus no detectable amounts of carbohydrates.

Table III

Enzymatic properties of Salivary kallikrein a)

---

Optimum activity : pH 8.0 - 8.5

No activity below pH 5.0

$K_m = 0.4 \text{ mM}$ ;  $V_{max} = 20 \text{ } \mu\text{mole/min /mg protein}$ ;  $k_{cat} \approx 13 \text{ s}^{-1}$

Inhibitors b) : Pancreatic Trypsin Inhibitor

Aprotinin

Diisopropyl fluorophosphate c)

Produces kinin in acid treated dog plasma as measured by contraction of guinea pig ileum and rat uterus.

Lowers blood pressure in rabbits.

Does not bind to  $\alpha_2$ -macroglobulin

---

a), In most experiments benzoyl-arginine ethyl ester was used as substrate, and the reactions were measured at pH 8.2.

b) Not inhibited by : soybean trypsin inhibitor and lima bean trypsin inhibitor.

c) DFP inhibition is reversible with time.

d) No digestion of azocasein.

## 2. Discussion

DEAE-Sephacel strongly bound about 87% of the arginine esterase activity that remained soluble after centrifugation and filtration of human whole saliva. From this DEAE-Sephacel fraction a very pure enzyme was obtained with a yield of about 35%. A study of the reaction with inhibitors and biological assays identified this arginine esterase as a kallikrein. The yield of pure enzyme from 500 ml of saliva was approximately 0.08 mg or 0.12% of the non-dialysable protein material in saliva. The purification was about 2,700 fold, when BAEE was used as substrate. This purification factor appears to be somewhat higher than that reported by others (Fujimoto et al., 1973, Modeer, 1977 and Wong et al., 1983). This may be due to the fact that HPLC was used in the final step of the purification reported here. The inactive protein fraction obtained by HPLC did not bind to the BPTI-Sepharose affinity column at pH 8.0, when applied by itself. It is therefore very likely that the salivary kallikrein binds other protein compounds strongly and that these associated proteins can be released only by slightly acid treatment or by separation on HPLC using acidic buffer, as is reported in this thesis.

Gel electrophoresis in the presence of SDS gave a Mw of 40,000 which is in agreement with values reported by Modeer (1977) and Wong et al., (1983). However, it should be noted that the reported molecular weight, as determined by gel filtration

differed widely from 28,000 (Fujimoto et al., 1973) to 38,000 (Modeer, 1977).

The molecular weight value obtained from sedimentation equilibrium was found to be 39,700. This value confirms that obtained by electrophoresis. The partial specific volume of kallikrein used in the calculation was 0.71 although a value of  $v = 0.713$  was calculated from the amino acid composition (Cohn and Edsall, 1943). The slightly lower value was decided upon to correct, at least partially, for the distinct possibility of electrostriction in view of the strong negative charge on the enzyme.

The esterolytic activity of human salivary kallikrein was inhibited by high substrate concentrations. But it is not uncommon to find, that while the Michaelis equation is obeyed at lower concentrations, the velocity falls off again at high concentrations. The decrease in reaction velocity at high substrate concentrations may be due to the enzyme substrate complex binding additional substrate molecules, forming ineffective complexes (Dixon and Webb, 1979). In the case of human salivary kallikrein it is possible that the enzyme is inhibited by the positive charge on the substrate when multi-substrate complexes are formed. Preliminary experiments with polylysine showed that the enzyme is inhibited due to precipitation. Precipitation of kallikrein by BAEE has never been observed. However the hydrolytic reaction is proportional with enzyme concentration only below 20  $\mu\text{g/ml}$  (or below  $5 \times 10^{-7} \text{ M}$ ). It is possible that at higher



concentrations the enzyme forms aggregates in the presence of BAEE. Of course at high substrate concentration, the chance of formation of ineffective complexes, with two or more substrate molecules combined with an active site, also increases. The Lineweaver-Burk plot is not affected because the graph spreads out the data for the lower substrate concentrations so that the greater part of the curve represents the region where Michaelis' law is obeyed (Dixon and Webb, 1979).

The deviation from Michaelis-Menten kinetics at high BAEE substrate concentration was also observed by Fiedler and Werle (1968) and Zuber and Sache (1974) for porcine pancreatic kallikrein. This deviation was also believed to be kinetically related to the binding of a second substrate molecule at a secondary binding site in the enzyme (Trowbridge et al., 1963).

The pH-rate profile of the salivary kallikrein with low concentrations of substrate (BAEE) shows that the enzyme is essentially inactive at pH below 5.0. Above pH 6.0 the activity increases rapidly and reaches a maximum at about pH 8.0 - 8.5. One could argue that a group with an apparent pK value of about 6 to 7 is involved in the activity. Such a group is usually identified as a histidine residue, see for example Bender and Kezdy (1964) for chymotrypsin and Gutfreund (1955) for trypsin.

At pH values above 8.5 the enzymatic activity decreases somewhat, but not significantly as is the case for trypsin. Similar observations were made by Fiedler and Werle (1968) for

porcine pancreatic kallikrein. These authors suggested that the BAEI inhibition of the enzymatic activity decreases at higher pH, probably because of a lower affinity of the secondary binding site at high pH. Thus the presence of secondary binding sites on the enzyme could explain both the substrate inhibition and the independence of the reaction velocity of the pH in the region above pH 8.5.

The human salivary kallikrein has a low isoelectric point, (pI =4.07) which confirms the data reported by Fujimoto et al. (1973). The enzyme was homogeneous as demonstrated by isoelectric focusing. This was used as one criteria for purity. Other criteria that were applied to demonstrate homogeneity, were PAGE at pH 9.5 and SDS-PAGE at pH 8.3. The protein produces a single band in either electrophoresis. Rechromatography on HPLC using Spherogel TSK 3000SW also gave a single peak.

The enzyme lost only 50% of its activity when heated at 100°C for 5 min in the presence of SDS. No other band was detected after electrophoresis in SDS gels, therefore there was no cleavage of the protein. Circular dichroism (CD) measurements, to be discussed in a following chapter, indicated no dramatic changes in the structure after this treatment. However, the CD measurements and the activity assays were conducted after the solution had cooled down, and thus do not provide information about protein structure at elevated temperatures in the presence of detergent.

DFP inhibited salivary kallikrein to a lesser extent than trypsin. Normally the substituted phosphoryl group is transferred to the enzyme with the formation of a phosphorylated enzyme and the liberation of hydrogen fluoride. The phosphorylated enzyme is a comparatively stable compound, which in the case of trypsin and chymotrypsin hydrolyses only at a negligible rate. In the case of kallikrein, however, the substituted enzyme hydrolyses at a measurable rate. Nevertheless the inhibition with DFP suggests that serine could be involved in the active site of kallikrein.

There are numerous reports in the literature describing the presence of plasma proteins in saliva (Schultze and Heremans, 1966). However no interaction could be detected between  $\alpha_2$ -macroglobulin and salivary kallikrein. Since plasma kallikrein is readily bound by  $\alpha_2$ -macroglobulin (Harpel, 1970) our results prove that salivary kallikrein is a different protein and that it belongs to the group of glandular kallikreins.

The experimental evidences presented earlier clearly indicate that the salivary kallikrein is not closely related to the plasma kallikrein, which has a much higher Mw and which consists of several subunits (Nagase and Barrett, 1981). On the other hand the salivary kallikrein has several properties in common with the human urinary kallikrein (Nustad et al., 1974). For example salivary kallikrein like the urinary kallikrein has no significant activity to casein, and it does not appear to react

with SBTI although it reacts with aprotinin and BPTI. The molecular weights reported for both salivary and urinary kallikrein vary widely and are therefore of little use for comparative purposes. It is, however striking that isoelectric focusing of salivary kallikrein demonstrated a single peak with an isoelectric point between 4.0 and 4.2 that is in a region of pH where the fraction with the highest isoelectric point among the multiple forms of urinary kallikrein has been observed (Hojima et al., 1975). Thus it remains possible that the salivary and urinary kallikreins are closely related.

It seems unlikely that human salivary kallikrein contains a major carbohydrate moiety. Chemical analyses for carbohydrates were considered negative, indicating less than 5% carbohydrate. Furthermore, the enzyme is not adsorbed by a Concanavalin A-Sepharose or a Wheat germ lectin-Sepharose column.

## CHAPTER 5

### ISOLATION AND CHARACTERIZATION OF CF SALIVARY KALLIKREIN

#### 1 Results

##### A. Isolation of CF salivary Kallikrein

The procedure followed was the same as that developed for the isolation and purification of salivary kallikrein from normal individuals. The details of this procedure were described in the previous chapter. The procedure is illustrated in a flow chart (Fig. 23). The yields of activity and the purification factors for the individual steps in the procedure are given in Table IV. From a comparison with Table II it can be noted that the results are very much the same as those observed with the normal saliva. Because of these similarities in the individual steps of the purification procedures for both kallikreins, the chromatograms are not shown, except for the HPLC result which is illustrated in Fig. 24. The HPLC of CF kallikrein preparation, after affinity chromatography on a BPTI-Sepharose column, shows two peaks. As is the case with the normal product, at least 90% of the arginine esterase activity emerges from the column between 23 and 26 min. These results are thus identical with those obtained with normal enzyme.

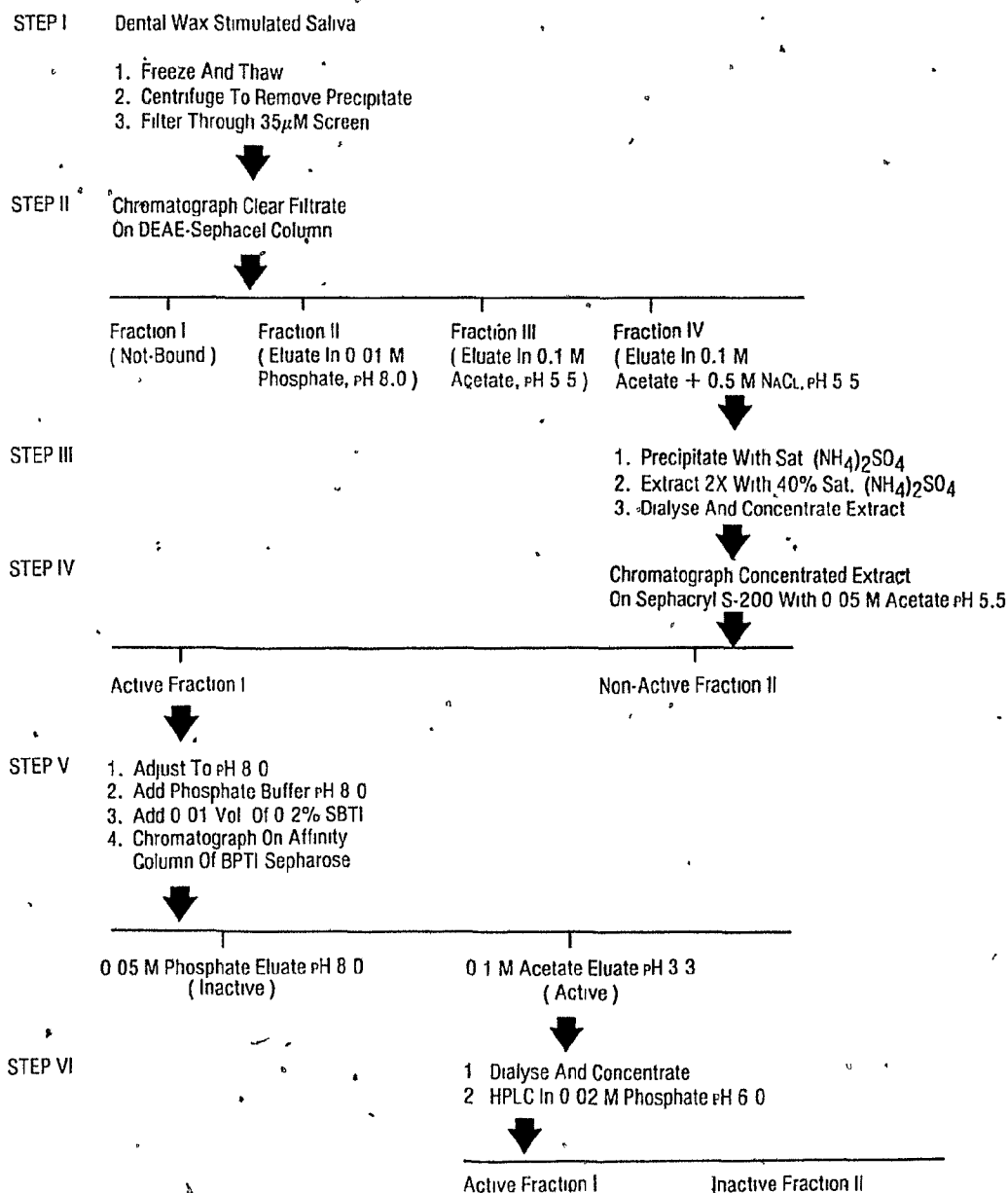


Figure 23. Flow chart for the purification of salivary kallikrein.

TABLE IV

Purification of CF salivary kallikrein

Preparation (Steps)	Total <sup>a)</sup> Protein (mg)	Activity <sup>b)</sup> (nmol/min/mg protein)	Yield (%)	Purification (fold)
1) Whole saliva, centrifuged and filtered	800.00	2.24	100.0	1.0
2) DEAE-Sephacel (Fraction IV)	95.94	14.53	77.8	6.5
3) Sephacryl S-200	15.96	70.8	63.1	31.6
4) BPTI-Affinity	0.60	1675.0	56.1	747.8
5) HPLC	0.0428	6579.0	15.7	2937.1

a) Data based on the protein assay by the Bio-Rad method  
(Bradford, 1976).

b) The substrate used was BAEE.

c) 500 ml of whole filtered saliva was used.

RELATIVE ABSORBANCE AT 280nm

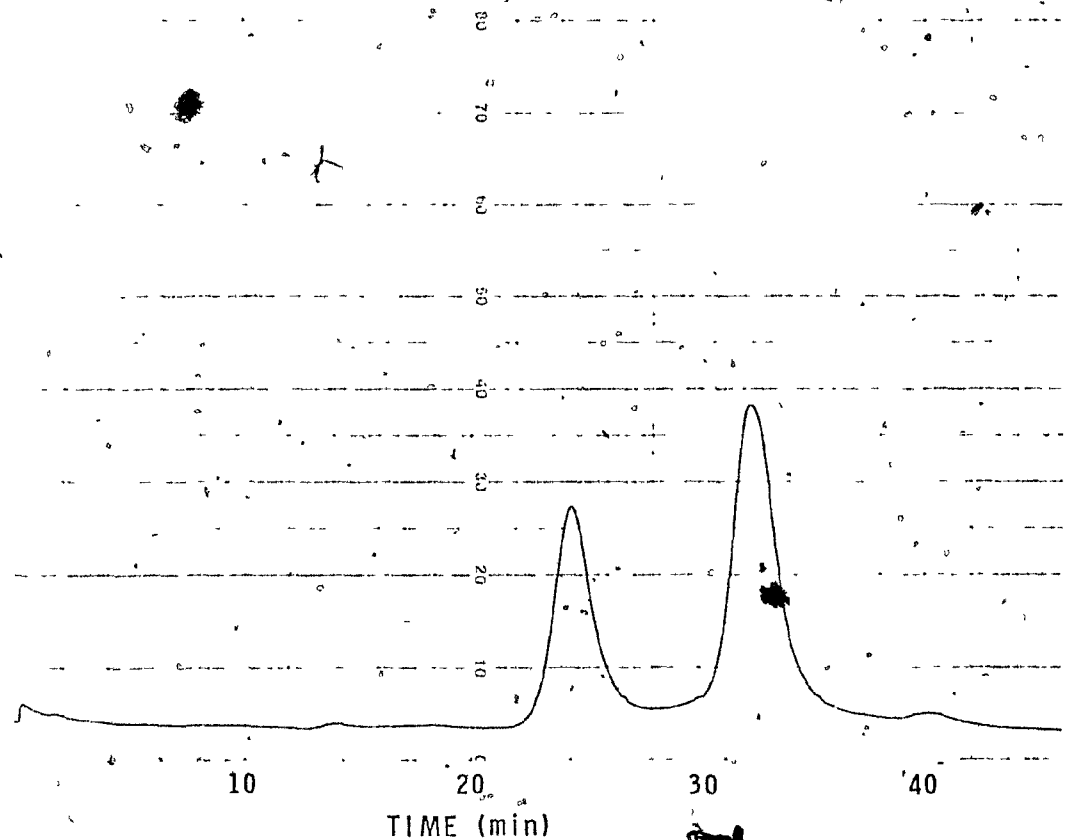


Fig. 24 : HPLC of CF salivary kallikrein on Sphergel 3000SW after BPTI-Affinity chromatography. The esterase activity was found in the first peak emerging from the column between 23 and 26 min



## B. Electrophoresis

### (i) Gel electrophoresis at pH 9.5

After polyacrylamide gel electrophoresis at pH 9.5 only one band could be observed upon staining with Coomassie Blue. The mobility of this band was identical to that of kallikrein from normal saliva, see Fig. 25.

### (ii) Gel electrophoresis in the presence of SDS

Gel electrophoresis at pH 8.3 in the presence of SDS gave a single band for the HPLC purified kallikrein of CF saliva, see Fig. 26. The mobility of the enzyme from CF patients was also the same as that of the normal individuals under these denaturing conditions. The value of  $M_w$  was calculated and found to be  $40,000 \pm 1,800$  D (depending on whether the  $M_w$  was determined at the leading or trailing edge of the band) which was the same as the  $M_w$  value of the kallikrein from normal individuals.

### (iii) Isoelectric focusing

A solution of the HPLC purified CF enzyme was subjected to isoelectric focusing in the range pH 2.5 to 5.0. A single arginine esterase activity peak with  $pI = 4.07$  was detected in the only protein peak observed by measuring the absorbance at 280 nm. The pattern obtained by isoelectric focusing is illustrated in Fig. 27.

Fig. 25 : Polyacrylamide gel electrophoresis at pH 9.5 of  
salivary kallikrein purified from normal (1) and CF  
saliva (2).

1

2

-109

Fig. 26 : Polyacrylamide gel electrophoresis at pH 8.3 in the presence of SDS : (1) mixture of standard proteins of given molecular weights; (2) purified kallikrein from normal saliva and (3) purified kallikrein from saliva of CF patients.

94 K 

67 K 

43 K 

30 K 

20.1 K 

14.4 K

1

2

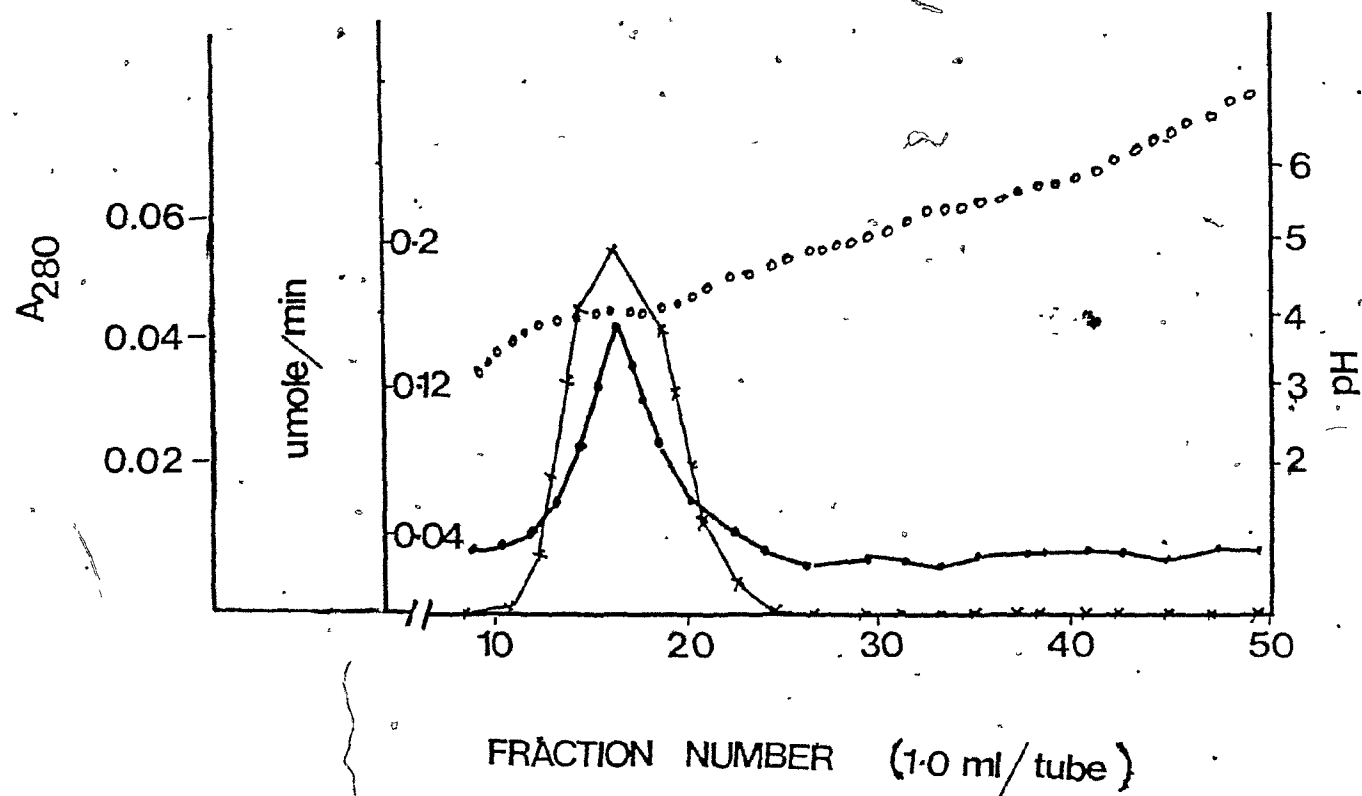
3





Fig. 27 : Isoelectric focusing, of purified salivary kallikrein obtained from CF, using Pharmalyte pH 2.5 to 5.0. The experiment was conducted in a sucrose density gradient at low temperature and at 500V for 24h.

The pH of each fraction was measured on a Radiometer Copenhagen, and the activity was assayed using BAEE.



### C. Enzymatic Properties

#### i) Biological activity

The enzyme caused an immediate lowering of blood pressure when injected intravenously into a rabbit. An amount of enzyme equivalent to 5ml saliva gave a reduction in blood pressure from 58mm Hg to 43mm Hg. The salivary kallikrein from CF patients did not cleave azocasein nor BAPNA.

#### ii) Variation of activity with CF kallikrein concentration.

The arginine esterase activity was assayed with 1.0 mM BAEE at pH 8.2 and 37°. When the activity was assayed, from the absorbance at 253 nm after 60 min of incubation it was found to be proportional with enzyme concentrations up to 16 µg per ml in the assay mixture. When the initial reaction velocities, measured during the first 10 min on a recording spectrophotometer, were plotted, the esterase activity was found to be proportional to an enzyme concentration of up to 20µg protein per ml with 1.0 mM BAEE as substrate (Fig. 28).

#### iii) pH optimum and stability

The pH optimum for the hydrolysis of BAEE by salivary kallikrein was found to be pH 8.5 -9.0 ( Fig. 29). At pH values lower than 4.5 there was absolutely no hydrolysis of the substrate. At pH values higher than 9.0 there was a small decrease in activity.

The enzyme was found to be stable over the pH range between



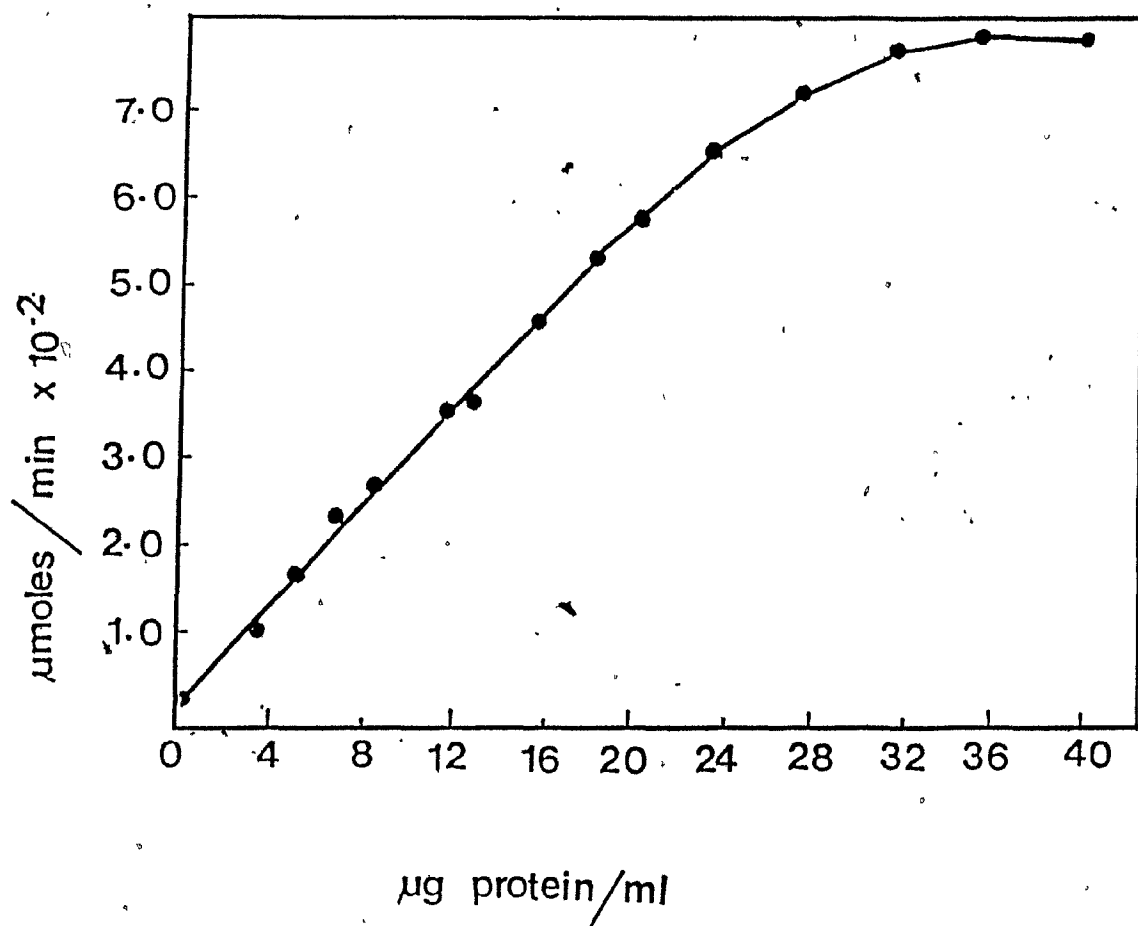


Fig. 28 : The rates of reaction at varying enzyme (CF) concentrations using 1.0 mM BAEE for substrate. The rates of hydrolysis of the substrate were measured during the initial 10 min at pH 8.2.

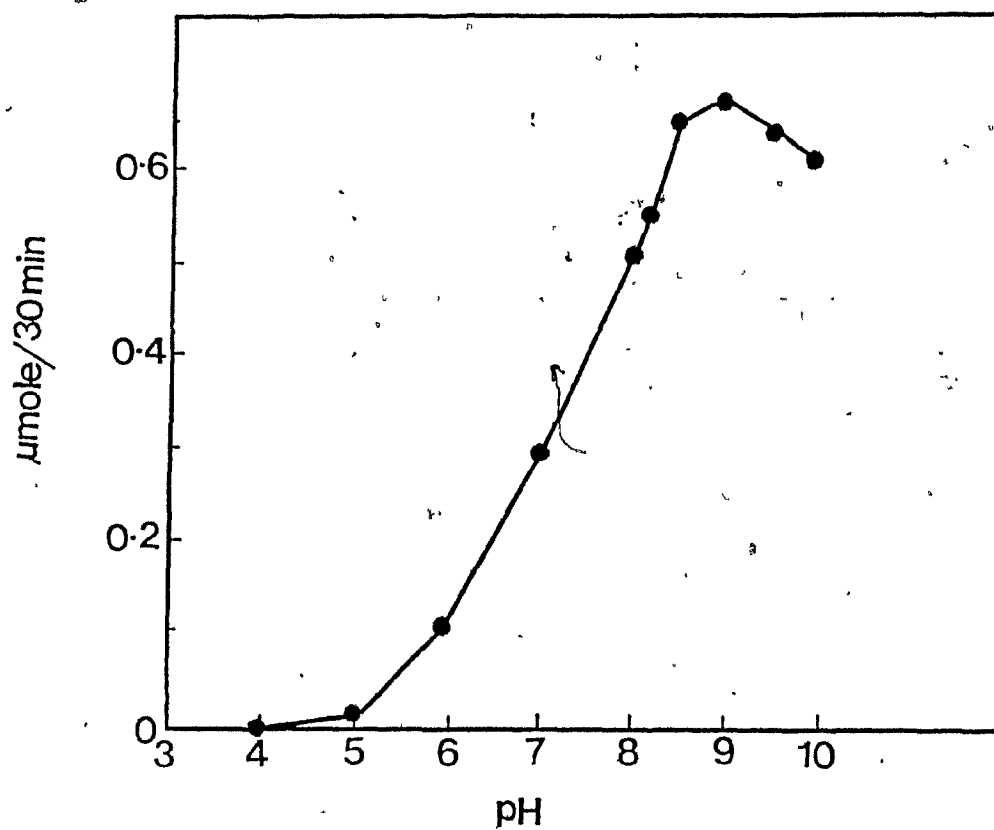


Fig.29 : The influence of pH on the hydrolysis of BAEE (1.0 mM) by 4  $\mu$ g CF salivary kallikrein at 37<sup>0</sup>. The conditions were similar to those used previously with the normal enzyme, that is sodium phosphate buffer was used below pH 7.0 and Tris-HCl above pH 7.0.

7 and 9 at room temperature. At lower pH values there was a slow decrease in enzyme activity but as much as 85% of the initial activity remained after exposure to pH 4.0 for 30 min. There was no significant loss of activity at pH 8.0 when the enzyme was heated at 40°C, 50°C or even at 60°C. The enzyme can be stored at 0 to 4°C for months without any significant loss in activity.

#### iv) Determination of $K_m$ and $V_{max}$

At low concentrations the reaction followed the kinetics predicted by the Michaelis-Menten theory. However, at high BAEE concentrations the velocities of the hydrolysis reaction deviated again from simple Michaelis-Menten kinetics as had been observed with enzyme from normal subjects. Values for the  $K_m$  and  $V_{max}$  for CF salivary kallikrein with the substrate BAEE were estimated from Lineweaver-Burk plots and found to be:  $K_m = 0.4\text{mM}$  and  $V_{max} = 21 \mu\text{mole/min/mg protein}$  (Fig. 30). From the value of  $V_{max}$  and a molecular weight of 40,000 the calculated  $k_{cat}$  was found to be about  $14 \text{ s}^{-1}$ . This value is the same as that found for the normal enzyme, for which  $k_{cat}$  was calculated to be  $13 \text{ s}^{-1}$ .

#### v) Inhibitor studies

The effects of various inhibitors on the CF kallikrein are shown in Table V. The arginine esterase activity was inhibited by bovine pancreatic trypsin inhibitor, aprotinin and diisopropylfluorophosphate but not by SBTI or LBTI.

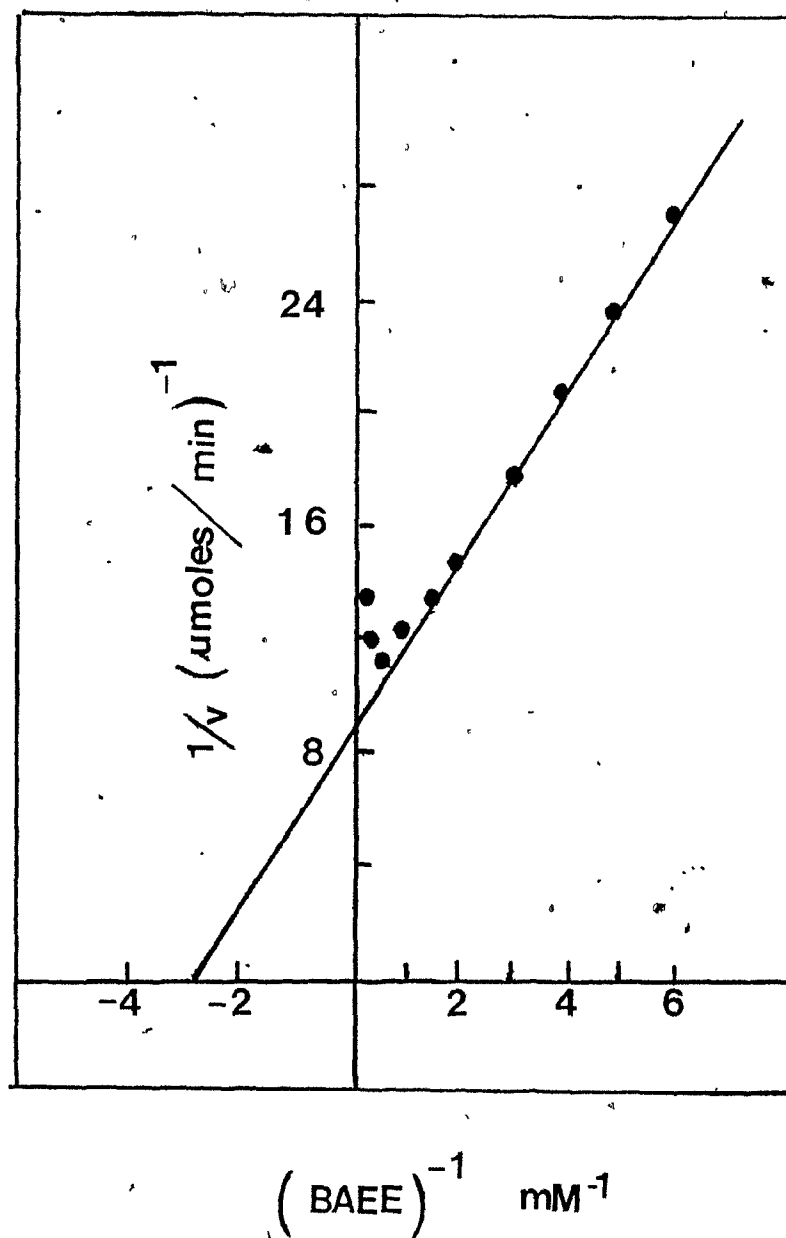


Fig. 30 : Lineweaver-Burk plot for the arginine esterase activity of CF salivary kallikrein (5  $\mu$ g). The reaction was carried out at pH 8.2 and at 37°C.

TABLE V

Enzymatic Properties of CF Salivary Kallikrein a)

---

Optimum activity : pH 8.5 - 9.0

No activity below pH 4.5

$K_m = 0.4 \text{ mM}$ ;  $V_{max} = 21 \text{ umole/min/mg protein}$ ;  $k_{cat} \approx 14 \text{ s}^{-1}$

Inhibited by b) : BPTI; aprotinin and DFP

Lowers blood pressure in rabbits, but does not digest azocasein

---

a) In most experiments BAEE was used as substrate, and the reactions were conducted at pH 8.2.

b) Not inhibited by : SBTI or by LBTI.

## 2. Discussion

The saliva of CF patients is very viscous and therefore very difficult to work with. Approximately 0.04mg or 16% of the non-dialysable arginine esterase activity in CF saliva, was obtained in a pure form from 500 ml of centrifuged whole CF saliva. This yield is lower than that obtained for the normal salivary kallikrein mainly because the HPLC step gave a lower yield. The purification was about 2900 fold, using benzoylarginine ethyl ester for substrate. This purification factor is slightly higher than that obtained for the normal salivary kallikrein. The differences in purification factor and yields are not believed to be significant.

The esterolytic activity of CF salivary kallikrein, like normal salivary kallikrein, was inhibited by high BAEE substrate concentrations and is 'pH-independent' above 9.0. This substrate inhibition perhaps can be explained by the binding of a second substrate molecule at a secondary binding site thus leading to the formation of inactive enzyme-substrate complexes as discussed in the previous chapter. The 'pH-independence' above 9.0 may be due to an increased affinity of the catalytic BAEE binding site in the deprotonated enzyme for the substrate.

As all substrates including BAEE, normally bind better to the correct enzyme-binding sites rather than to the secondary sites, substrate inhibition is rarely severe enough at low concentrations to interfere with the analysis of initial velocity

data. The possibility that the BAEE preparation was contaminated was therefore investigated. Many substrates, and their competitive inhibitors are structurally and chemically related. As such, it is not surprising that contamination of a substrate by inhibitors may occur during the chemical synthesis of the substrate. To check for possible contaminants we recrystallized the BAEE preparation from ethanol. The enzymatic reactions were carried out again, this time with the recrystallized substrate. The results were the same as before, that is the arginine ester-ase activity was still inhibited at high BAEE concentration. Thus the possibility that this inhibition is caused by contaminants in the substrate does not appear likely, supporting the hypothesis of multiple BAEE binding sites on the enzyme.

The pH-rate profile of the reaction of CF salivary kallikrein with BAEE is similar to that obtained for normal salivary kallikrein except for a slight shift in pH optimum, which perhaps is as much as 0.5 pH higher in the case of CF kallikrein.

In general the results of studies on the effects of pH on enzyme activity are difficult to interpret with certainty. Even in the simplest system the pK values obtained are relatively complex molecular constants rather than simple group constants. From the rapid rise in activity between pHs 6 and 7 it can be speculated that an imidazole ring, namely that of histidine, is at or near the active site.

Although it is generally recognized that enzymes may be

unstable at extremes of pH, it is sometimes forgotten that the same may be true for substrates. Not only could the substrate concentrations be diminished by spontaneous break-down, but their breakdown products are likely to be enzyme inhibitors, since they share some molecular features with the real substrates. Therefore the pH stability of BAEE was checked by using control experiments and we found that the BAEE was stable over the pH range used.

The analysis of progress curves form a very valuable part of enzyme kinetics provided that it is carried out cautiously and sensibly. The artificial substrate BAEE, however, does not mimic the physiological response of the natural substrate of kallikrein, for example, kininogen. Therefore we confined ourselves to initial rates and simple kinetic arguments.

The Lineweaver-Burk plots for normal and CF salivary kallikreins gave values for  $K_m$  and  $V_{max}$  that were similar, despite the fact that these constants could only be obtained by a long extrapolation in view of the substrate inhibition.

The kinetic results discussed above, suggest that the normal and CF kallikrein are identical. Further support for the identity comes from HPLC and isoelectric focusing as well as from their behaviour during gel electrophoresis, both at pH 9.5 and under denaturing conditions in the presence of SDS. In addition the latter method confirmed that both kallikreins, from normal and from CF saliva, have a molecular weight of 40,000 D.



In the next chapter we shall discuss further physico-chemical evidence pointing to identical kallikreins in saliva of normal subjects and CF patients.

## CHAPTER 6

### PHYSICO-CHEMICAL COMPARISON OF SALIVARY KALLIKREIN OBTAINED FROM NORMAL INDIVIDUALS AND CF PATIENTS.

#### 1. Results

##### A. Amino Acid Composition

The amino acid compositions of the kallikreins from both normal and CF saliva were determined and are given in Table VI. The compositions are very similar. Both enzymes show a large excess of acidic over basic residues and this confirms the low isoelectric points that were observed before. The enzymes contain relatively low percentages of apolar residues.

Both enzymes are low in sulfur-containing residues.

The N-terminal amino acid in the kallikreins was found to be serine.

##### B. Fluorimetry

The excitation spectra for salivary kallikrein from normal individuals showed a maximum at 286 nm after correction for the variation in light intensity output of the Xenon source.

The corrected emission spectra for normal kallikrein obtained by excitation at 275 nm, the wavelength of maximum excitation of ~~acetyl~~ acetyl tyrosine ethyl ester, and at 286 nm which is the maximum for the enzyme are shown in Fig. 31. The two spectra

Table VI

The Amino Acid Composition

<u>Amino Acids</u>	<u>CF mole%<sup>a)</sup></u>	<u>Normal mole%</u>
Aspartic acid	10.8	10.8 $\pm$ 0.7
Threonine	5.3	5.2 $\pm$ 0.2
Serine	11.5	11.1 $\pm$ 0.7
Glutamic Acid	13.6	15.3 $\pm$ 2.1
Proline	4.7	5.9 $\pm$ 0.6
Glycine	12.4	13.3 $\pm$ 0.8
Alanine	6.6	6.5 $\pm$ 0.3
Valine	6.3	5.4 $\pm$ 0.2
Cysteine	0.7	0.4 $\pm$ 0.3
Methionine	1.1	1.1 $\pm$ 0.4
Isoleucine	3.0	2.6 $\pm$ 0.2
Leucine	7.4	7.6 $\pm$ 0.3
Tyrosine	2.4	2.3 $\pm$ 0.2
-Phenylalanine	3.7	3.4 $\pm$ 0.1
Lysine	3.1	3.3 $\pm$ 0.3
Histidine	2.9	2.6 $\pm$ 0.3
Arginine	3.3	2.8 $\pm$ 0.4
Tryptophan <sup>b)</sup>	1.0	1.0

a) Values are the average of 3 determinations for CF kallikrein and 4 determinations for the enzyme obtained from controls. The standard deviation is shown for the 4 determinations on the normal salivary kallikrein.

b) Tryptophan was determined indirectly, by the method of Goodwin and Morton (1946).

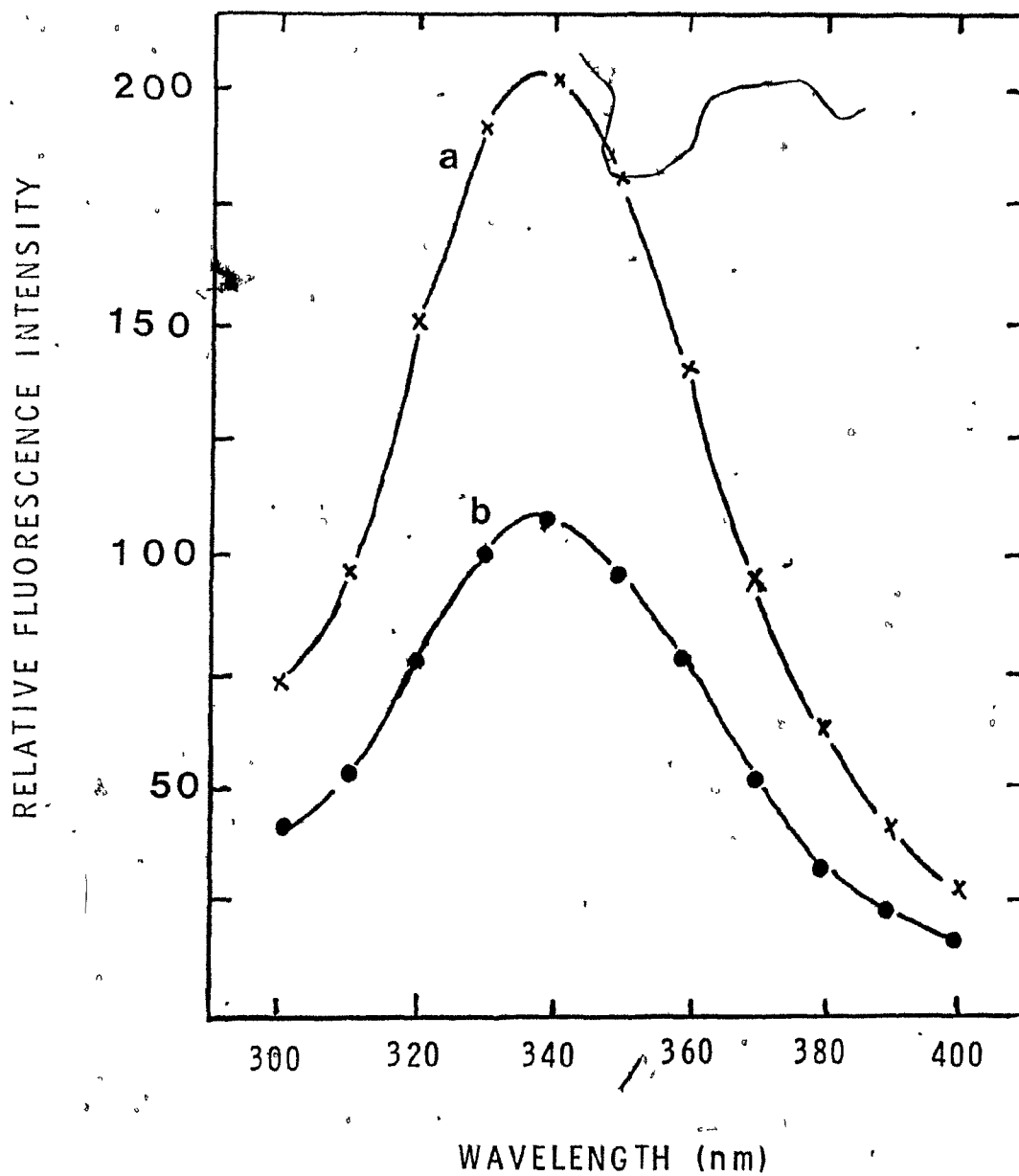


Fig.31 : Corrected fluorescence spectra for salivary kallikrein

The protein concentration was about 50 $\mu$ g protein per ml of 0.01 M phosphate buffer, pH 7.2. The experiments were carried out in a 3mm square cuvette using excitation at 286 nm (a) and 275 nm (b).

show maximum emission at 338 nm. The maximal emissions for the ethyl esters of N-acetyl tryptophan and N-acetyl tyrosine were found to be at 351 and 304 nm respectively.

The corrected, fluorescent intensities of kallikrein at 4 different wavelengths were calculated from spectra measured at 4 different wavelengths of excitation. The data were set down in a 4x4 matrix as shown in Fig. 32 and the rank analysis is shown in Table VII. The nine independent 2x2 submatrices of this matrix gave determinant/permanent ratios varying between 0.0065 and 0.1675. The latter value is about 3 x the experimental error. On the other hand the 4 independent 3x3 submatrices had determinant/permanent ratios of between 0.0003 and 0.003 or within the experimental error. The calculations therefore show that two different fluorophores, e.g., tyrosine and tryptophan residues, contribute to the intrinsic fluorescence of the salivary kallikrein obtained from normal individuals.

Fig. 33 illustrates the corrected emission spectra for purified CF and normal kallikrein obtained by excitation at 286 nm. The wavelength of maximum intensity is again found at 338 nm, and the spectrum appears to be the same as that observed for the enzyme from normal saliva. Furthermore the relative fluorescence intensities are directly proportional to the absorbances at 286 nm, of the normal and CF enzyme solutions.

An example of matrix analysis of the fluorescence data of an

Fig. 32 : Corrected fluorescence intensities of a solution of normal kallikrein in 0.02 M phosphate buffer pH 7.2.

The emissions were measured at 4 different wavelengths each at 4 different excitation wavelengths.

The data are shown in a 4x4 matrix and 4 independent 3x3 submatrices.

Emission Wavelength ( nm )

<u>Excitation Wavelength ( nm )</u>			
275	282	286	292
310	84	155	161
330	165	276	322
350	160	209	303
370	87	91	160

275 282 286 292

Emission Wavelength ( nm )

310	84	155	161	155	161	156
330	165	276	322	276	322	308
350	160	209	303	209	303	279
330	165	276	322	276	322	308
350	160	209	303	209	303	279
370	87	91	160	91	160	132

Table VII

Rank analysis of corrected fluorescence spectra of normal salivary kallikrein.

Submatrix	Determinant	Permanent	Ratio, a)
4 x 4	1.4580 x 10 <sup>7</sup>	74.2838 x 10 <sup>8</sup>	1.963 x 10 <sup>-3</sup>
3 x 3	x 10 <sup>4</sup>	x 10 <sup>7</sup>	x 10 <sup>-3</sup>
	5.0420	4.10745	1.230
	-2.6600	7.4696	0.356
	1.1485	3.4952	0.330
	-18.2786	5.9210	0.080
2 x 2	x 10 <sup>3</sup>	x 10 <sup>4</sup>	x 10 <sup>-2</sup>
	-2.391	4.8759	4.90
	5.474	9.4346	5.80
	-0.644	9.9820	0.65
	-9.675	5.7745	16.75
	16.330	15.0926	10.82
	-3.486	18.3126	1.90
	-3.623	2.9120	12.44
	5.867	6.1013	9.62
	-4.644	8.436	5.49

a) Ratio is Determinant divided by Permanent. The lowest ratios are found for the 3 x 3 square submatrices and the rank of the matrix is therefore 2.



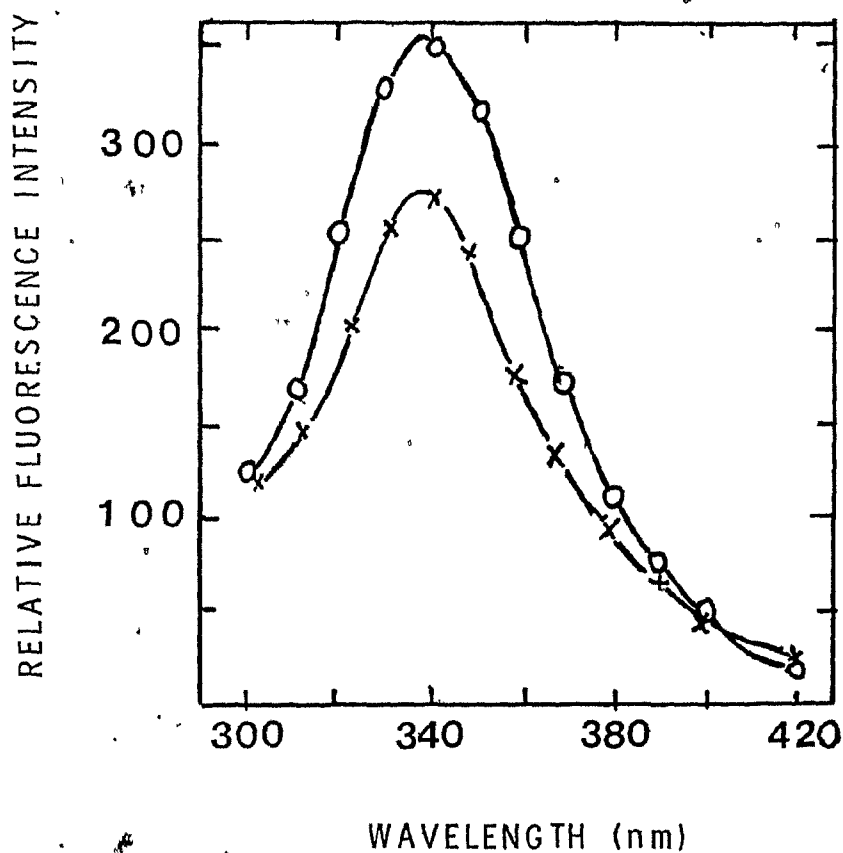


Fig. 33 : The corrected fluorescence spectra for normal salivary kallikrein (O—O) and kallikrein purified from CF saliva (X—X). The emission spectra were obtained by excitation of solutions in 0.01 M phosphate buffer pH 7.2 using 3 mm square cuvettes and an excitation wavelength of 286 nm. The absorbances at 286nm were 0.357 for the normal enzyme and 0.287 for the CF enzyme.

experiment with a preparation of the CF enzyme is shown in Fig. 34 and Table VIII. The results once more indicate the combined contribution of tyrosine and tryptophan residues to the fluorescence of the enzyme.

The fluorescence of normal kallikrein in phosphate buffer, pH 7.2 can be quenched by iodide. The quenching of the fluorescence in the presence of 0.1 M and 0.3 M KI is shown in Fig. 35.

### C. Circular Dichroism

The circular dichroism (CD) spectra for normal and CF kallikreins in the far ultraviolet, below 250 nm are quite similar, see Fig. 36A. The spectra show a minimum at about  $197 \pm 1$  nm with  $(\theta) = -12,200 \pm 1,300$ . A computer fit of this curve with 4 reference spectra, for example, those for  $\alpha$ -helix, pleated sheet,  $\beta$ -turns and random coils, indicated no  $\alpha$ -helix structure, as shown in Table IX. The data, however indicate 40% pleated sheet and 15%  $\beta$ -turn structure. The calculated curve, using the data shown in Table IX, give a relatively poor fit.

The kallikreins are quite stable to heat and SDS. When the normal enzyme is heated at  $100^{\circ}\text{C}$  for 5 min it loses 50% of its activity. The resulting spectrum, shown in Fig. 36B, shows a slight increase in ellipticity in the 220 to 230 nm range.

Emission Wavelength (nm)	Excitation Wavelength (nm)			
	275	282	286	292
310	81	136	143	136
330	144	227	263	289
350	134	209	253	238
370	76	114	171	119

Fig. 34 : A 4x4 matrix representing the corrected fluorescence intensities of CF kallikrein measured with a 3 mm square cuvette.

The enzyme was dissolved in 0.01M phosphate buffer pH 7.2 at a concentration of 121  $\mu\text{g/ml}$ .

Table VIII

Rank analysis of corrected fluorescence spectra of CF salivary kallikrein.

Submatrix	Determinant	Permanent	Ratio a)
4 x 4	$9.3000 \times 10^6$	$48.1628 \times 10^8$	$1.95 \times 10^{-3}$
3 x 3	$\times 10^4$	$\times 10^7$	$\times 10^{-3}$
	8.302	27.505404	0.30
	18.4399	50.105885	0.368
	6.202	25.245558	0.25
	7.531	44.980623	0.17
2 x 2	$\times 10^3$	$\times 10^4$	$\times 10^{-2}$
	1.195	3.7971	3.152
	3.307	6.8227	4.847
	5.559	7.7095	7.211
	0.322	6.0514	0.532
	2.464	11.2398	2.192
	10.523	13.5711	7.754
	0.608	3.1160	1.951
	0.627	5.8311	1.075
	0.3451	6.3665	5.421

a) Ratio is Determinant divided by Permanent. The lowest ratios are found for the 3x3 square submatrices and the rank of the matrix is therefore 2.

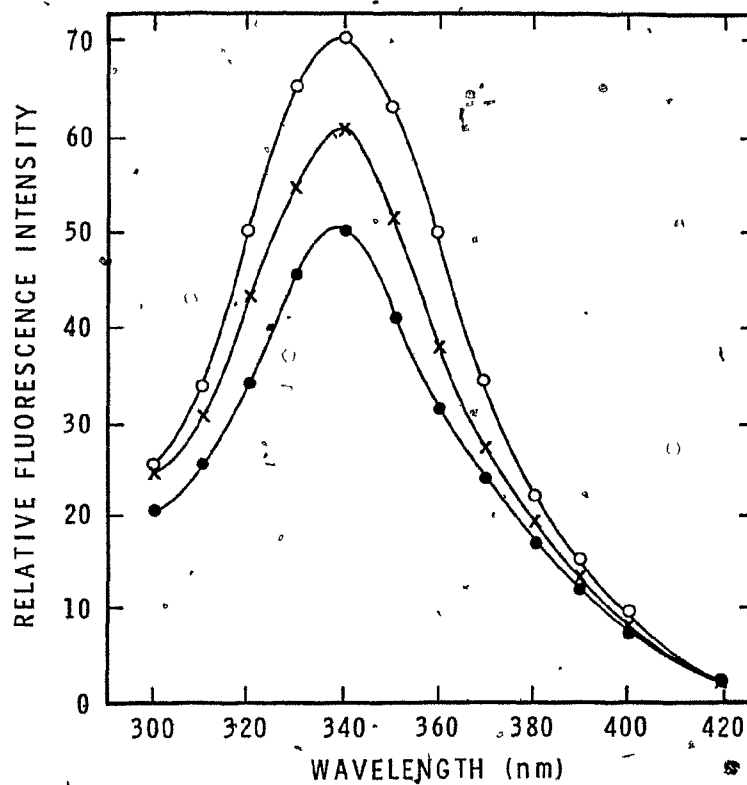


Fig. 35 : The corrected fluorescence of normal salivary kallikrein in 0.01 M phosphate buffer, pH 7.2 (○—○); phosphate buffer with 1 mM sodium arsenite and 0.1 M potassium iodide (x—x) or 0.3 M potassium iodide (●—●). Excitation was done at 286 nm.

Fig. 36 A: The CD spectra for Normal (—) and CF kallikreins (---) in 0.01 M phosphate buffer, pH 7.2. The calculated spectrum for 0%  $\alpha$ -helix, 41% pleated sheet and 15%  $\beta$ -turn is also shown (-.-).

B: The CD spectrum of normal kallikrein in 0.01 M phosphate buffer, pH 7.2 plus 0.5% SDS (—). The enzyme had lost about 50% of its activity during 5 min heating at 100°C. The calculated spectrum (---) for 0%  $\alpha$ -helix, 51% pleated sheet and 8%  $\beta$ -turn is also shown.

$[\theta] \times 10^{-3} \text{ deg} \cdot \text{cm}^2 \cdot \text{d mole}^{-1}$

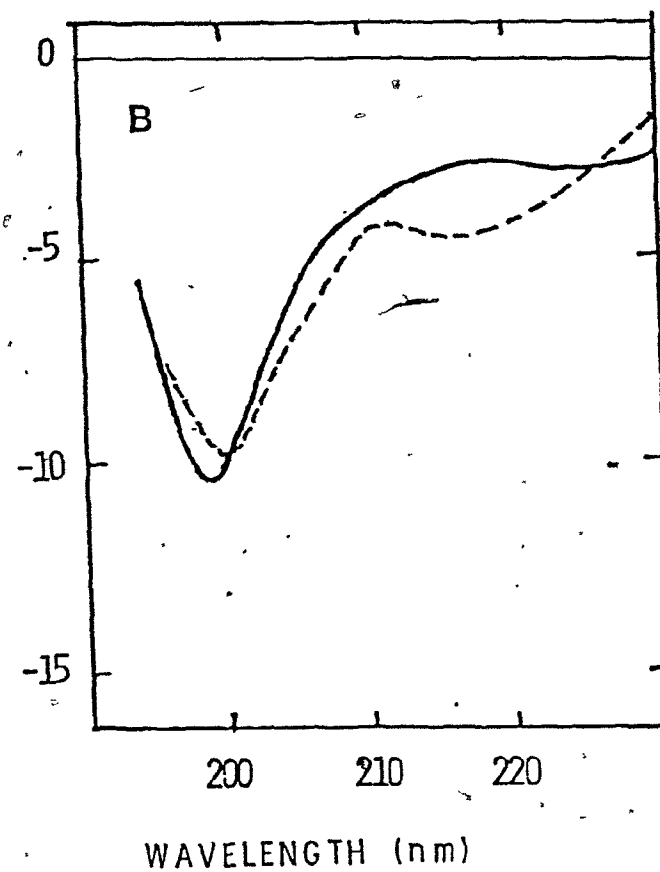
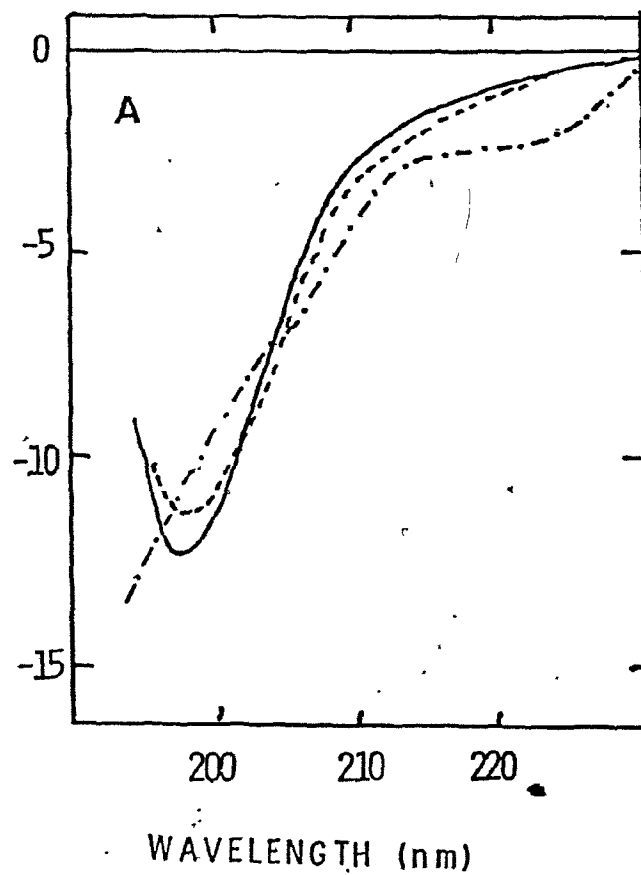


Table IX

Secondary structure of normal and CF salivary kallikreins.

Preparation	$\alpha$ -helix %	pleated sheet %	$\beta$ -turn %	Unordered %
Normal, pH 7.2	0	41	15	44
Normal in SDS	0	51	8	41
CF, pH 7.2	0	38	13	49



However, a computer analysis indicated that this is due to increased pleated sheet structure rather than the formation of  $\alpha$ -helix. In fact the structural changes brought about by this treatment do not appear as dramatic as normally observed. The calculated curve, again using the data of Table IX, shows an improved fit, but this is still not very satisfactory.

## 2. Discussion

The salivary kallikreins purified from normal individuals and CF patients are very similar in structure and composition.

The amino acid compositions indicate that both proteins are very acidic. This has been confirmed by isoelectric focusing which gave isoelectric points of  $4.07 \pm 0.05$  for both enzymes.

The enzymes have ratios of polar amino acids (aspartic and glutamic acid, lysine, histidine and arginine) over non-polar amino acids (alanine, valine, leucine, isoleucine, phenylalanine, methionine and tryptophan) of about 1.2. These values are fairly high and are reflected in the high solubility of the enzymes in aqueous solutions.

The enzymes have identical mobilities during electrophoresis in polyacrylamide gels under non-denaturing conditions, and also in gels containing SDS. The molecular weights have been calculated from electrophoresis experiments in the presence of SDS and a value of  $40,000 \pm 1,800$  has been found. This molecular weight has also been confirmed for the enzyme from normal saliva using sedimentation equilibrium.

The enzymatic properties of the enzymes are also similar except for a slight variation in the pH optimum of the activity and in the  $K_m$  value for BAEE calculated by extrapolation of Lineweaver-Burk plots. The latter variation is not surprising in view of the inhibitory effect of the substrate. This inhibitory effect is evident at BAEE concentrations of 1 mM and up and could

interfere with the regression analysis applied to the Lineweaver-Burk plots.

The salivary kallikreins show relatively weak circular dichroism. The spectra exhibit maximum negative ellipticity at 197 nm. Curve fitting by computer analysis indicates that no  $\alpha$ -helix structure is present in the enzymes. On the other hand the analysis shows large amounts of pleated sheet structure, despite the high glutamic acid contents, which would favour  $\alpha$ -helix rather than pleated sheets.

The general approach to the estimation of secondary structure is based on the premise that the CD spectra can be adequately described by a linear combination of  $\alpha$ -helix, pleated sheet,  $\beta$ -turn and unordered structure. When this hypothesis is applied to salivary kallikreins it is found that the computed closest fit does not resemble the observed spectra. In the presence of SDS, where the normal enzyme has lost about 50% of its activity during heating, the computed closest fit appears much improved, yet the overall structural parameters did not change dramatically.

These results may be used to argue against the adequacy of the 4-component model for the estimation of secondary structure in salivary kallikrein. The results shown in Table IX should therefore be interpreted with a great deal of caution. However, the main CD finding is that, normal and Cf salivary kallikreins apparently have similar conformations.

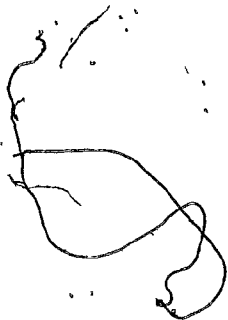
The fact that the normal and Cf salivary kallikreins possess

very similar conformations has further been confirmed by a study of the intrinsic fluorescence of the enzymes. Salivary kallikreins show relatively good fluorescence when excited at wavelengths between 270 and 295 nm. Solutions of normal and CF kallikreins with the same absorbances at 275 nm exhibit identical fluorescence intensities. The corrected fluorescence spectra show a maxima at 338 nm regardless of the wavelength of excitation. The wavelength of maximum fluorescence is lower than that of the reference compound, N-acetyl tryptophan ethyl ester which was found to be at 351 nm.

This shift could be due to the fact that the tryptophan residues are partly internal or that the fluorescence spectrum contains contributions of other fluorophores, e.g., tyrosine residues. Although the corrected spectrum does not show visible evidence of tyrosine fluorescence, which would occur at lower wavelengths, their presence has been demonstrated by an algebraic analysis of the spectrum. This algebraic analysis which depends on a rank analysis of a data matrix is considered reliable because the fluorescence spectra of model compounds such as the N-acetylated ethyl esters of tyrosine and tryptophan, or the spectra of the kallikrein do not show wavelength shifts upon changing the excitation wavelength. Thus tyrosine fluorescence does occur but is likely partly obscured by energy transfer to tryptophan.

It has been shown that potassium iodide quenches the fluo-

rescence of normal kallikrein to some extent. This suggest that at least some of the aromatic amino acid residues are located on the outer surface of the protein.



### SUMMARY AND CONCLUSIONS

The work presented in this thesis was undertaken in order to investigate whether or not glandular or tissue kallikrein, specifically salivary kallikrein is involved in the pathogenesis of CF. In order to carry out this investigation a method had to be developed to purify the protein very quickly and efficiently. The results of this study can be summarised as follows:

- 1) Whole centrifuged saliva, whether from normal individuals or from CF patients, can be fractionated by DEAE-Sephacel chromatography. Four fractions are obtained only one of which exhibits arginine esterase activity. No gross differences in this fraction could be detected between CF and normal saliva despite the dramatic difference in viscosity. The direct fractionation of whole saliva is a convenient method for the isolation of kallikrein, because whole saliva is a more readily available source than the secretions of specific glands. Therefore this method offers the advantage of speed in collecting and fractionating salivary kallikreins.
- 2) The yield of pure kallikrein is 0.077 mg from 500 ml of normal saliva. This represents a yield of 35% of the arginine esterase activity. In contrast the yield of activity from 500 ml of whole CF saliva supernatant is only 16% or 0.044 mg of enzyme. The data indicate that the CF saliva contains a normal amount of this soluble enzyme.
- 3) The kallikreins lower the blood pressure as demonstrated by

intravenous injection into a rabbit. They also produce kinins in acid treated dog plasma which in turn contract the smooth muscle of isolated rat uterus. Their esterolytic activities are inhibited by Aprotinin and BPTI but not by SBTI and LBTI. The esterolytic activity is reversibly inhibited by DFP but not by  $\alpha_2$ -macroglobulin which also does not bind to the salivary kallikrein.

- 4) The arginine esterase activities of the kallikreins are inhibited by high BAEE substrate concentrations and are 'pH-independent' above pH 9.0. It is speculated that this 'pH-independence' and substrate inhibition are due to binding of substrate molecule at secondary site on the enzymes.
- 5) The arginine esterase activity of the enzyme purified from CF patients is similar to that from normal individuals except for a slightly higher pH, by about 0.5 units, than the normal enzyme. This is not believed to be of any significance because the enzyme obtained from CF patients and non-CF subjects both have similar  $K_m$  and  $V_{max}$  values.
- 6) Human salivary kallikrein obtained from the whole saliva of normal individuals and CF patients have similar amino acid compositions. The amino acid compositions indicate that the enzymes are rather acid proteins. Their isoelectric points are indeed low and are  $4.07 \pm 0.05$ . Neither of the proteins contains detectable amounts of carbohydrates.

- 7) The normal and CF kallikreins have identical electrophoretic mobility in polyacrylamide gels. The enzymes have identical molecular weights of  $40,000 \pm 1,800$  D, and identical optical activities which indicate the absence of  $\alpha$ -helical structure. The CD studies, however, suggested the presence of a relatively large amount of pleated sheet structure in both enzymes. They also exhibit identical fluorescence spectra which again suggests identical conformations.
- 8) HPLC of preparations that had previously been chromatographed on a BPTI-Sepharose affinity column suggest that both normal and CF kallikreins bind a similar protein. It is believed that this association is broken when the pH is adjusted to pH 6.0 or lower.

The purified salivary kallikrein reported here resembles that of Wong et al. (1983) but differs from that of Hoffman et al. (1983). The latter also reported on the presence of more than one salivary kallikrein in man as did Brandzaeg et al. (1976) for rats, Fiedler et al. (1970) and Fritz et al. (1977) for pig. In 1975, Hojima et al. also described a microheterogeneity in human salivary kallikrein that was isolated by the method of Fujimoto et al. (1973). We do not find any evidence-suggesting multiple forms or microheterogeneity in purified human salivary kallikrein. It may be important to point out that the initial fractionation of whole saliva supernatant results in a quick



separation of the kallikrein from other potentially harmful factors in the saliva. Furthermore the isolated kallikrein is kept at a pH of 5.5 most of the time, where autolysis should be minimal. Thus we eliminated a number of possible factors which could well lead to apparent microheterogeneity.

When interpreting results of studies on biological enzymes with artificial substrates it should always be remembered that the artificial substrates are much less bulky than their ill-defined polymeric natural substrates, and also that an enzyme capable of binding a polymer is likely to bind a small molecule in many ways. Thus instead of a single enzyme-substrate complex that breaks down to products, there may be in addition numerous 'non-productive complexes'. Therefore the results obtained with synthetic substrates like BAEE should be interpreted with great care.

The physiological function of salivary kallikrein remains an enigma and its natural substrate is as yet not defined. Thus further studies will have to be carried out in order to demonstrate its function, mechanism and site of action.

The initial fractionation of centrifuged and filtered whole saliva on DEAE-Sephacel and subsequent gel electrophoresis does not reveal any gross differences between CF patients and normal individuals. This is rather surprising in view of the much increased viscosity of the CF saliva (which in some cases appears almost jelly-like). On the other hand our observation confirms

those reported by Gugler et al. (1967) and Tabachnik et al. (1981) who also did not detect any significant differences between proteins of control and CF patient saliva. These researchers supported the notion that a quantitative rather than a qualitative difference is associated with the secretory processes.

We have concluded that the salivary kallikrein in CF is not defective, and that its concentration in the CF saliva is quite normal.

Scientists have been battling with the problem of CF for many years and with the advances in technology and knowledge we are hopeful that all the pieces of the CF puzzle will snap into place someday soon. Thus I would like to conclude with a quote from the 'Great Master'-Aristotle : "The search for truth is in one way hard and in another easy. For it is evident that no one can master it fully nor miss it wholly. But each adds a little to our knowledge of Nature, and from all the facts assembled there arises a certain grandeur".

BIBLIOGRAPHY

- Albano, J., Bhoola, K.D., Heap, P.F. and Lemon, M.J. (1976) J. Physiol. (Lond.) 258, 631-658
- Albano, J., Bhoola, K.D. and Kingsley, G. (1977) J. Physiol. (Lond.) 267, 35-36P
- Aldaheff, J.A. (1978) Clin. Genet. 14, 189-201
- Allars, H.M., Blomfield, J., Rush, A.R. and Brown, J.M. (1976) Pediatr. Res. 10, 578-584
- Amouric, M., Lechene de la Porte, P. and Figarella, C. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 515-519
- Armstrong, D., Dry, R.M., Keele, C.A. and Märkhäm, J.W. (1952) J. Physiol. (Lond.) 117, 4P
- Armstrong, D. (1970) in Bradykinin, Kallidin and Kallikrein (E.G. Erdos, ed.) Handb. Exp. Pharmacol., Vol. 25, pp.434-481, Springer-Verlag, New York
- Baker, A.P. and Chakrin, L.W. (1977) Mod. Probl. Pediat. 19, 99-109: Mucus Secretion in Cystic Fibrosis (G.G. Forstner ed.) S. Karger. Basel. Munchen. Paris. London. New York.
- Barbero, G.J. and Chernick, W.S. (1966) in Third International Conference on Research on Pathogenesis of Cystic Fibrosis of the Pancreas (Mucoviscidosis) (P.A. di Sant'Agnese, ed.) Lancaster, Pa, Wickersham Printing Company pp. 208-222
- Barton, S., Sanders, E.J., Schachter, M. and Uddin, M. (1975) J. Physiol. (Lond.) 251, 363-369
- Bendayan, M and Orstavik, T.B. (1982) J. Histochem. and Cytochem 30, 58-66
- Bender, M.L. and Kezdy, F.J. (1964) J. Am. Chem. Soc. 86, 3704-3714
- Bennick, A. (1977) Biochem. J. 163, 229-239
- Bhoola, K.D., Morley, J., Schachter, M. and Smaje, L.H. (1965) J. Physiol. (Lond.) 179, 172-184
- Bhoola, K.D. and Ogle, C.W. (1966) J. Physiol. (Lond.) 184, 663-672

Bhoola, K.D. (1968) *J. Physiol. (Lond.)* 196, 431-445

Bhoola, K.D. (1970) in *Bradykinin and related kinins*, (F. Sicuteri, M. Rocha e Silva, eds.) *Adv. Exp. Med. Biol.* Vol 8, pp. 615-619, Plenum Press, New York-London

Bhoola, K. D., Dorey, G. and Jones, C.W. (1973) *J. Physiol. (Lond.)* 235, 503-522

Bhoola, K. D., Lemon, M. and Matthews, R. (1979) in *Bradykinin, Kallidin and Kallikrein* (E.G. Erdos ed.) *Handb. Exp. Pharmacol.* Vol. 25 Suppl. pp.489-523, Springer-Verlag, New York

Bradford, M.N. (1976) *Anal. Biochem.* 72, 248-254

Brandtzaeg, P., Gautvik, K.M., Nustad, K. and Pierce, J.V. (1976) *Br. J. Pharmacol.* 56, 155-167

Burgen, A.S.V. and Seeman, P. (1958) *J. Biochem.* 36, 119-143

Bury, A.F. and Barret, A.J. (1982) *Pediatr. Res.* 16, 613-620

Catanzaro, O.L., Seeber, A.M. and Beraldo, W.T. (1978) in *Kinins II* (S. Fujii, H. Moriya and T. Suzuki, eds.), *Adv. Exptl. Med. Biol.* 120A, pp. 451-454, Plenum Press, New York and London

Chang, C.T., Wu, C-S.C. and Yang, Y.T. (1978) *Anal. Biochem.* 91, 13-31

Chen, R.F. (1966) *Anal. Biochem.* 14, 497-499

Chervenka, C.H. (1969) *A Manual of Methods for the Analytical Ultracentrifuge*, pp. 39-64, Spinco Division of Beckman

Cleason, G., Friberger, P., Knos, M. and Eriksson, E. (1978) *Haemostasis* 7, 76-78

Coburn, M.D., Coburn, L.A. and Solomons C.L. (1974) *Am. Rev. Respir. Dis.* 110, 368-369

Cohn, E.J. and Edsall, J.T. in *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions* (E.J. Cohn and J.T. Edsall, eds.), pp. 370-381, Harner, New York (reprinted 1965)

Collier, H.O.J. (1970) in *Bradykinin, Kallidin and Kallikrein* (E.G. Erdos, ed.) *Handb. Exp. Pharmacol.*, Vol. 25, pp. 409-420. Springer-Verlag, New York

- Colman, R.W., Mattler, L. and Sherry, S. (1969) J. Clin. Invest. 48, 23-31
- Colman, R.W., Girey, G.D., Zarest, R. and Talamo, R.C. (1971) Prog. Hematol. 7, 255-298
- Conover, J.H. and Conod, E.J. (1978) Biochem. Biophys. Res. Commun. 83, 1595-1601
- Cutting, J.A. (1973) Anal. Biochem. 54, 386-394
- Dann, L.G. and Blau, K. (1978) Lancet 2, 405-407
- Dawes, C. (1974) Inter'l J. Chronobiol. 2, 253-279
- Dearborn, D.G. (1976) in Cystic Fibrosis Projections into the Future (J.A. Mangos and R.C. Talamo, eds.) pp. 179-191, Intercontinental Medical Book Comp. New York
- di Sant'Agnese, P.A., Darling, R.C., Perera, G.A. and Shea, E. (1953) Pediatrics 12, 549-562
- di Sant'Agnese, P.A. and Talamo, R.C. (1967) New Eng. J. Med. 277, 1287-1294, 1344-1352, 1399-1408
- di Sant'Agnese, P.A., Grossman, H., Darling, R.C. and Denning, C.R. (1968) Pediat. Res. 2, 464-478
- Dixon, M. and Webb, E.C. (1979) Enzymes (3rd edition) pp. 126-132 Academic Press, New York San Francisco
- Dubois, M. Gillis, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956) Anal. Chem. 28, 350-356
- Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80-91
- Emmelin, N.J. and Henriksson, K.G. (1953) Acta. Physiol. Scand. 30, 75-82
- Erdos, E.G., Tague, L.L. and Miwa, I. (1968) Biochem. Pharmac. 17, 667-674
- Fazekas de St. Groth, S., Webster, R.G. and Datyner, A. (1963) Biochim. Biophys. Acta. 71, 377-391
- Fiegel, R.J. and Shapiro, B.L. (1979) Nature 278, 276-277

Felberg, W. and Lewis, G.P. (1964) J. Physiol. (Lond.) 171, 98-108

Fiedler, F. and Werle, E. (1968) European J. Biochem. 7, 27-33

Fiedler, F., Muller, B. and Werle, E. (1969) European J. Biochem. 10, 419-425

Fiedler, F., Muller, B. and Werle, E. (1970) Hoppe-Seyler's Z. Physiol. Chem. 351, 1002-1006

Fink, E. and Schleuning, M. (1982) Hoppe-Seyler's Z. Physiol. Chem. 36, 1331-1340

Frey, E.K. (1926) Arch. Klin. Chir. 142, 663-668

Fritz, H., Fiedler, F., Dietl, T., Warwas, M., Truscheit, E., Klob, H.J., Mair, G. and Tschesche, H. (1977) in Kininogenases-Kallikrein; Vol 4 (G.L. Haberland, J.W. Rohen and T. Suzuki, eds.) pp. 15-28, Stuttgart, New York Schattauer

Fritz, H. (1983) in Kinins-III Part A (H.Fritz, N. Back, G. Dietze and G. Haberland, eds.), Ad. Expt. Med. Biol. Vol 126A, pp. 1-9, Plenum Press, New York and London

Fujimoto, Y., Moriya, H. and Moriwaki, C. (1973) J. Biochem. 74, 239-252

Ganrot, P.O. (1966) Clin. Chim. Acta. 14, 493-501

Garrett, J.R. (1967) Archs. Oral Biol. 12, 1417-1425

Gautvik, K.M. (1970) Acta. Physiol. Scand. 79, 174-187

Gautvik, K.M. and Kriz, M. (1974a) Acta. Physiol. Scand. 92, 95-105

Gautvik, K.M., Berg-Oestavik, T. and Nustad, K. (1974b) in Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease, Fogarty Internat'l Center Proc. No. 27, (J.J. Pisano and R.F. Austin, eds.), pp. 335-337, DHEW Publication No. 76-791, National Institute of Health

Goldsmith, G.H., Stern, R.C., Saito, H. and Ratnoff, O.D. (1977) J. Lab. Clin. Med. 89, 131-134

Gordon, A.H. (1969) in Laboratory Techniques in Biochemistry and Molecular Biology Vol. 1, Part F (T.S. Work and E. Work, eds.), pp. 21-41, North Holland, Amsterdam

Goodwin, T.W. and Morton, R.A. (1946) Biochem. J. 40, 628-632

Gryglewski, R. and Vane J.R. (1972) Brit. J. Pharmacol. 45, 449-457

Gugler, E., Pallavicini, C.J., Swerdlow, H. and di Sant'Agnese, P.A. (1967) J. Pediatr. 71, 585-588

Gutfreund, H. (1955) Trans. Faraday Soc. 51, 441-446

Hallinan, F. (1981) Medical Hypothesis 7, 793-800

Hamberg, M. and Samuelsson, B. (1974) in Prostaglandin Synthetase Inhibitors (H. Robinson and J.R. Vane, eds.), pp 107-119, New York, Raven Press

Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Proc. Nat'l Acad. Sci. U.S.A. 72, 2994-2998

Harpel, P.C. (1970) J. Exp. Med. 132, 329-352

Hilton, S.M. and Lewis, G.P. (1955) J. Physiol. (Lond.) 128, 235-248

Hilton, S.M. and Lewis, G.P. (1956) J. Physiol. (Lond.) 134, 471-483

Hoffman, W., Junk, A. and Geiger, R. (1983) Hoppe-Seyler's Z. Physiol. Chem. 364, 413-432

Hojima, Y., Matsuda, Y., Moriwaki, C. and Moriya, H. (1975) Chem. Pharm. Bull. 23, 1120-1127

Horton, E.W. (1959) J. Physiol. (Lond.) 149, 267- 282

Keele, C.A. and Armstrong, D. (1964) in Substances Producing Pain and Itch, London: Edward Arnold Ltd

Kaemmler, U.K. (1970) Nature 227, 680-685

Lamm, O. (1929) Z. Physik. Chem. A143, 177

Lands, W.E.M., LeTellier, P.R., Rome, L. and Vanderhoek, J.Y. (1974) in Prostaglandin Synthetase Inhibitors, (H. Robinson and J.R. Vane eds.), pp. 1-8 New York, Raven Press

Leme, J.G., Hamamura, L., Leite, M.P. and Rocha e Silva, M. (1973), Br. J. Pharmacol. 48, 88-96

Lieberman, J. Littenberg, G.D. (1969) Pediat. Res. 3, 571-578

Lieberman, J. (1974) Am. Rev. Respir. Dis. 109, 399-401

Lineweaver, H. and Burk, D. (1934) J. Amer. Chem. Soc. 56, 658-666

Lottspeich, F. (1980) Hoppe-Seyler's Z. Physiol. Chem. 361, 1829-1834

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275

Mangos, J.A., McSherry, N.R. and Benke, P.J. (1967a) Pediat. Res. 1, 436-442

Mangos, J.A. and McSherry, N.R. (1967b) Science 158, 135-136

Mangos, J.A. and McSherry, N.R. (1968) Pediat. Res. 2, 378-384

Mangos, J.A., Bargman, G.J., Martinez, J.R. and Rennert, O.M. (1976) in Cystic Fibrosis a Projection into the Future (J.A. Mangos and R.C. Talamo, eds.), pp. 311-336, Symposia Specialists Medical Books, Stratton Intercontinental Medical Book Cop. New York

Mann, R. and Geiger, R. (1977) in Kininogenases 4 (G.L. Haberland, J.W. Rohen and T. Suzuki, eds.) pp. 55-61, Schattauer, New York, Stuttgart

Manning, D.C., Snyder, S.H., Kachur, J.F., Miller, R.J. and Field, M. (1982) Nature 299, 256-259

Martinez, J.R., Camden, J. and Boney, F. (1981) Pediatr. Res. 15, 1439-1442

McGiff, J.C. (1980) Clin. Sci. 59, 105s-116

Melhuish, W.H. (1962) J. Opt. Soc. Am. 52, 1256-1258



Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Science 211, 1437-1438

Michaelis, L. and Menten, M.L. (1913) Biochem. Z. 49, 333-369

Mills, I.H. and Ward, P.E. (1975) J. Physiol. (Lond.) 246, 695-707

Modeer, T. (1977) Acta. Odontol. Scand. 31, 31-39

Morita, T., Kato, H., Iwanaga, S., Takada, K., Kimura, T. and Sakakibara, S. (1977) J. Biochem. 82, 1495-1498

Moriya, H., Yamazaki, K. and Fukushima, H. (1965) J. Biochem. 58, 201-207

Nadler, H.L., Rao, G.J.S. and Taussig, L.M. (1978) in The Metabolic Basis Of Inherited Diseases, 4th ed. (J.B. Stanbury, J.B. Wyngaarde and D.S. Fredrickson, eds.) pp. 1683-1710, New York, McGraw-Hill

Nagase, H. and Barrett, A.J. (1981) Biochem. J. 193, 187-192

Nustad, K., Gautvik, K.M. and Pierce, J.V. (1974) in Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease Fogarty International Center Proc. No. 27, (J.J. Pisano and K.F. Austen, eds.), Ch. 11, DHEW Publication No. 76-791, National Institute of Health

Nustad, K., Orstavik, T.B. and Gautvik, K.M. (1977) Microvasc. Res. Abstr.

Ole Moi Yoi, O., Pinkus, G.S., Spragg, J. and Austen, K.F. (1979) N. Engl. J. Med. 300, 1289-1294

Palade, G. (1975) Science 189, 347-358

Palm, S., Schill, W.B., Wallner, O., Prinzen, R. and Fritz, H. (1975) in Kinins, (F. Sicuteri, N. Rack and G.L. Haberland, eds.) Adv. Exp. Med. Biol. 70, 271-180

Piper, P.J. and Vane, J.R. (1969a) Nature 223, 29-35

Piper, P.J. and Vane, J.R. (1969b) in Prostaglandins, Peptides and Amines (P. Mantefazza and E.W. Horton eds.), pp.15-19, Academic Press: London, New York

Proud, D., Bailey, G.S., Nustad, K. and Gautvik, K.M. (1977) Biochem. J. 167, 835-838

Quinton, P.M. (1983) Nature 301, 421-422

Rao, G.J.S. and Nadler, H.L. (1972a) J. Pediatr. 80, 573-576

Rao, G.J.S., Posner, L.A. and Nadler, H.L. (1972b) Science 177, 610-611

Rao, G.J.S. and Nadler, H.L. (1974) Pediatr. Res. 6, 684-686

Rao, G.J.S. and Nadler, H.L. (1975) Pediatr. Res. 9, 739-741

Richman, J.B.Y. and Verpoorte, J.A. (1981) Can. J. Biochem. 59, 519-523

Roth, J., Bendayan, M. and Orci, L. (1978) J. Histochem. Cytochem. 26, 1074-1081

Sato, M., Funakoshi, I., Hayashi, K. and Yamashina, J. (1982) J. Biochem. 9, 1337-1345

Schachter, M. (1969) Physiol. Rev. 49, 509-547

Schachter, M. (1970) in Bradykinin, Kallidin and Kallikrein (E.G. Erdos, ed.), Handb. Exp. Pharm. Vol. XXV, pp. 400-408, Springer-Verlag

Schachter, M. (1980) Pharmacol. Rev. 31, 1-17

Schultze, H.E. and Heremans, J.F. (1966) Molecular Biology of Human Proteins- with special reference to plasma protein; Vol. I, pp. 762-772, Elsevier Publishing Company, Amsterdam-London-New York

Schwert, G.W. and Takenaka, Y. (1955) Biochem. Biophys. Acta. 16, 570-575

Spock, A., Heick, H.M.C., Cress, H. and Logan, W.S. (1967) Pediatr. Res. 1, 173-175

Staszewska-Barczak, J. and Vane, J.R. (1967) Br. J. Pharmacol. 30 655-677

Stewart, J.M., Roblero, J. and Ryan, J.W. (1970) in Bradykinin and related kinins, (F. Sicuteri, M. Rocha e Silva, eds.) Adv. Exp. Med. Biol. Vol 8, pp. 175-182

Sutcliffe, C.H., Style, P.P. and Schwartz, V. (1968) Proc. Roy. Soc. Med. 61, 297-300

Tabachnik, N.F., Blackburn, P. and Cerami, A. (1981) J. Biol. Chem. 256 7161-7165

Terragno, N.A. and Terragno, A. (1979) In Bradykinin, Kallidin and Kallikrein (E.G. Erdos ed.) Handb. Exp. Pharmacol. Vol. 25, Suppl. pp. 401-435, Springer-Verlag, New York

Thomas, G. and West, G.B. (1973) J. Pharm. Pharmacol. 25, 747-748

Trautschold, I. (1970) in Bradykinin, Kallidin and Kallikrein (E.G. Erdos ed.) Handb. Exp. Pharmacol. Vol. 25, pp. 52-81, Springer-Verlag, New York

Trowbridge, C.G., Krehbiel, A. and Laskowski Jr., M. (1963) Biochemistry 2, 843-850

Uddin, M. and Tyler, D.W. (1981) Experientia 37, 872-878

Vane, J.R. (1964) Brit. J. Pharmacol. Chemother. 23, 360-373

Vane, J.R. (1969) Brit. J. Pharmacol. 35, 209-242

Vane, J.R. and Ferriera, S.H. (1974) in Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease, Fogarty Internat. Center Proc. No. 27 (J.J. Pisano and K.F. Austen, eds.), Ch. 31, DHEW Publication No. 76-791, National Institute of Health

Vane, J.R. (1964) Brit. J. Pharmacol. Chemother. 23, 360-373

Vane, J.R. (1969) Brit. J. Pharmacol. 35, 209-242

Vane, J.R. and Ferriera, S.H. (1974) in Chemistry and Biology of the Kallikrein-Kinin System in Health and Disease, Fogarty Internat. Center Proc. No. 27 (J.J. Pisano and K.F. Austen, eds.), Ch. 31, DHEW Publication No. 76-791, National Institute of Health

Vargaftig, B.P. and Dao, N. (1972) J. Pharm. Pharmacol. 24, 159-161

Warren, L. (1959) J. Biol. Chem. 234, 1971-1975

Weber, G. (1961) Nature 190, 27-29

Weber, K. and Osborn, M. (1975) in The Proteins (H. Neurath, R.L. Hill and C.L. Boeder, eds.), 3rd edition, Vol. I, pp. 179-223, Academic Press, New York

Webster, M.E. and Pierce, J.V. (1961) Proc. Soc. Expt'l Biol. Med. 107, 186-191

Webster, M.E., Marès-Guia, M. and Diniz, C.R. (1970) in Bradykinin, Kallidin and Kallikrein, (E.G. Eros ed.) Handb. Exp. Pharmacol., Vol. 25, pp. 131-135, Springer-Verlag, New York

Weisman, U.N., Boat, T.F. and di Sant'Agnese (1970) J. Pediatr. 76, 444-451

Weisman, U.N., Boat, T.F. and di Sant'Agnese, P.A. (1972) Lancet 2, 510-512

Werle, E. and von Roden, P. (1939) Biochem. Z. 301, 328-337

Wong, R.S.C., Madapallimattan, G. and Bennick, A. (1983) Biochem. J. 211, 35-44

Wood, R.E., Boat, T.F. and Doershuk, C.F. (1976) Am. Rev. Resp. Disease. 113, 833-878

Wuepper, K.D. and Cochrane, C.G. (1972) J. Exp. Med. 135, 1-20

Zuber, M. and Sache, E. (1974) Biochemistry 13, 3098-3109