PLASMA ARGININE ESTERASE ACTIVITY
IN CYSTIC FIBROSIS OF THE PANCREAS

by

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ABSTRACT

Using a micro-method for the determination of plasma arginine esterase activity, the values for soy-bean trypsin inhibitor (STI)-inhibited arginine esterase activity in patients with cystic fibrosis, obligate heterozygotes and age matched control individuals have been investigated. The mean of STI-inhibited activity was lowest for cystic fibrosis patients while the mean for normal controls was the highest. The mean of STI-inhibited activity for the heterozygotes was midway between the values of the patients and the normal individuals. The deficiency of arginine esterase activity was statistically significant for both cystic fibrosis patients and heterozygotes.

By ion-exchange chromatography on DEAE-Sephadex, arginine esterase activity in chloroform-ellagic acid treated plasma from cystic fibrosis patients and control subjects has been resolved into two component fractions. The smaller peak of activity was eluted at approximately 0.02 M NaCl while the bulk of the activity was eluted at an NaCl concentration of 0.15 M. Similar elution patterns were obtained in both cases of control and cystic fibrosis plasma, though a reduction in size

of the two activity peaks was observed in cystic fibrosis plasma. No iso-enzyme deficiency was detected using anion-exchange chromatography. Hence, the deficiency of arginine esterase activity in the cystic fibrosis plasma was apparently entirely quantitative rather than qualitative.

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I. INTRODUCTION

Cystic fibrosis of the pancreas is a generalized hereditary disorder of children and adolescents manifesting primarily by dysfunction of exocrine glands. The classic features are chronic bronchiolar obstruction and infection of the lungs, steatorrhea, azotorrhea, increase in the salinity of sweat, malnutrition and growth failure. The basic defect remains unknown.

A. HISTORY

Early reports described various clinical manifestations of the disease, but it was not until 1938 that cystic fibrosis of the pancreas was recognized by Anderson as a specific disease entity because of the anatomic finding of extensive pancreatic involvement (1). However, the recognition that many of the pathological changes were produced by increased viscosity of mucous secretions led to the term mucoviscidosis (51) which is still widely used in Europe. Since the discovery by di Sant'-Agnese (41) in 1953, that in cystic fibrosis the salinity of sweat is increased and that the disease is a generalized exocrinopathy with various abnormalities of exocrine secretion, a shortened form of the original name, cystic fibrosis, has become the most common name

used.

B. GENETICS AND INCIDENCE

Cystic fibrosis is transmitted as an autosomal recessive trait (37). At birth, both sexes are equally affected (132), even though more males than females survive past the age of puberty (37, 131). Parents and other heterozygous carriers are unaffected. The disease occurs without regard to maternal age (37), birth order (14) or season of the year (96).

Cystic fibrosis is the most common of all inherited diseases in Caucasian children. Many estimates of the incidence of cystic fibrosis have been made, with figures ranging from 1 in 2,000 to 1 in 3,700 live births (37, 81, 82, 105, 138). Thus, the frequency of heterozygous carriers in the Caucasian population is approximately 3 - 5% implying that about 1 in 500 marriages has the potential for producing an affected child (102).

Cystic fibrosis has a striking racial incidence. It occurs approximately equally in all groups of Caucasians, very infrequently in Orientals, and is rare in Negroes (45).

C. CLINICAL FEATURES

Cystic fibrosis may present in the neonatal period as intestinal obstruction by thickened meconium requiring surgical relief. This condition is called meconium ileus and has an incidence of 10-15% in cystic fibrosis patients (48).

During the first year of life, most patients have frequent large, foul stools, a large appetite, poor weight gain and develop abdominal distension. Steatorrhea may be severe with fecal fat averaging 4 to 5 times normal (102).

Pulmonary symptoms of wheezing, coughing and steatorous breathing may appear at any time after birth, but most frequently in the first year of life (122). Viral infections affecting the lungs often develop into bacterial pneumonia. The common bacterial invaders are *Staphylococcus aureus* (76) and *Pseudomonas aeruginosa* (47). Pulmonary involvement later in childhood may be suggested by barrel-chest deformities and digital clubbing. Chronic pulmonary disease dominates the clinical picture and determines the fate of the patient. It is present in almost all patients at some time in the course of their disease. Death may occur as a result

of broncho-pneumonia, asphyxia, cardiorespiratory failure associated with cor-pulmonale or acute pulmonary infection on top of severe chronic lung disease. A summary of the symptoms and signs of cystic fibrosis is shown in Figure 1.

Normal growth is retarded in the cystic fibrosis patient, with body weight below that expected for height or age, and even linear growth is sometimes depressed (137). These children are therefore small and appear malnourished. The extremities are thin and the abdomen distended. With improved medical treatment, many cystic fibrosis patients have survived to adulthood. The median age of death in 1970 was 15 years (46), in contrast to a median age of death of 8 months before any treatment was available (145).

D. PATHOGENESIS

Cystic fibrosis is produced by a gene with pleiotropic effects, i.e. the homozygous gene state manifests itself in multiple defects of seemingly unrelated origin (102). Most research work has concentrated on the exocrine system, in which the secretory fluids have many abnormal properties which exacerbate the clinical picture. Nevertheless, it has been

Figure 1: A summary of the symptoms and signs of cystic fibrosis (66).

PRIMARILY DUE TO PULMONARY LESION

EARLY SYMPTOMS AND SIGNS OF LOW GRADE CHRONIC PULMONARY INFECTION

- Increased respiratory rate
- Dry, hacky, non-productive cough
- Prolonged expiration phase
- Decreased activity

MODERATE

- Change in personality—more introverted and irritable
- Decreased appetite—may still be good but not voracious
- Decreased exercise tolerance
- Scattered or localized wheezes, musical rhonchi, rales
- Signs of emphysema—
 - increased A-P diameter
 - diminished area of cardiac dullness
 - depressed diaphragms
 - palpable liver border
- Failure to gain or grow or weight loss
- Repeated episodes of respiratory infection

ADVANCED

- Listless, limitation of normal activity, easily fatigued
- Marked decrease in appetite associated with weight loss
- Growth failure—stunting
- Chronic, paroxysmal, productive cough often associated with vomiting
- Muscular weakness—flabby
- Increased respiratory rate, shortness of breath on exertion, orthopnea, dyspnea
- Noisy respiration—wheezing, bubbling, audible rales
- Cyanosis, digital clubbing
- Marked emphysema—
 - marked increase in A-P diameter—barrel chest—pigeon breast
 - limited respiratory excursion of thoracic cage
 - depressed diaphragms
 - hyper-resonance over entire chest
 - decreased exchange
- Diffuse and localized rales and rhonchi
- Fever, tachycardia, toxicity
- Rounded shoulders, forward position of head, poor posture
- Hemoptysis, signs of atelectasis, pneumothorax and lung abscess
- Signs of cardiac failure—edema, enlarged tender liver, venous distention
- Visual impairment and eye ground changes

PANCREATIC AND NUTRITIONAL

- Meconium ileus
- Poor weight gain despite voracious appetite
- Distended abdomen
- 3-4 bulky, greasy, floating, foul-smelling stools per day
- Rectal prolapse
- Palpable loops of bowel especially in right lower quadrant
- Cramps and excessive foul flatus
- Malnutrition, poor muscle tone, small flabby muscles, lack of subcutaneous fat
- Hypoproteinemia with generalized edema

SIGNS OF HYONATREMIA AND HYPOCHLOREMIA

- Loss of sodium and chloride by sweating
 - Mild—forehead tastes salty
 - Severe—muscle cramps, weakness, shock

SIGNS OF BILIARY CIRRHOSIS AND PORTAL HYPERTENSION

- Firm, nodular liver—often palpable in midline
- Splenomegaly
- Hypersplenism—decreased WBC and platelets, anemia
- Hematemesis and melena from esophageal varices

difficult to focus attention on any one biochemical abnormality for investigation. The following is a subjective selection of what I consider to be some of the relevant research done on cystic fibrosis.

1. Sweat Glands

The increased sodium and chloride concentrations of eccrine sweat are the most constant and clearly defined abnormalities that have been detected in cystic fibrosis. In 98% of patients the secretion of chloride and sodium is increased two to five times above normal levels (41, 66). Sweat potassium is also increased, but to a lesser degree than sodium and chloride (41, 44). This characteristic finding is present at birth and throughout life and appears to be unrelated either to the severity of the underlying disease or to the extent of organ involvement. Excessive salt loss in sweat due to sudden rise in environmental temperature may lead to severe sodium depletion and vascular collapse (41, 44).

Many studies have been performed to determine the cause of elevated salt concentration in cystic fibrosis sweat. There appear to be no morphological differences between the sweat glands of cystic fibrosis

patients and normal subjects (59, 108). The sweat gland is of normal size in cystic fibrosis (7). There are no differences in the rates of sweating (50, 95, 133) or in concentrations of organic constituents (19, 57).

However, both by direct micropuncture studies (125, 126) and indirect calculations (50, 56, 135), there is an indication that the duct of the sweat gland in cystic fibrosis has a greatly reduced capacity to reabsorb sodium. Sweat from patients with cystic fibrosis contains a heat-labile, non-dialyzable factor which inhibits sodium reabsorption in the duct of the rat parotid gland (99) and in the normal sweat gland (78). Whether this factor produces the defect in sodium reabsorption is unknown.

2. Glycoproteins and Calcium

Glycoprotein metabolism in cystic fibrosis has been the subject of intensive investigation. Apparent increased viscosity of mucous secretions in cystic fibrosis organs and glands has suggested a defect in this area of metabolism to many investigators. In the duodenal fluid and rectal mucus of patients with cystic fibrosis, there are glycoproteins of abnormal chemical structure which are easily denatured and rendered

insoluble (39, 43, 120). The total amount of glycoprotein is normal, but the carbohydrate moieties contain an increase in fucose and a decrease in sialic acid (39, 120). It has been postulated that the ratio of fucose to sialic acid may determine glycoprotein solubility and viscosity and may be a factor in the pathogenesis of cystic fibrosis (93). Similar compositional changes in glycoprotein also occur in salivary gland (97). Most recently, Vaze et al have demonstrated a marked increase in glycoprotein in the platelets of cystic fibrosis patients (141). The sialic acid-protein ratio (1:50) is much lower in the patients' platelets when compared to controls (1:15). However, the presence of abnormal glycoproteins is not unique in cystic fibrosis. Patients with fucosidosis also exhibit an accumulation of fucose containing glycoproteins in all tissues due to the lack of an acid lysosomal hydrolase, α - fucosidase (49). Increased chloride and sodium contents of sweat and mixed saliva have been reported in some patients with this disease.

Glycoproteins from the bronchial tree of cystic fibrosis patients have been studied by Lamblin and co-workers (85). Their data suggest that these glycopro-

teins are more acidic and a fucose-rich glycoprotein is not found in the cystic fibrosis sputum. Low levels of fucose in mucous glycoproteins may be important because of a recent promising finding that cystic fibrosis sera do not possess the property for addition of ^3H -fucose to cell membrane surfaces (18).

The submaxillary gland of cystic fibrosis patients secretes a glycoprotein-rich saliva which is abnormal in appearance and composition as demonstrated by Mandel et al and Chernick et al (16, 97). The fluid is turbid and contains a higher fucose, sialic acid, hexose, total protein and calcium content (17). This is in contrast to the clear secretion in unaffected persons. Cystic fibrosis saliva clears with the addition of chelating agents, while calcium added to normal saliva will increase its turbidity (65). An increase in calcium levels of many glycoprotein-rich biologic fluids is indeed one of the most constant findings in cystic fibrosis. An effect of divalent ions on the solubility of glycoproteins is recognized, and Kwart and Sashoua suggested that calcium might form a link between glycoproteins and amino acids (84). Svennerholm (139) and Lev et al (88) also suggested that calcium might play

an important part in decreasing the solubility of glycoproteins in cystic fibrosis. The precipitation of a relatively insoluble calcium-glycoprotein complex may explain some of the pathological and clinical findings in cystic fibrosis.

Effects of calcium on the permeability of membranes are also recognized. An interesting theory has been proposed by Gibson and associates (60). This concerns the supposition that mucus has a biological function of inhibiting passive flows of water and small ions. The theory claims that cystic fibrosis is a disease in which mucus is hyperpermeable to water and fails to perform this function adequately, and that this failure is caused by an excess secretion of calcium from exocrine glands. However, we still do not know whether the fundamental molecular abnormality in cystic fibrosis resides within the cell or within a circulating substance affecting cell function.

3. Pancreas

Pathological changes in the pancreas may be observed during fetal life. At first, small casts of eosinophilic material may be found in the acini which become irregular with cellular proliferation. The casts

then appear in the ducts which become distended and occluded by fibrous tissue. Cyst formation and acinar atrophy are followed by infiltration with adipose tissue. After 2 years of age the pancreas may be unrecognizable except as islets clustered in fibrous or adipose tissue (2, 11). Localized foci of biliary obstruction and fibrosis are common findings in the liver at necropsy (66).

Untreated pancreatic deficiency results in progressive malnutrition, marked steatorrhea and azotorrhea, abnormal fat in the stools and deficiencies of lipid soluble vitamins. Approximately 10-15% of patients do not develop signs of pancreatic deficiency. The steatorrhea and azotorrhea of cystic fibrosis are due to the deficiency of pancreatic lipases, trypsin, chymotrypsin and carboxypeptidases (45, 93).

Hadorn and associates have studied pancreatic function in patients with cystic fibrosis who do not have steatorrhea (67, 68). Enzyme secretion in response to pancreozymin was almost normal, but secretin response was much decreased. In contrast, children with pancreatic insufficiency without cystic fibrosis had a more normal

secretion response but deficient enzyme secretion (69). In 8 of 10 patients with cystic fibrosis, the viscosity of duodenal juice was increased after secretin stimulation. The bicarbonate concentration did not rise, and chloride concentration remained high as the rate of flow increased. The same findings have been reported by Rick (118). Hadorn concludes that decreased secretin response may contribute to the obstructive pathology in the pancreas and also may indicate a primary abnormality of the duct cells.

4. Lung

Chronic obstructive lung disease is the most serious aspect of cystic fibrosis. Pulmonary difficulties issue from the inadequate removal of mucous secretions from the tracheobronchial tree and upper respiratory tract (40). The mucous glands are distended by accumulated viscid mucus (2) which may obstruct one or more bronchi (144) in a progressive dilatation leading to bronchiectasis. Pulmonary-function studies in various laboratories have confirmed the clinical findings of airway involvement. Sequential testing of a patient with cystic fibrosis usually shows increasing obstruction of the airways as indicated by peak expiratory rate, mid-maximal

expiratory flow rate, and 1 sec. forced expiratory volume. Vital capacity decreases with age and functional residual capacity is elevated (8). Pulmonary hypertension and cor pulmonale are frequent complications (63, 107).

The analysis of bronchial mucus, although contaminated with bacteria and inflammatory exudate, shows a decreased content of sodium and chloride in cystic fibrosis patients (93). The ratio of fucose to sialic acid in the glycoprotein is decreased (110). These relationships are opposite to those observed for salivary glands (41) and duodenal fluid (39, 120).

Since chronic pulmonary disease in cystic fibrosis patients is probably the result of the presence of abnormally viscous mucus in the respiratory passages, it could naturally be assumed that the mucociliary function in cystic fibrosis might be disturbed. However, studies by Sanchis et al (123) and Newhouse et al (109) indicated that mucociliary clearance was normal in cystic fibrosis subjects, as judged by the removal of deposited labelled aerosols from the lungs. Most recently, Rossman and co-workers has shown that cystic fibrosis serum inhibits mucociliary clearance when applied to nasal mucosa previously treated with IgE to

induce an inflammatory response and disrupt the mucous barrier (121). Normal serum has no effect on mucociliary activity under the same conditions.

5. Blood

Erythrocytes

The sodium and potassium concentrations and the morphology of erythrocytes are normal in cystic fibrosis (4, 93, 94). In studies of the sodium efflux from these cells, Balfe et al found a small decrease in ouabain-sensitive sodium transport and a marked reduction in the ouabain-insensitive transport of sodium (4). This latter was caused by deficiencies in both the ethacrynic acid-sensitive and -insensitive portions. Paradoxically, they noted decreased ouabain-sensitive (Na^+/K^+) ATPase in cystic fibrosis erythrocyte membranes, whereas the ouabain-insensitive portion was normal. Lapey and Gardner however, in a similar study, obtained rather different results in that there was a decrease in the ethacrynic acid-sensitive portion of Na^+ efflux in the erythrocytes of cystic fibrosis males and postpubertal females or heterozygotes (87). Horton et al reported normal (Na^+/K^+) and Mg^{2+}) ATPase activities but decreased (Ca^{2+}) ATPase in cystic fibrosis homozygotes (75). The

degree of abnormality was directly proportional to the clinical severity of disease. Cole and Dirks observed normal ouabain-sensitive ATPase and diminished ouabain-insensitive (Mg^{2+})-ATPase in cystic fibrosis red blood cell membranes (20). Later, Cole and Sella studied the effect of saliva from children with cystic fibrosis on an ATP hydrolyzing enzyme system. Significant decrease in the activities of the Na^+/K^+ activated ouabain-sensitive component and a Ca^{2+} -activated component of ATPase was detected. They concluded that there was a factor in the saliva of cystic fibrosis patients which interferes with active cation transport as a result of inhibition of one or more components of the ATP hydrolyzing enzyme system.

More recently, McEvoy et al demonstrated normal levels of the Mg^{2+} -dependent and $\text{Ca}^{2+}+\text{Mg}^{2+}$ -dependent ATPase activities in the erythrocyte membranes of cystic fibrosis patients (103). Feig et al also reported that the total, ouabain-sensitive, and ouabain-insensitive (Na^+/K^+) ATPase and (Ca^{2+}) ATPase activities were indistinguishable from that of normal control subjects (52). Hence, they concluded that intrinsic ATPase and monovalent cation transport functions are normal in

cystic fibrosis red blood cells.

Schmoyer and Baglia examined membrane associated ATPase activities in human red blood cells following the addition of spent fibroblast culture media from normal and cystic fibrosis cell lines (124). In comparison to normal and unspent media, the cystic fibrosis media consistently depressed the Na^+/K^+ ATPase, Mg^{++} ATPase as well as Ca^{++} ATPase activities. However, these observations have not been confirmed by Applegarth et al (3).

Leukocytes

Danes et al demonstrated metachromasia in cultured leukocytes from both cystic fibrosis homozygotes and heterozygotes upon staining with toluidine blue (35). Danes and Bearn proposed that this probably reflects uptake of heparin from the culture-medium by pinocytosis and not, as in the genetic mucopolysaccharidoses, stored mucopolysaccharide (33). This hypothesis has received support from work of Robertson et al (119). Using ^{35}S -heparin they demonstrated an increased uptake of heparin in short-term cystic fibrosis white blood-cell cultures which correspond to the observations made on the basis of staining.

Studies of short-term leukocyte cultures using $\text{Na}_2^{35}\text{SO}_4$ incorporation into glycosaminoglycans (G.A.G.) have been done by Candill et al (15). The amount of $\text{Na}_2^{35}\text{SO}_4$ incorporation into G.A.G. material found in both the incubation medium and the cells from cystic fibrosis patients is significantly increased over normals. However, there is no morphologic evidence for accumulation of G.A.G. in vivo (42), and their urinary excretion was found to be normal in 27 patients with cystic fibrosis (31).

Plasma and Serum

Sera from cystic fibrosis patients and heterozygotes contain a factor which causes the disruption of normal ciliary beat in rabbit tracheal explants (136) and the total cessation of the ciliary beat of oyster gills (12). This substance is associated with a nondialyzable, heat-labile, cation with an apparent molecular weight of 125,000 to 180,000 daltons (13, 136). In fact, the cystic fibrosis factor is a smaller molecule with a molecular weight between 1,000 and 10,000 daltons (28) which is bound to proteins or immunoglobulins as discussed later. This ciliary dyskinesia factor is presumed to be similar to a substance in cystic fibrosis sweat (99) and

saliva (100) which inhibits the reabsorption of sodium in the rat parotid gland and human sweat gland (98). Work on this ciliary dyskinesia factor has been hampered by the non-quantitative nature of the ciliary effect and the seasonal and regional inaccessibility of the ciliated tissue (10, 13). Furthermore, these bioassays do not distinguish between an individual who is homozygous and one who is heterozygous for cystic fibrosis (10, 13, 102, 136).

Early attempts at fractionation on gel filtration columns suggested that the anticiliary factor was absorbed to several serum proteins (136). Bowman's group reported a consistent association of the factor with IgG-containing fractions (13). Danes et al later demonstrated that the cystic fibrosis factor activity is class specific for human IgG, subclass for IgG1 and IgG2. Interaction occurs with the constant region of heavy chains but not with K and λ - light chains (36). By isoelectrofocusing, Wilson et al identified a cationic protein which a pI (isoelectric point) of 8.41. Like the ciliary dyskinesia factor, it had a molecular weight of less than 10,000 daltons and a stoichiometric relation to IgG (147, 150). Conover and co-workers (24)

have modified the original rabbit trachea assay of Spock et al for anticiliary activity and claimed that their method reliably distinguished normal control serum from that of heterozygotes and cystic fibrosis patients. However, this test did not distinguish clearly between the heterozygote and homozygote.

The reported presence of anticiliary factor(s) in such diverse tissue as lymphocytes and fibroblasts is additional evidence that cystic fibrosis may not be simply a disorder of exocrine glands. More likely, the factor(s) is a normal cellular or extracellular constituent which is overproduced or ineffectively catabolized in cystic fibrosis (38).

6. Complement

Several groups have attempted to establish a relation between the ciliary dyskinesia factor and one of the complement components. Conover and his colleagues reported elevated levels of the C3 component of complement in patients with cystic fibrosis and in obligate heterozygotes (27). They also observed physicochemical similarities between the cystic fibrosis factor and C3a (anaphylatoxin), a fragment of C3 (26). Purified C3a had anticiliary activity when bound to IgG. Moreover,

they showed that the anticiliary activity is inactivated by antibody to C3a (9). Hence, cystic fibrosis may be a complement-caused or complement-mediated disorder, in which a C3a-like molecule either alone or by complexing with IgG gives rise to the physiological and pathological state of the disease (25). Once C3a (or CF factor) is complexed to IgG, it is protected from its inhibitor(s). Danes et al showed that the cystic fibrosis factor is bound mostly to IgG₁ (and IgG₂)(36). Molenaar et al also demonstrated that C3a links to IgG, and suggested that the activity of C3a might be evolved on the surface of an immune complex (106). Later, Conover et al assumed that a defect in the anaphylatoxin inactivator (AI) or another enzyme similar to carboxypeptidase B must occur in cystic fibrosis to explain the accumulation of C3a or CF factor. However, a defect in AI need not be postulated if C3a is protected from AI when it is complexed to IgG. The findings of Conover et al (27) has been confirmed by Hann et al (70), but refuted by Lieberman (90) and Scanlin et al (33).

E. THE BASIC DEFECT IN CYSTIC FIBROSIS

What, then, is the basic defect in cystic fibrosis? Is it an inborn error of glycoprotein metabolism, as has been postulated in the past? What is the relation of the electrolyte abnormality to the physicochemical and perhaps structural abnormality of mucous secretions? Where and how do the electrolytes and glycoproteins interact with each other so as to give rise to a defect present in exocrine glands throughout the body? Does the ciliary dyskinesia factor modify exocrine function, giving rise to symptoms, or is it just a secondary manifestation of the disease?

At present, no one hypothesis can be made to reconcile and place into a rational pattern all the diverse and often conflicting observations so far advanced. As more insight is gained into the biochemical background of cystic fibrosis, the probability increases that the disease is genetically heterogeneous. This is supported by work on cultured cell metachromasia (34), serum-hexosaminidase levels (29), and sulphate incorporation of leukocytes from patients with cystic fibrosis (15). However, the identity of the defective gene(s) remains a complete mystery. One hopes that from the varied

laboratory investigations a unified theory of pathogenesis of cystic fibrosis will emerge but this has not yet happened.

F. PLASMA ARGININE ESTERASE ACTIVITY IN CYSTIC FIBROSIS

Several theories for the pathogenesis of abnormal exocrine secretions in patients with cystic fibrosis of the pancreas have been proposed (45). Because of the potential role of bradykinin in the function of glandular tissues and in the mediation of electrolyte transport, an investigation of the kallikrein-kinin system in cystic fibrosis was undertaken by Lieberman and Littenberg in 1969 (92). Figure 2 shows the pathway of bradykinin formation. Factor XII (Hageman factor) is converted by exposure to a foreign surface (e.g. glass or collagen) to a protelytic enzyme factor XII a. Activated factor XII, though possessing potent coagulant activity has relatively low activity in initiating kinin formation. For efficient conversation of prekallikrein to kallikrein, factor XII a must first be proteolytically split to polypeptide fragments that are more potent prekallikrein activators. The molecular changes accompanying the conversion of human plasma prekallikrein to kallikrein is not yet elucidated.

Figure 2: Kallikrein - kinin system.

Plasma kallikrein is a proteolytic and esterolytic enzyme with arginine and lysine esterase activity. There are also a number of tissue kallikreins that differ from plasma kallikrein in behaviour toward substrates and inhibitors, electrophoretic mobility and immunochemistry. Plasma kallikrein acts on kininogens to release bradykinin. It also catalyzes a positive feedback reaction that converts factor XII a to a more potent prekallikrein activator. The activity of kallikrein is inhibited by three plasma proteins - α_2 -macroglobulin, C1 esterase inactivator and, in the presence of heparin, antithrombin III (21). These inhibitors normally guard against the profound effects of bradykinin release on the organism.

Bradykinin exists in plasma in very low concentration (less than 3 ng per milliliter as determined by radioimmunoassay). This low level is the result of the balance struck between simultaneous processes of production and inactivation. Kininases are carboxypeptidases which destroy the plasma bradykinin. Kinins have striking cardiovascular effects, the most pronounced of which is the generalized dilatation of peripheral arterioles. Intravenous administration of bradykinin in man produces an increase in cardiac output, an increase in

stroke frequency and volume, a decrease in systemic arterial blood pressure and total systemic resistance. Kinins also increases capillary permeability, produce pain, and influence migration of leukocytes (21).

The glandular tissues in which the kinins were first detected are characterized by rapid alterations in blood flow (53, 73). The kinins are also found to be the mediators of electrolyte transport in some tissues (55). Thus, a study of the kallikrein-bradykinin system is of interest in the search for the basic defect in cystic fibrosis.

Lieberman and Littenberg evaluated the bradykinin-forming system in plasma, urine, and saliva. They failed to yield any evidence for excessive formation or impaired inactivation of bradykinin in cystic fibrosis (92). However, mixed saliva of patients with cystic fibrosis was found to contain significantly higher kallikrein activity than that of normal controls. It seems likely that this increase in salivary kallikrein accompanies the overall increase of organic constituents shown to exist in saliva of patients and it is doubtful that the increased kallikrein content of saliva is the abnormality underlying the pathogenesis of cystic fibrosis (92).

The findings of Lieberman and Littenberg are in conflict with other workers' observations as discussed below.

In 1972, Rao and Nadler began investigations on cystic fibrosis in order to explain the presence of the cationic factor inhibitory to sodium transport in the salivary gland of patients (113). The accumulation of this macromolecular factor may be due to the deficiency of an enzyme or enzymes which normally hydrolyse the factor to an inactive product. Since the cationic factor resembles polylysine which is also polycationic, it would be expected to be hydrolysed by enzymes similar to trypsin. Rao and Nadler tried to compare the level of trypsin-like activity in the saliva of patients with cystic fibrosis, their siblings, and their parents with that in the control subjects (113).

Trypsin-like activity was assayed by following the hydrolysis of α -N-benzoyl-L-arginine ethyl ester (BAEE). Since esterases may contribute to the hydrolysis of BAEE, the activity was also assayed in the presence of excess soybean trypsin inhibitor (STI). The differences in activity in the presence and absence of STI were taken to represent trypsin-like activity. In the saliva of cystic fibrosis patients, the enzyme activity was reduced

to 1/10 of the control value. Although the trypsin-like activity in the plasma of heterozygotes was significantly different from that in either controls or affected children, the individual heterozygote could not be reliably identified in each case. Attempts to assay the activity with more specific substrates such as BAA (α -N-benzoyl-L-arginine amide) or BAPNA (α -N-benzoyl-DL-arginine-p-nitroanilide) were not successful, hence, the presence of trypsin itself was excluded. The inhibition of trypsin-like activity by STI, together with the inability of salivary extract to hydrolyse BAA and BAPNA suggested that the trypsin-like activity might be similar in a number of its properties to those of kallikreins. Since Lieberman and Littenberg (92) have shown previously that the total kallikrein in cystic fibrosis saliva is increased in parallel with protein secretion, Rao and Nadler suggested that the kallikrein fraction known to be inhibited by soybean trypsin inhibitor could be deficient in patients with cystic fibrosis.

Since kallikreins are present in human plasma, Rao and co-workers then extended their studies to include the determination of kallikrein activities in the plasma of controls and patients with cystic fibrosis (114). They assayed kallikrein activity as arginine

esterase activated by treating plasma with chloroform and ellagic acid. The results were expressed as micro-moles of α -N-(p-toluenesulfonyl)-L-arginine methyl ester (TAME) utilized per hour per ml plasma. Significant differences between the levels of total kallikrein and STI-inhibited kallikrein activity between the plasma of control individuals and cystic fibrosis patients (Table 1) were found.

Mixing plasma from controls and patients with cystic fibrosis either before or after activation yielded the expected intermediate levels of kallikrein activity. This result excluded the possibility that an inhibitor was present, and that the factors needed to activate pre-kallikrein were absent in cystic fibrosis plasma. The presence of trypsin was excluded since plasma from controls and cystic fibrosis patients did not hydrolyse BAPNA. Also, initial experiments showed that the levels of carboxypeptidase and kallikrein inhibitor, two other components of the kinin system, were not significantly different in the control plasma compared to that in patients with cystic fibrosis. All these findings indicated that the observed deficiency appeared to be restricted to kallikreins (114).

Table 1. Kallikrein activity in plasma as measured by Rao, Posner and Nadler. The results are expressed as micromoles of TAME utilized per hour per milliliter of plasma. (114)

SAMPLE	N	<u>Total activity</u>		<u>STI-inhibited activity</u>		<u>STI-resistant activity</u>	
		M $\pm$ S.D.	Range	M $\pm$ S.D.	Range	M $\pm$ S.D.	Range
NORMAL ADULTS	15	43.3 $\pm$ 17.7	20.2-73.5	30.2 $\pm$ 15.3	12.9-69.1	13.1 $\pm$ 13.1	1.1-51.6
NORMAL CHILDREN	20	41.2 $\pm$ 7.6	30.5-55.1	23.2 $\pm$ 6.2	14.1-35.6	17.9 $\pm$ 5.4	10.1-31.4
CYSTIC FIBROSIS (CF ₁)	20	16.8 $\pm$ 6.9	2.4-27.6	9.6 $\pm$ 4.2	0.5-17.6	7.2 $\pm$ 4.0	1.9-17.2
CYSTIC FIBROSIS (CF ₂)	17	26.6 $\pm$ 12.3	9.9-50.4	13.0 $\pm$ 6.9	3.4-29.5	13.3 $\pm$ 8.7	3.0-32.5
PARENTS	27	32.5 $\pm$ 10.4	14.2-64.0	18.4 $\pm$ 8.4	6.1-47.2	14.8 $\pm$ 8.8	1.8-30.0
ALL CONTROLS	35	42.1 $\pm$ 13.0	20.2-73.5	26.2 $\pm$ 11.6	12.9-69.1	15.9 $\pm$ 9.8	1.1-51.6
ALL CYSTIC FIBROSIS	37	21.3 $\pm$ 10.9	2.4-50.4	11.2 $\pm$ 5.9	0.5-29.5	10.0 $\pm$ 7.2	1.9-32.5

In 1974, Lieberman reported that the findings of Rao and co-workers could not be reproduced in his laboratory (91). However, Lieberman's arginine esterase assay differed from that used by Rao et al in three aspects: (i) Lieberman used BAEE as the substrate instead of TAME, (ii) he used a buffer of pH 7.1 rather than 7.6, (iii) Rao et al included 0.15M sodium chloride in the substrate mixture while Lieberman did not. Nevertheless, Lieberman claimed that he obtained identical values for activity with the modified substrate as with the substrate used by Rao and associates when assaying plasma arginine esterase.

Rao and Nadler later claimed to have qualitative and quantitative differences in arginine esterase activity in the plasma from controls and cystic fibrosis patients (111). Using DEAE-Sephadex column chromatography, chloroformellagic acid treated plasma from control subjects could be separated into two fractions of arginine esterase activity (Figure 3) (111). The first peak which comprised over 60% of the total activity was completely inhibited by STI. The second peak consisted of arginine esterase activity which was almost totally resistant to inhibition of STI. In the plasma of all cystic fibrosis patients tested, a

Figure 3: Elution profile of arginine esterase activity in plasma from control subject and cystic fibrosis patient. 3.0 ml fractions were collected and assayed for activity. The dashed line represents the NaCl gradient. (Results of Rao and Nadler (6)))

a reduction of the size of peak I was found. The size of peak II was similar to that observed in controls.

Electrofocusing on polyacrylamide gels and subsequent staining for arginine esterase activity revealed seven bands in plasma samples from all the controls (Figure 4) (111). In plasma samples from eleven patients with cystic fibrosis, only five bands were found. In eight samples, band D was missing; in two samples, band E was missing; and in one sample, band C was missing. This finding may be explained by genetic heterogeneity of the disease. The possibility that cystic fibrosis may not be a single entity but rather a group of closely related genetic abnormalities with similar pathological consequences has been discussed by many authors (29, 34, 38).

The present study was undertaken in an attempt to confirm and possibly extend the observations made by Rao and co-workers. The activity of plasma arginine esterase in cystic fibrosis was investigated utilizing enzymologic, chromatographic and isoelectrofocusing techniques. The general objective was to look for qualitative and quantitative differences in arginine esterase activity in plasma and fractions of plasma from patients

Figure 4: Photograph and diagrammatic representation of arginine esterase separated by polyacrylamide gel electrophoresis.

with cystic fibrosis, parents of patients and normal control subjects. Part of this work is published in the Clinica Chimica Acta (Appendix I).

II. MATERIALS AND METHODS

A. Subjects

1. Cystic Fibrosis Patients

Nineteen clinically ascertained patients with cystic fibrosis (CF) were chosen for the study from individuals who attended the Cystic Fibrosis Clinic located at the Health Centre for Children, Vancouver General Hospital and from in-patients at the Children's Hospital in Vancouver. The diagnosis was confirmed in all patients by the clinical history and the presence of an elevated concentration of chloride in the sweat (58). The severity of the disease was assessed using the Shwachman scoring system (130) which is shown in Appendix II. The patients had Shwachman scores which ranged from 38 to 94, and ages which ranged from 4 to 26 years. Ten of the patients were male and nine were female.

Another group of 11 patients with CF were chosen for the column chromatographic and isoelectrofocusing studies. These patients also attended the Cystic Fibrosis Clinic at Vancouver General Hospital or were in-patients of the Children's Hospital. They were also diagnosed by their clinical histories and elevated sweat chloride tests. The age of this group ranged from 5-24 years.

2. Control Subjects

Fifty individuals who had no known family history or clinical history indicative of cystic fibrosis were studied and assayed for arginine esterase activities. They were divided into two groups-children and adult controls.

The control children group consisted of 16 individuals who ranged in age from 1 to 15 years. They were selected from in-patients at the Children's Hospital in Vancouver and outpatients who attended the Metabolic Investigation Unit at the Children's Hospital. They all had no known family history or clinical history indicative of cystic fibrosis.

The 34 adult control subjects were chosen at random from the clinical, laboratory and secretarial staffs of the Children's Hospital. They were all clinically healthy and ranged in age from 16-42 years.

Another group of 13 normal, healthy adults were selected for the chromatographic and isoelectrofocusing studies. They were selected from the staff of Children's Hospital and ranged in age from 20 to 42 years.

3. Heterozygous Carriers

Nineteen obligate heterozygotes were chosen

among parents of cystic fibrosis children. Seventeen of the carriers were female and two were male. The age of the heterozygous carriers of cystic fibrosis ranged from 32 to 42 years.

B. Blood Samples

Informed consent was obtained from each subject before collection of blood. In the case of minors, their parents were informed of the reason for the study.

Venous blood was collected from each subject into a plastic syringe. For the micro-assay of arginine esterase activity, 1.8 ml of blood was immediately mixed with 0.2 ml of 3.8% (w/v) sodium citrate in a polypropylene tube. The blood was centrifuged at 3,000 r.p.m. for 15 min. at 4°C. The plasma samples were used immediately or stored at -20°C. All samples were handled in plasticware and contact with glass surfaces was avoided.

In the case of macro-assays of arginine esterase activity, studies on ion-exchange column chromatography and isoelectrofocusing, 9.0 ml of blood was collected and mixed with 1.0 ml of 3.8% (w/v) sodium citrate. Plasma was separated by centrifugation at 3,000 r.p.m. for 15 min. at 4°C and frozen in polypropylene tubes at -20°C until use.

C. Macro-assay of Arginine Esterase Activity

1. Reagents

(a) Chloroform

(b) Ellagic acid 0.1 mM

- obtained from Aldrich chemical Co., Inc., Milwaukee Wis.

- 3.4 mg of ellagic acid was suspended in 0.10 M phosphate buffer, pH 7.6, containing NaCl at a concentration of 0.15 M.

(c) α -N-(p-toluene-4-sulfonyl)-L-arginine methyl ester (TAME) 0.015 M

- obtained from BDH Chemicals Ltd., Poole, England.

- 57.5 mg of TAME was dissolved in 10.0 ml of 0.10 M phosphate buffer, pH 7.6 containing 0.15 M NaCl.

(d) Trichloroacetic acid (TCA), 10% aqueous

(e) Soybean Trypsin Inhibitor (STI)

- obtained from Grand Island Biological Co., Grand Island, New York.

-10.0 mg of STI was dissolved in 1.0 ml of normal saline.

(f) Potassium permanganate, 2% aqueous.

(g) Sodium sulfite, 10% aqueous.

(h) Chromotropic acid working reagent.

- In a 1-liter volumetric flask, 200 ml of cold water and 100 ml of 2% aqueous chromotropic acid (British Drug Houses Ltd., Poole, England).
- 600 ml of cold, concentrated sulfuric acid was then added. When cooled to flask calibration temperature, the mixture was diluted to 1000 ml with water. The working reagent was kept in the refrigerator.
- (i) Trichloroacetic acid, 5% aqueous (for standards and blanks)
- (j) Methanol
 - obtained from Matheson Colman and Bell. Manufacturing Chemicals, Norwood, Ohio.
 - 2.02 ml of methanol was added to a 50 ml volumetric flask and made up to the mark with 5% TCA. 0.10 ml of this solution was diluted to 50 ml with 5% TCA. The final solution had a concentration of 2.0 umoles/ml.

2. Procedure

The macro-assay of arginine esterase activity was essentially the method described by Rao, Posner and Nadler (114). Measurement of esterase activity was accomplished by quantitation of the methanol released in

the hydrolysis of the substrate, TAME, using a modification of the procedure of Siegelman et al (134). The hydrolysis of TAME by arginine esterase is shown in Figure 5. The methanol released is oxidized to formaldehyde by potassium permanganate. After the removal of excess permanganate by sodium sulfite, the formaldehyde is coupled with chromotropic acid to yield a purple-coloured compound which absorbs maximally at 580 nm.

All samples were handled in polypropylene containers. The plasma was treated with an equal volume of cold chloroform and mixed with an electric rotator at approximately 10 r.p.m. for 5 minutes at 4°C. The mixture was then centrifuged at 2,000 r.p.m. for 30 minutes at 4°C and the plasma was separated. The arginine esterase in the plasma fraction was activated by incubation with an equal volume of 0.1 mM ellagic acid suspension at 25°C for 15 minutes. 0.3 ml of this plasma was transferred to tubes containing 0.7 ml of 0.015 M TAME in 0.10 M phosphate buffer (pH 7.6) with NaCl at a concentration of 0.15 M. The reaction mixture (final volume, 1.0 ml) was incubated at 37°C for 15 minutes, and the reaction was terminated by the addition of 1.0 ml of 10% trichloroacetic acid. The mixture was centrifuged at 2,000 r.p.m.

Figure 5: Hydrolysis of TAME by arginine esterase

for 10 minutes at 4°C.

0.5 ml of supernatant was added to a 10-ml graduated tube. Other tubes containing either 0.5 ml methanol standard or 5% TCA (Blank) were also set up. 0.1 ml of potassium permanganate was added to each tube, mixed and allowed to stand at room temperature for 1 min. 0.1 ml of sodium sulfite was added and mixed until decolourized. (If decolourization was not complete, additional sodium sulfite solution was added dropwise until decolourized.) 4.0 ml chromotropic acid working reagent was added and mixed with a Vortex mixer. The mixture was boiled for 15 minutes followed by cooling in an ice-bath. Water was added to make the final volume to 5.0 ml. The sample was then read at 580 nm in a 1-cm cuvette on a Gilford 2000 spectrophotometer.

By reference to a standard methanol curve, (an example is given on Figure 6) the micromoles of ester hydrolyzed were calculated and expressed as μ moles of TAME utilized per hour per ml of plasma.

The macro-assay for arginine esterase activity was also performed in the presence of soybean trypsin inhibitor. 20 μ l of the STI solution was added to 1.0 ml of the chloroform treated plasma to give a final con-

Figure 6: An example of one of the methanol standard curves.

centration of 200 $\mu\text{g/ml}$ of plasma. The enzyme activity measured in the presence of STI was expressed as STI-resistant arginine esterase activity. The enzyme activity assayed without STI was expressed as the total activity. The difference in activity in the presence and absence of STI was taken to represent STI-inhibited arginine esterase activity.

D. Micro-assay of Arginine Esterase Activity

1. Reagents

Same as the reagents used in the macro-assay.

2. Procedure

Arginine esterase activity in the plasma samples were measured using techniques modified from the macro-assay, with most volumes scaled down to approximately one-fifth. The plasma, in polypropylene tubes, was treated with an equal volume of cold chloroform and mixed using an electric rotator at approximately 10 r.p.m. for 5 minutes at 4°C . The mixture was then centrifuged at 2,000 r.p.m. for 30 minutes at 4°C , after which the plasma was separated, and incubated with an equal volume of 0.1 mM ellagic acid suspension at 25°C for 15 minutes. 50 μl of the activated plasma was then added to a tube containing 150 μl of 0.10 M phosphate buffer, pH 7.6 with

0.15 M NaCl and 0.015 M TAME. The reaction mixture was terminated by the addition of 200 μ l of 10% trichloroacetic acid, and the mixture centrifuged at 2,000 r.p.m. for 10 minutes at 4°C. Methanol in the supernatant was assayed using the method of Siegelman et al (134) modified by using one-fifth of the reported volumes of the samples and reagents.

100 μ l of supernatant was transferred to a 5-ml graduated tube. 20 μ l of potassium permanganate was added to each tube, which was then mixed and allowed to stand at room temperature for 1 minutes. 20 μ l of sodium sulfite was added and the solution was mixed until it decolourized. 0.8 ml chromotropic acid working reagent was added and mixed with a Vortex mixer. The mixture was boiled for 15 minutes and then cooled in an ice-bath. Water was added to make the final volume 1.0 ml and the absorbance at 580 nm was recorded.

The effect of STI on total arginine esterase activity was also studied. 4 μ l of the STI solution was added to 0.2 ml of the chloroform treated plasma to give a final concentration of 200 μ g/ml. The rest of the procedure was the same as mentioned above for micro-assay. The enzyme activity was expressed as STI-inhibited

arginine esterase activity (the difference between the values obtained by assaying in the presence and absence of STI). One unit of arginine esterase activity was defined as the number of micromoles of TAME hydrolyzed per hour per ml plasma.

E. Assessment of the Micro-assay Technique

The micro-assay technique was validated by studying the effects of different concentrations of ellagic acid and STI, and varying the incubation times for ellagic acid and chloroform.

1. Effect of Storage on Enzyme Activity

Plasma samples were obtained from 2 female control subjects A and B (ages 21 and 26 respectively) and stored in 0.5 ml aliquots at -20°C . The micro-assay of arginine esterase activity was performed on Day 1 (the day when the blood samples were collected), and also on the 3rd, 4th, 8th, and 10th days after the storage of plasma samples.

2. Effect of Chloroform on Enzyme Activity

18 ml of blood from a normal subject was added to a polypropylene tube containing 2.0 ml of 3.8% sodium citrate. The plasma was separated by centrifugation at

3,000 r.p.m. for 15 minutes at 4°C, and divided into aliquots of 0.5 ml. An equal volume of cold chloroform was added to the plasma and the resulting dispersion mixed at 4°C using an electric rotator at approximately 10 r.p.m. for various periods of time up to 15 minutes. The mixture was centrifuged at 2,000 r.p.m. for 5 minutes at 4°C, the plasma separated and arginine esterase activity assayed as indicated previously.

3. Effect of Ellagic Acid on Enzyme Activity

(i) Concentration of Ellagic Acid

Ellagic acid suspensions were prepared in 5 different concentrations - 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM and 0.5 mM using 0.10 M phosphate buffer, pH 7.6, containing NaCl at a concentration of 0.15 M. To each 0.2 ml aliquot of the Chloroform-treated plasma, an equal volume of the ellagic acid solution was added. The samples were then incubated at 25°C for 15 min. Incubation was also carried out in the absence of ellagic acid. Arginine esterase activity was measured as stated in the procedure for micro-assay.

(ii) Incubation with Ellagic Acid

An equal volume of 0.1 mM ellagic acid suspension

was added to 1.0 ml of chloroform-treated plasma, and the mixture incubated at 25°C. At various times, 50 µl of the mixture was transferred to a tube sitting in an ice bath and containing 150 µl of 0.10 M phosphate buffer, pH 7.6 with 0.15 M NaCl and 0.015 M TAME. The micro-assay of arginine esterase activity was then performed.

4. Effect of Soybean Trypsin Inhibitor on Enzyme Activity

STI solution was prepared in various concentrations - 0, 2.5, 5.0, 7.5, 10.0 and 15.0 µg/ml of normal saline. 4 µl of the STI solution with different concentrations was added to each 0.2 ml chloroform-treated plasma, and the solutions allowed to stand at room temperature for 10 minutes. Ellagic acid was then added and the arginine esterase assay was performed.

5. Day-to-day Variation of Enzyme Activity

Blood was collected from a normal subject (male, age 30) on three separate days. Blood was also taken 7 and 11 days later. Arginine esterase activity of all the plasma samples was determined on the 11th day.

6. Within-day Variation of Enzyme Activity

Blood samples were obtained from a normal

subject (male, age 25) at different times on the same day. Blood was taken at 8:00 a.m. (fasting blood sample) 11:00 a.m. (before lunch), 2:00 p.m. (after lunch), and 4:30 p.m.

F. Preparation of Columns

Sephadex G-50 (fine, particle size 20-80 microns) DEAE-Sephadex (A-50, total capacity 3.5 ± 0.5 meq/g) and Sephadex columns, K 9/15 (of internal diameter 0.9cm) and K 15/30 (of internal diameter of 1.5 cm) were purchased from Pharmacia (Canada) Ltd., Dorval, Quebec.

1. Preparation of Sephadex G-50 Columns

Sephadex G-50 was allowed to swell in excess 5 mM phosphate buffer, pH 8.0 and left to stand for at least $1\frac{1}{2}$ hours on a boiling water-bath. Two columns with different sizes (K 9/15 and K 15/30) were used, and filled with the phosphate buffer. The cooled swollen gel was then poured carefully into the column down a glass rod. While the column was allowed to flow freely, the addition of slurry was continued until a bed height of 13 cm was obtained for the K 9/15 column; in the case of the K 15/30 column, a bed height of 25 cm. A buffer reservoir was connected to the column. The gel bed

stabilized and equilibrated with 5 column volumes of buffer at 4°C.

The total bed volume of the K 9/15 column was 9.5 ml while the bed volume of the K 15/30 column was ca 40 ml. Each gram of the original dry Sephadex G-50 occupied a packed volume of ca 10 ml. With a pressure head (distance between the top of the buffer reservoir and the top of the column packing) of 25 cm of buffer, the flow rate was about 35-40 ml/hour for both columns.

2. Preparation of DEAE-Sephadex Column

DEAE-Sephadex A-50 was mixed with excess 5mM phosphate buffer, pH 8.0. The mixture was stirred at intervals and allowed to settle and swell in a boiling water-bath for 1½ hours. The supernatant was aspirated and the buffer replaced. The suspension was thoroughly stirred and an aliquot, removed by Pasteur pipette, introduced under the buffer surface in a Sephadex column (K 9/15) and the slurry expelled. While the column was allowed to flow freely, the addition of slurry was continued until a bed height of 13-14 cm was obtained. The prepared column was attached to a reservoir of buffer and allowed to run overnight at 4°C. Columns were not re-used in this work. A new column was prepared for each

experiment.

The total bed volume of such a column was 9.5 ml. Using a head of 30 cm of eluant, the flow rate varied between 24 and 36 ml/hour from one column to another.

G. Preparation of Samples for Chromatography and Isoelectrofocusing

An equal volume of cold chloroform was added to 5.0 ml plasma. The dispersion was mixed with an electric rotator at ca. 10 r.p.m. for 5 minutes at 4°C. After centrifugation at 2,000 r.p.m. for 30 minutes, 3.0 ml of plasma was removed and treated with an equal amount of 0.1 mM ellagic acid suspended in distilled water. The mixture was incubated at 25°C for 15 minutes and then chilled to 4°C in an ice-bath. 1.06 g. of solid ammonium sulfate was added under stirring to an ammonium sulfate concentration equivalent to 25% saturation at 4°C. After 15 minutes, the mixture was centrifuged at 3,500 r.p.m. for 15 minutes and the precipitate discarded. 2.33 g. of solid ammonium sulfate was added to the supernatant under stirring (55% saturation) and the mixture centrifuged at 150,000 r.p.m. for 20 mins. at 4°C. The precipitate was dissolved in about 1.5 ml of

distilled water, and desalted on a column of Sephadex G-50 equilibrated with 5mM phosphate buffer, pH 8.0 at 4°C. 1.0 ml fractions were collected and absorbances at 280 nm (A₂₈₀) of the Sephadex G-50 fractions were recorded. The fractions included in the A₂₈₀ peak were pooled. The total volumes ranged from 7.6-9.0 ml. 0.2 ml of this pooled eluate was removed for arginine esterase assay and another 0.1 ml was used for polyacrylamide gel electrofocusing analyses. The rest of the Sephadex G-50 eluate was used for further chromatographic studies.

Desalting of the solutions with Sephadex G-50 columns was performed on 24 activated plasma samples - 13 samples from normal individuals and 11 from CF patients. 5 normal and 3 CF plasma samples were desalted on a column of size K 9/15, and then analyzed by DEAE-Sephadex column chromatography. Another 8 plasma samples from normal controls and 8 from CF patients were desalted on a Sephadex G-50 column (K 15/30). The eluates collected from this larger column were chromatographed on DEAE-Sephadex.

The desalting abilities of the two columns (K 9/15 and K 15/30) of Sephadex G-50 were assessed.

The concentration of protein in the eluted fractions was measured by reading absorbance at 280 nm (A_{280}), with a Gilford 2000 spectrophotometer. The amount of ammonium ion in the fractions was assayed by means of a commercial test designed to measure ammonium with alkaline phenol - hypochlorite reagents (details of test are shown below).

Measurement of Ammonium ion in the Eluted Fractions

1. Reagents

(a) Phenol colour reagent

- obtained from Hyland, Div. Travenol Lab. Inc., Costa Mesa, California.
- contained sodium nitroferricyanide 0.5% (w/v) and phenol.

(b) Alkali-Hypochlorite reagent

- obtained from Hyland, Div. Travenol Lab. Inc., Costa Mesa, California.
- contained sodium hypochlorite 1.5% (w/v), and sodium hydroxide 17.9% (w/v).

(c) Nitrogen Standard

- obtained from Hyland, Div. Travenol Lab., Inc. Costa Mesa, California.

-contained ammonium sulfate 70.7 mg/dl, and sulfuric acid 18 M.

(d) Ammonium sulfate standard

- obtained from Fisher Scientific Co., Fair Lawn, N.J.
- 1 M solution was prepared by dissolving 1.32 g $(\text{NH}_4)_2\text{SO}_4$ in 10 ml of deionized water.
- 1, 2, 3, 5 and 10 mM solutions were prepared by dilution of the 1M stock.

2. Procedure

The eluted fractions were diluted 100 times by adding 20 μl of the fractions to 2.0 ml of deionized water. Into the tubes for blank, standard, control and duplicate tests, 200 μl of deionized water was added. 20 μl of the samples were transferred to each tube and the tube mixed. 1.0 ml Phenol reagent was added followed by 1.0 ml Alkaline-Hypochlorite reagent and the tube mixed immediately using a Vortex mixer. All tubes were incubated at 37°C for 15 mins. 5.0 ml of deionized water was then added to each tube and absorbance at 560 nm was recorded. By reference to the standard curve, the concentration of ammonium sulfate in each eluted fraction could be calculated.

H. DEAE-Sephadex Column Chromatography

DEAE-Sephadex chromatography was performed on 13 normal control and 11 CF plasma at 4°C. The Sephadex G-50 eluate of the ammonium sulphate precipitate was applied to the DEAE-Sephadex column in portions, by pipette, until all had been added. During this time, the column was open but special care was taken to ensure that the top of the packed gel never became dry. The column was then attached to a gradient system of two identical 250 ml conical flasks connected by polyethylene tubing. One of these, the mixing vessel, was stirred magnetically. From this latter vessel a tube carried the eluting fluid to the column.

The gradient system consisted of 200 ml of 5 mM phosphate buffer, pH 8.0 in the mixing vessel and 200 ml of 5 mM phosphate buffer, pH 8.0 containing 0.6M NaCl in the donor vessel. The column was allowed to flow freely under a head of ca 30 cm of eluting solution. 2.0 ml fractions were collected by a LKB Ultrorac 7000 fraction collector. At the completion of the NaCl Gradient, the gel bed showed a 30% shrinkage.

I. Analysis of Eluted Fractions

Arginine esterase activities in the eluted fractions were determined using a modified micro-method with TAME as the substrate. For the chromatography of 5 normal and 3 CF plasma samples, the enzyme activity of the samples and fractions from Sephadex column was also measured spectrophotometrically using BAEE as substrate. The amount of protein in all eluted fractions was estimated by reading the absorbance at 280 nm on a Gilford 2000 spectrophotometer. The concentration of chloride in the eluted fractions was measured using a Corning chloride meter 920 M.

1. Arginine esterase assay using TAME

(i) Reagents

(a) α -N-(p-toluenesulfonyl)-L-arginine methyl ester

(TAME) 0.033M

-obtained from BDH Chemical Ltd., Poole, England.

- 128 mg of TAME was dissolved in 10.0 ml of 0.10 M phosphate buffer, pH 7.6 containing 0.15 M NaCl.

(b) Soybean Trypsin Inhibitor (STI)

- obtained from Worthington Biochemical Corp., Freehold, N.J.

- 20.0 mg of STI was dissolved in 1.0 ml of 0.10 M phosphate buffer, pH 7.6 containing 0.15M NaCl.

(c) Trichloroacetic acid (TCA), 20% aqueous.

(d) Trichloroacetic acid (TCA), 40% aqueous.

(ii) Procedure

200 μ l of the DEAE-Sephadex fractions and 100 μ l of the Sephadex G-50 eluates were used for the arginine esterase assay. 100 μ l of the TAME solution was added to each tube which was then incubated at 37°C for 15 min. 100 μ l of 20% TCA was added to terminate the reaction. The total volume of the final mixture was 400 μ l. At the end of incubation, the reaction mixtures of the DEAE-Sephadex fractions contained:

200 μ l of eluted fraction

100 μ l of substrate solution (.033 M TAME in 0.10 M phosphate buffer, pH 7.6 containing 0.15 M NaCl).

100 μ l of 20% TCA.

The contents of the reaction mixtures of Sephadex G-50 eluates at the end of the incubation were:

100 μ l of Sephadex G-50 eluate

100 μ l of substrate solution (TAME)

100 μ l of 5 mM phosphate buffer, pH 8.0

100 μ l of 20% TCA

The contents of reaction mixtures of the chloroform-ellagic acid treated plasma samples at the end of incubation consisted of:

50 μ l of activated plasma

100 μ l of substrate solution (TAME)

150 μ l of 5 mM phosphate buffer, pH 8.0

100 μ l of 20% TCA

The contents of the reagent blanks were:

200 μ l of 5 mM phosphate buffer pH 8.0

100 μ l of substrate solution (TAME)

100 μ l of 20% TCA

The mixtures were then centrifuged at 2,000 r.p.m. for 10 mins. at 4°C and methanol in the supernatant assayed in exactly the same procedure as indicated in part (D) for the micro-assay of arginine esterase activity.

The above enzyme assay was also performed in the presence of STI. 50 μ l of the STI solution was added to an appropriate amount of eluate. The mixture was allowed to stand at room temperature for 15 mins. 100 μ l of the substrate solution (.033 M TAME in 0.10 M

phosphate buffer, pH 7.6 containing 0.15 M NaCl) was added and the mixture incubated at 37°C for 15 mins. At the end of the reaction, 50 µl of 40% TCA was added. The final volume of the mixture was 400 µl. At the end of incubations, the reaction mixtures of the DEAE-Sephadex fractions contained:

- 200 µl of eluted fraction
- 50 µl of STI solution
- 100 µl of substrate solution (TAME)
- 50 µl of 40% TCA

The reaction mixtures of Sephadex G-50 eluates at the end of incubation consisted of:

- 100 µl of Sephadex G-50 eluate
- 50 µl of STI solution
- 100 µl of 5 mM phosphate buffer, pH 8.0
- 100 µl of substrate solution (TAME)
- 50 µl of 40% TCA

The reaction mixtures of chloroform-ellagic acid treated plasmas at the end of incubation consisted of:

- 50 µl of activated plasma
- 50 µl of STI solution
- 150 µl of 5 mM phosphate buffer, pH 8.0
- 100 µl of substrate solution (TAME)
- 50 µl of 40% TCA

The rest of the assay was the same as the procedure for measurement of arginine esterase activity in the absence of STI.

2. Arginine Esterase Assay Using BAEE

(i) Reagents

(a) α -N-benzoyl-L-arginine ethyl ester (BAEE) 5mM

- obtained from Sigma Chemical Co., St. Louis, Mo.
- 17.4 mg of BAEE was dissolved in 10.0 ml of water

(b) Soybean Trypsin Inhibitor (STI)

- obtained from Worthington Biochemical Corp., Freehold, N.J.
- 10.0 mg was dissolved in 1.0 ml of normal saline.

(c) Phosphate buffer, pH 7.6

- 0.10 M sodium phosphate, pH 7.6 containing 0.15 M NaCl.

(d) α -N-Benzoyl-L-arginine 5mM

- obtained from Sigma Chemical Co., St. Louis, Mo.
- 13.9 mg was dissolved in 10.0 ml of phosphate buffer, pH 7.6

(ii) Procedure

Arginine esterase activity was assayed by

following the hydrolysis of BAEE (113). The reaction mixture of DEAE-Sephadex eluted fractions in a cuvette contained 100 μ l of the eluted fraction and 800 μ l of 0.10 M phosphate buffer, pH 7.6 containing 0.15 M NaCl. The cuvette was kept at 37°C and the reaction initiated by the addition of 100 μ l of 5 mM BAEE, also at 37°C. The increase in absorbance at 253 nm (A_{253}) was followed against a blank not containing the eluate (900 μ l of phosphate buffer and 100 μ l of BAEE solution).

In the case of Sephadex G-50 eluates, the reaction mixture contained:

50 μ l of eluate

850 μ l of 0.10 M phosphate buffer, pH 7.6 containing 0.15 M NaCl.

100 μ l of BAEE solution

When assaying the enzyme activity in the chloroform-ellagic acid treated plasma, the reaction mixture contained:

50 μ l of activated plasma

850 μ l of phosphate buffer, pH 7.6

100 μ l of BAEE solution

A series of Benzoyl-arginine standards were prepared and absorbances at 253 nm recorded. The standard graph is shown in Figure 7, the change in

Figure 7: An example of a standard graph of Benzoyl-arginine.

absorbance per min. was corrected to provide units indicating the number of micromoles of BAEE utilized per hour per ml of plasma or eluate.

The effect of STI was evaluated by first mixing the eluate or plasma sample with 100 μ l of STI solution. 0.10 M phosphate buffer, pH 7.6 containing 0.15 M NaCl was added to make a final volume of 900 μ l. This mixture in a cuvette was allowed to stand at 37°C for 10 mins. 100 μ l of BAEE solution was then added to initiate the reaction. The enzymatic reaction was recorded in the Gilford spectrophotometer at 37°C. The reaction mixtures of DEAE-Sephadex eluted fractions contained:

- 100 μ l of eluted fraction
- 100 μ l of STI solution
- 700 μ l of phosphate buffer, pH 7.6
- 100 μ l of BAEE Solution

The reaction mixtures of Sephadex G-50 eluates consisted of:

- 50 μ l of eluate
- 100 μ l of STI solution
- 750 μ l of phosphate buffer, pH 7.6

100 μ l of BAEE solution

The reaction mixtures of chloroform-ellagic acid treated plasmas consisted of:

50 μ l of activated plasma

100 μ l of STI solutions

750 μ l of phosphate buffer, pH 7.6

100 μ l of BAEE solution

J. Isoelectrofocusing of Sephadex G-50 Eluate

Polyacrylamide gel isoelectrofocusing (PAG-IEF) of the Sephadex G-50 eluate of activated plasma was carried out in a disc gel electrophoresis apparatus (Buchler Polyanalyst) and in Brinkman thin layer IEF double chamber. Isoelectric focusing is a procedure developed by Vesterberg and Svensson (143) to separate proteins (and other ampholytes) according to their different isoelectric points. In principle, the method is quite similar to electrophoresis but differs from it in that it is not carried out at a specific pH value. A gradient of pH is used and this is accomplished by using synthetic polyamino-polycarboxylic acids which possess subtly different isoelectric points. In an electric field these carrier ampholytes (one trade name

of which is 'Ampholines') quickly form a gradient of pH from anode to cathode. On this gradient a mixture of proteins can be separated. The added proteins move towards the anode or cathode according to their net charge but eventually become stationary at a point in the pH gradient equal to their isoelectric point.

In this investigation, isoelectric focusing was performed using both vertical gel tubes (Buchler Polyanalyst) and flat gel slabs (by Brinkman thin layer - IEF double chamber). The techniques used for detecting protein and arginine esterase activity were similar for gel tubes and gel slabs.

1. Reagents

(a) Ampholines pH 5-8, 40% (w/v)

- obtained from LKB-Produkter, Bromma, Sweden.

(b) Polyacrylamide stock solution

- acrylamide and N, N'-methylene-bisacrylamide were purchased from Eastman Kodak Co., Rochester, New York.

- 33.0 g of acrylamide and 1.0 g of methylene-bisacrylamide were dissolved in 100 ml of distilled water.

(c) NNN'-tetramethyl-ethylene-diamine (TEMED) 5% (w/v)

-obtained from Eastman Kodak Co., Rochester, New York.

(d) Ammonium persulfate 10% (w/v)

(e) Electrode solutions for gel slab:

anode: 0.2 M sulfuric acid

cathode: 0.3 M ethylene diamine

(f) Electrode solutions for disc gel tubes:

anode: 0.2% sulfuric acid

cathode: 0.4% diethanolamine

(g) Sucrose 40% (w/v)

(h) Ampholyte solution, 0.75%

-0.38 ml of 40% (w/v) Ampholine was diluted to 20 ml with distilled water.

(i) Solutions for detection of Protein:

Fixing solution: 12% (w/v) trichloroacetic acid,
5% (w/v) sulfosalicylic acid,
in ethanol-water-acetic acid
(6:13:1, by volume)

Staining solution: 0.1% (w/v) Coomassie brilliant
blue G (obtained from Sigma
Chemical Co.) in ethanol-water-
acetic acid (9:9:2, by volume)

Destaining solution: ethanol-water-acetic acid

(6:13:1, by volume)

Preserving solution: 5% acetic acid

(j) Reagents for detection of arginine esterase activity:

(i) B-nicotinamide-adenine dinucleotide (NAD^+)

- obtained from Sigma Chemical Co., St. Louis,
Mo. Product no. N-7254.

(ii) Phenazine methosulfate (PMS)

- obtained from Sigma Chemical Co., St. Louis,
Mo.

(iii) Nitro Blue Tetrazolium (NBT)

- obtained from Sigma Chemical Co., St. Louis,
Mo.

(iv) Yeast alcohol dehydrogenase (ADH)

- obtained from Sigma Chemical Co., St. Louis,
Mo. Product no. A-7011.

(v) N- α -benzoyl-L-arginine ethyl ester (BAEE)

- obtained from Sigma Chemical Co., St. Louis,
Mo.

(vi) Cobalt chloride (Co Cl_2)

- obtained from Mallinckrodt Chemical Works,
St. Louis, Mo.

(vii) Tris-EDTA-borate buffer, 0.08 M, pH 9.2

-Tris (Hydroxy methyl) aminomethane (THAM) and Disodium ethylenediamine tetraacetic acid (EDTA) were obtained from Fisher Scientific Co., Fair Lawn, New Jersey.

-9.69 g. of THAM and 1.86 g of EDTA were dissolved in 600 ml of distilled water.

0.08 M Boric acid was added dropwise until pH of the solution became 9.2. Distilled water was then added to make the final volume of 1000 ml.

(viii) Methanol-acetic acid-water, 5:1:5 by volume.

(ix) Acetic acid 7%.

1. Electrofocusing Using Brinkman Thin-Layer IEF Chamber

(i) Preparation of Polyacrylamide Gel Plates

The PAG polymerization chamber consisted of:

- (a) Frame with locking screws
- (b) Plastic plate with rubber spacer strips
- (c) Two 140 x 200 mm glass plates (the thin one was 1 mm thick and used as carrier plate, the thick one (3 mm) was used to reinforce the carrier plate).

The gel solution contained the following:

- (a) 0.9 ml 40% ampholyte (pH 5-8)

(b) 7.5 ml polyacrylamide stock solution

(c) 0.25 ml 5% TEMED

(d) 41.1 ml distilled water

(e) 0.25 ml 10% ammonium persulfate

The mixture containing the reagents (a)-(d) was introduced in a vacuum flask (125 ml) and mixed by magnetic stirrer. The solution was degassed for about 3 minutes during stirring. Ammonium persulfate was then added and the mixture was stirred for a few seconds. This solution results in a gel with T=5% and C=3%, containing 0.75% (w/v) ampholyte.*

The gel solution was transferred into the polymerization chamber by a pipette. Once the chamber was properly filled, the polymerization solution was overlaid with distilled water (3-5 mm) to obtain a straight edge, and it was left overnight at 4°C. When the gel slab was to be used, the PAG layer, together with a glass plate was removed and placed into the pre-cooled (5°C) thin-layer IEF chamber.

*% T = grams of acrylamide + grams of bisacrylamide
per 100 ml of solution

% C = 100 times grams of bioacrylamide/100 ml solution/
T (Nomenclature according to Vesterberg and Nice
(142))

(ii) Isoelectric Focusing

The electrode solutions consisted of 0.2M sulfuric acid for the anode and 0.3 M ethylene diamine for the cathode. Filter paper strips impregnated with the appropriate electrode solution were placed on both ends (200 mm side) of the gel-free glass plate and pushed against the gel to ensure contact. The platinum ribbon electrodes were then placed on the outer edge of the paper strips. The gel was prefocused for 1 hour at 10 mA to remove the residual ammonium persulfate.

After prefocusing, 20 μ l of the Sephadex G-50 eluates were pipetted onto 5 x 10 mm pads of Whatman no. 3 MM chromatography paper, which were placed on the gel in a line parallel to the anode and at a distance of approximately 1.5 cm from the anodic end of the gel. Electrofocusing was performed at 4°C starting with a voltage of 100 volts. The voltage was increased by 100 volts every 15 minutes until the final voltage of 800 volts was attained. The total time used for electrofocusing was about 4 hours.

2. Tube Electrofocusing Using Disc Gel Electrophoresis Apparatus

(i) Preparation of Polyacrylamide Gels

Composition of the gel solution was similar to the one used in polyacrylamide gel slabs. 1.2 ml of the gel mixture was introduced into the electrophoresis tube (75 mm long with internal diameter of 5 mm) containing 50 μ l of 40% sucrose at the bottom to ensure a flat surface. The end of the electrophoresis tube was closed with a rubber tube cap. The length of the gel was ca. 65 mm. Distilled water was laid on top of the gel surface to obtain a straight edge. The gels were left overnight at 4°C to allow complete polymerization. When the gels were to be used, the water was removed and replaced by a layer of 0.75% ampholine. The tubes were mounted in a disc gel electrophoresis apparatus (Buchler Polyanalyst).

(ii) Isoelectrofocusing

The anode compartment contained 600 ml of 0.2% H_2SO_4 while the cathode compartment contained 200 ml of 0.4% diethanolamine. The whole apparatus was cooled by passing running water at a temperature of 4°C through the outer jacket of the column. The gels were pre-run at 200V for 1 hour. Ammonium persulfate was removed from the gel by this prefocusing.

After the pre-run, a mixture of 50 μ l of Sephadex G-50 eluate of the activated plasma and 50 μ l of 40% sucrose was introduced under the layer of ampholine. Electrofocusing was performed at 4°C starting with the voltage of 200 volts. The voltage was increased by 200 volts every 15 minutes until the final voltage of 800 volts was attained. Focusing was continued for 3-4 hours, by which time, the current dropped to ca 5 mA. Following this, each gel was displaced from its tube by passing a small amount of water between the gel and the inside surface of the tube by means of a long-tipped syringe.

3. Detection of Protein in Polyacrylamide Gels

The gels were fixed overnight in a solution of 12% TCA, and 5% sulfosalicylic acid, in ethanol-water-acetic acid (6:13:1). This removes the ampholytes and precipitates the proteins which were stained subsequently with 0.1% Coomassie brilliant blue G in ethanol-water-acetic acid (9:9:2) for 1½ hours. Successive destaining washes of ethanol-water-acetic acid (6:13:1) were then performed until the gel background was clear. The gels were stored in 5% acetic acid.

4. Detection of Arginine Esterase Activity in Polyacrylamide Gels

This method was modified from the technique of Fujimoto and associates (54). Detection of arginine esterase activity is based on the formation of tetrazolium salt by the electron released with alcohol dehydrogenase from ethanol which is liberated from BAEE by the activity of arginine esterase, as illustrated in Figure 8.

Two different concentrations of the staining solution were used. The first one (solution A) was exactly the same concentration as used by Rao and Nadler (111), while the second one (solution B) was three times the previous concentration for all the components except alcohol dehydrogenase which was ten times more concentrated. The compositions of the staining solutions were:

	<u>Solution A</u>	<u>Solution B</u>
(a) NAD	100 mg	200 mg
(b) PMS	10 mg	20 mg
(c) NBT	30 mg	60 mg
(d) ADH	5 mg	33 mg
(e) BAEE	250 mg	500 mg
(f) Co Cl ₂	300 mg	600 mg
(g) Tris-EDTA-borate buffer	150 ml	100 ml

Figure 8: The reactions involved in the detection of arginine esterases with the formazan system.

The gels were immersed in the arginine esterase staining solution and kept in the dark at 37°C for 1½ hours. The gels were then fixed in methanol-acetic acid-water (5:1:5) overnight and destained in 7% acetic acid until the bands were clearly visible on a pale background.

K. Methods of Data Analysis

$$1. \text{Coefficient of variation} = \frac{S}{\bar{x}} \times 100\%$$

S = Standard deviation

$\bar{x}$ = mean

$$2. 95\% \text{ confidence limit} = \bar{x} \pm (t_{.05, n-1}) \cdot \frac{S}{\sqrt{n}}$$

n = number of observations from the sample

If the 95% confidence limits of one group do not include the mean of another group, those two groups are likely to be significantly different.

3. Test for normal distribution of the enzyme activities in each population:

(i) Chi-square test

$$\text{chi-square} = \chi^2 = \sum_{i=1}^k \frac{(n_i - e_i)^2}{e_i}$$

K = number of classes

n_i = observed frequency

e_i = expected frequency

Null hypothesis: The observed distribution constitutes a sample from a population having the theoretical (normal) distribution.

In general, a large χ^2 value indicates a poor fit while a small χ^2 value indicates a good fit. A CHIPROB is the probability of obtaining a χ^2 value as large as the one calculated for the sample. If $\text{CHIPROB} > 0.5$, the null hypothesis is not rejected. However, if $\text{CHIPROB} < 0.05$, the null hypothesis is rejected with the conclusion that the observed distribution is not normally distributed.

(ii) Kolmogorov-Smirnov test

This goodness of fit test is based on cumulative frequencies as follows:

$$D = \max |S_n(x) - F(x)|$$

$$S_n(x) = \frac{K}{N} \quad \text{and} \quad F(x) = \frac{K_i}{N}$$

K = number of observations $\leq x$ in the observed distribution

K_i = number of observations $\leq x$ in the theoretical distribution.

N = total number of observations in the observed distribution

D is the maximum deviation which can be used along with a critical value from standard Kolmogorov-Smirnov tables to determine whether or not the observed and theoretical (normal) distributions differ significantly. Hence, for $D < KS(.05)$ there is no significant differences between the two distributions at a level of significance of 0.05. For $D > KS(.05)$ the observed distribution is significantly different from a normal distribution.

4. Student's t-test

(i) Values assuming only the normality of the parent population:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

$\bar{x}_i$ = arithmetic means of the sample i

s_i^2 = estimated variance of sample i

n_i = number of observation from sample i

PROB (t) = probability of obtaining a value of t whose
|value| is greater than or equal to |value|

of the one calculated, given that the two population means are equal. If $\text{PROB}(t) < .05$, it is concluded that the sample means are significantly different.

$$\begin{aligned} \text{DF}(t) &= \text{degrees of freedom} \\ &= \frac{\left(\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2} \right)}{\frac{\left(\frac{S_1^2}{n_1} \right)}{n_1 - 1} - \frac{\left(\frac{S_2^2}{n_2} \right)}{n_2 - 1}} \end{aligned}$$

(iii) Values assuming the population variances are equal:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{n_1 + n_2}{n_1 n_2 (n_1 + n_2 - 2)} \left[(n_1 - 1) S_1^2 + (n_2 - 1) S_2^2 \right]}}$$

$\text{PROB}(t)$ = similar to (i) above

$$\text{DF}(t) = n_1 + n_2 - 2$$

5. Correlation Coefficient (r) between two variables,

x and y.

$$r = \frac{\sum (x - \bar{x}) \cdot (y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}}$$

III. RESULTS

A. Assessment of the Micro-Assay Technique

1. Effect of Chloroform

Treatment of plasma with chloroform to remove the arginine esterase inhibitor was the major source of variability in the assay. The effect of chloroform rotation time on arginine esterase activity is shown in Figure 9. The enzyme activity reached a steady level at between 2 and 10 minutes of mixing with the electric rotator. Enzyme activity began to fall as the time of rotation was longer than 10 mins. Since 5-minutes was near the center of the plateau on the graph, it was chosen for the plasma arginine esterase assay.

2. Effect of Ellagic Acid Concentration

Early work had been done using ellagic acid in solution in dilute sodium hydroxide. Very low arginine esterase activity was obtained for the plasma from normal controls. Later, ellagic acid was prepared in suspension in 0.10 M phosphate buffer, pH 7.6 containing 0.15 M of NaCl. With this technique, more reproducible results were obtained.

Figure 9: EFFECT OF CHLOROFORM ON ENZYME ACTIVITY

A study of the effect of ellagic acid concentration on arginine esterase was performed. Results were plotted on a graph presented on Figure 10. In the absence of ellagic acid, the enzyme activity was very low. With the addition of ellagic acid suspension to activate the esterase, higher activity was recorded. The amount of ellagic acid present did not affect the enzyme activity. Since Rao and co-workers used 0.1 mM in their assay, 0.1 mM was chosen for the micro-method of arginine esterase assay.

3. Effect of Ellagic Acid Incubation Time

The time course of activation following the addition of ellagic acid to plasma obtained from a normal subject is shown on Figure 11. Maximal activation of the arginine esterase was achieved after 5-10 mins. of exposure to ellagic acid. Thereafter, a gradual decay of arginine esterase activity was observed. 15 minutes of incubation with ellagic acid was used because Rao et al used the same incubation time in their original assay.

Figure 10: EFFECT OF ELLAGIC ACID CONCENTRATION ON ENZYME ACTIVITY

Figure 11: EFFECT OF ELLAGIC ACID INCUBATION TIME
ON ENZYME ACTIVITY

4. Effect of Soybean Trypsin Inhibitor Concentration

A study of the effect of STI concentration on arginine esterase activity was performed and results are presented on Figure 12. Enzyme activity was the highest in the absence of STI. With the addition of STI, enzyme activity began to decline. As the concentration of STI added reached 100 µg/ml plasma, the enzyme activity began to level off. Increase in STI to concentration higher than 100 µg/ml plasma did not have any significant effect on enzyme activity. In the arginine esterase assay, 200 µg of STI was added per ml of chloroform treated plasma.

5. Reproducibility of the Micro-Assay

The micro-method for arginine esterase activity of a plasma sample obtained from a normal adult was performed 10 times on the same day. Enzyme activities measured from the ten runs were 60.1, 60.5, 60.8, 61.4, 62.4, 63.8, 64.1, 66.7, 67.2, and 67.4 units. The mean value obtained was 63.4 with a standard deviation of 285. The coefficient of variation of the micro-assay was $\pm 4.5\%$.

The micro-assay of arginine esterase activity was also carried out on two plasma samples on different

Figure 12: EFFECT OF SOYBEAN TRYPSIN INHIBITOR CONCENTRATION ON ENZYME ACTIVITY

days. Results are presented on Table 2. The enzyme activity units were reproducible at ± 10 or $\pm 17\%$ respectively over a ten day storage period.

Blood was taken from the same normal subject on three separate days. The arginine esterase activity of the plasma samples was assayed and the results were presented in Table 3. Little variation of the enzyme activity was detected for plasma samples collected on different days.

Blood samples were also collected from an adult subject at different times on the same day. Results obtained for the enzyme assay of the four plasma samples are shown in Table 4. This experiment indicated that the method was not subject to gross variations for the time of sampling.

B. A Comparison of the Micro-Assay With the Macro-Assay of Arginine Esterase Activity

The arginine esterase activities of 26 plasma samples (from both controls and patients) were measured using both the macro-and micro-assays. The actual values of STI-inhibited enzyme activity of all samples assayed were presented in Table 5. The correlation between the

Table 2: Effect of storage on arginine esterase activity.

Day of enzyme assay	Units of STI-inhibited enzyme activity	
	A	B
1	26.8	37.1
3	26.6	36.2
4	26.3	
8	27.8	44.8
10	38.3	36.5
Mean	29.2	38.6
S.D.	5.1	4.1
Coefficient of variation	17.4%	10.6%

Table 3: Day-to-day variation of arginine esterase activity.

Day of blood collection	Units of enzyme activity
1	36.0
7	35.6
11	33.0
Mean	34.9
Standard deviation	1.6
Coefficient of Variation	$\pm 4.7\%$

Table 4: Within-day variation of arginine esterase activity.

Time of blood collection	Units of enzyme activity
8:00a.m.	14.8
11:00a.m.	18.9
2:00p.m.	17.8
4:30p.m.	13.5
Mean	16.3
Standard deviation	2.5
Coefficient of variation	$\pm 15.5\%$

Table 5: A comparison of the arginine esterase activities measured by the micro-assay and macro-assay.

Sample No.	STI-inhibited arginine esterase activity (μ moles tame hydrolysed /hr. /ml plasma)	
	Micro assay	Macroassay
1	30.6	32.8
2	30.1	13.6
3	22.9	22.1
4	48.0	46.9
5	36.8	31.2
6	19.8	20.3
7	60.2	61.8
8	10.9	8.0
9	49.0	29.9
10	25.3	17.6
11	14.7	11.2
12	40.6	31.2
13	11.2	8.5
14	25.0	20.8
15	95.0	101.3
16	61.8	86.4
17	42.6	49.3
18	51.5	54.4
19	11.8	9.3
20	8.0	9.1
21	33.3	21.9
22	18.6	17.3
23	11.2	6.9
24	25.0	16.8
25	28.8	19.2
26	13.8	9.9
Mean	31.8	29.1
S. D.	20.3	24.3
Variance	411.3	592.2

Table 6:

ARGININE ESTERASE ACTIVITY IN PLASMA

The results are expressed as micromoles of TAME utilized per hour per ml. of plasma.

SAMPLE	NO.	TOTAL ACTIVITY	STI-INHIBITED ACTIVITY	STI-RESISTANT ACTIVITY
		Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.
(A) CONTROLS	50	53.8 $\pm$ 15.8	48.2 $\pm$ 14.8	5.6 $\pm$ 3.9
Adults	34	54.7 $\pm$ 14.0	50.1 $\pm$ 13.7	4.9 $\pm$ 3.1
♂ adults	16	50.4 $\pm$ 11.0	46.9 $\pm$ 10.0	3.5 $\pm$ 2.2
♀ adults	18	58.5 $\pm$ 15.5	52.4 $\pm$ 14.9	6.1 $\pm$ 3.3
Children	16	51.7 $\pm$ 19.5	44.8 $\pm$ 18.2	7.0 $\pm$ 5.1
♂ children	7	43.6 $\pm$ 19.9	37.3 $\pm$ 19.5	6.4 $\pm$ 6.5
♀ children	9	58.0 $\pm$ 17.7	50.7 $\pm$ 16.0	7.4 $\pm$ 4.0
(B) CYSTIC FIBROSIS	19	34.4 $\pm$ 12.0	30.4 $\pm$ 10.6	3.9 $\pm$ 2.9
I. Grouped according to sex :				
♂ patients	10	32.8 $\pm$ 9.3	29.6 $\pm$ 8.8	3.2 $\pm$ 2.5
♀ patients	9	36.2 $\pm$ 14.9	31.5 $\pm$ 12.8	4.7 $\pm$ 3.2
II. Grouped according to age :				
Ages < 16	11	30.8 $\pm$ 9.6	27.6 $\pm$ 8.2	3.3 $\pm$ 3.1
Ages $\geq$ 16	8	39.3 $\pm$ 14.5	34.5 $\pm$ 12.7	4.8 $\pm$ 2.5
(C) Heterozygotes	19	43.1 $\pm$ 14.0	37.1 $\pm$ 15.0	6.0 $\pm$ 5.0
Fathers of CF	2	34.6 $\pm$ 3.6	31.6 $\pm$ 3.2	2.9 $\pm$ 0.4
Mothers of CF	17	44.1 $\pm$ 14.4	38.9 $\pm$ 17.0	6.4 $\pm$ 5.2

enzyme activity measured by both assays is shown graphically in Figure 13. The correlation coefficient between the two assays was calculated, and the significance of each correlation coefficient was assessed by a Student's t-test.

The levels of enzyme activity obtained using the microassay were found to be significantly correlated to the levels measured by the macro-assay. The correlation coefficient between the two methods was 0.947.

C. Comparisons Between Arginine Esterase Activities of the Cystic Fibrosis Patients, Control Subjects and Heterozygotes Using the Micro-Assay

The individual values for arginine esterase activity obtained from 50 control subjects, 19 patients with cystic fibrosis and 19 obligate heterozygotes were expressed as total activity, STI-inhibited activity and STI-resistant activity. The means and standard deviations of the arginine esterase activities in the plasma of patients with cystic fibrosis, their parents (obligate heterozygotes) and normal controls are shown in Table 6. The actual values of STI-inhibited activity of all individuals measured are presented in Figure 14. The

Figure 13: The correlation between the arginine esterase activities measured by the micro-assay and the macro-assay.

Figure 14: Distribution of STI-inhibited arginine esterase activity in the plasma samples. (The bars represent the means of each group.)

differences in STI-inhibited arginine esterase levels between the groups are shown graphically in Figure 15. To obtain slightly more accuracy in comparing group means, Student's "t" values were calculated and presented in Table 7. The gaussian distribution of enzyme activities within each population is shown in Figure 16. It was confirmed using the Chi-square test and Kolmogorov-Smirnov test for goodness of fit. The significant differences found using Student's t-test (Table 7) confirm the differences that can be seen visually (the 95% confidence limits of one group do not include the mean of another group (Figure 15)).

The mean level of STI-inhibited arginine esterase activity in the patients with cystic fibrosis was approximately two-thirds of the mean level of activity in age-matched control subjects, while the mean heterozygote activity was midway between that of normal controls and patients. However, the values were scattered over a wide range for each group. The activity in CF patients was significantly lower than that of control groups in both children and adults ($p < 0.03$). The difference between the activities in obligate heterozygotes and the activities in control subjects was also

Figure 15: The mean(●) and 95% confidence limits(I) for each

TABLE 7

Differences of STI-inhibited arginine esterase activities in the plasma of control subjects, patients with cystic fibrosis and obligate heterozygotes. The "t" values were obtained by using the Student "t" test.

STI-INHIBITED ARGININE ESTERASE ACTIVITY (μ moles of TAME hydrolyzed/hour/ml of plasma)							
<u>SUBJECT</u>	<u>AGE</u>	<u>NO.</u>	<u>MEAN $\pm$ S.D.</u>	<u>RANGE OF ACTUAL VALUES</u>			<u>PROBABILITY (t)</u>
C.F. [†] Children	4 - 15	11	27.6 $\pm$ 8.2	13.5	-	42.2	<0.003*
Control Children	1 - 15	16	44.8 $\pm$ 18.2	12.2	-	82.2	
C.F. [†] Adults	16 - 26	8	34.5 $\pm$ 12.7	18.9	-	52.5	<0.028*
Control Adults	16 - 26	21	47.6 $\pm$ 12.9	26.8	-	71.6	
Obligate Heterozygotes	32 - 42	19	37.1 $\pm$ 15.0	6.4	-	65.3	<0.002*
Control Adults	27 - 46	13	53.4 $\pm$ 12.6	30.0	-	81.2	
C.F. [†] Adults	16 - 26	8	34.5 $\pm$ 12.7	18.9	-	52.5	<0.65**
Obligate Heterozygotes	32 - 42	19	37.1 $\pm$ 15.0	6.4	-	65.3	

*difference significant

**difference not significant

†C.F. = cystic fibrosis

Figure 16: Distribution of STI-inhibited arginine esterase values in the populations studied.

significant ($p < 0.002$). However, the difference between the activities of patients with CF and their parents was not significant. Arginine esterase activity was unrelated to sex or age of the individual tested.

D. Correlation Between the Plasma Arginine Esterase Activities of Cystic Fibrosis Patients and Their Parents

Plasma arginine esterase activities of 8 pairs of CF patient-mother values are presented in Table 8. The correlation coefficient was calculated to be 0.773 (significant at the 0.05 level). Hence, there seems to be slight correlation between the enzyme levels of the CF patients and parents in the same family. If the patient has a below-average enzyme activity, his mother is likely to have a low level of enzyme activity and vice versa.

E. Correlation Between the Plasma Arginine Esterase Activity and Shwachman Score of the CF Patients

Table 9 shows the correlation between arginine esterase activity and Shwachman score of the CF patients. The Shwachman scores for the patients varied from 38 to 94 while the range of enzyme activities was 13.5-52.5 units. There was no obvious correlation between arginine esterase activity and Shwachman score of the CF patient.

Table 8: Correlation between the STI-inhibited arginine esterase activities of CF patients and their parents.

Family no.	Patients with CF			Parents of CF patients		
	Sex	Age	Units of enzyme activity	Sex	Age	Units of enzyme activity
1	F	7	13.5	F	30	8.0
2	F	16	28.1	F	38	22.4
3	F	16	19.9	F	36	32.0
4	M	11	31.4	F	36	52.2
5	M	13	27.9	F	42	33.3
6	M	6	21.8	F	36	32.0
7	M	13	28.8	F	42	40.4
8	M	15	26.2	F	38	42.2

Table 9: Correlation between STI-inhibited arginine esterase activity and Shwachman scores of the CF patients.

Patient	Sex	Age	Units of arginine esterase activity	Shwachman score
N. H.	F	16	28.1	86
D. F.	M	15	42.2	62
K. F.	F	19	18.9	81
D. B.	F	16	52.5	94
R. E.	M	26	30.1	71
J. C.	F	15	19.9	67
J. S.	M	10	21.2	81
A. W.	M	11	31.4	68
B. G.	M	14	27.9	64
P. U.	M	6	21.8	89
D. S.	F	15	35.2	89
J. M.	M	12	28.8	78
W. D.	M	14	26.2	46
J. M.	F	4	35.2	38
K. B.	F	24	46.1	74
M. E.	M	17	19.5	83
M. P.	F	17	33.9	78
C. D.	F	7	13.5	72
G. V.	M	19	46.7	75

Hence, the enzyme activity was not related to the severity of the disease.

F. DEAE-Sephadex Column Chromatography of Plasma From Cystic Fibrosis Patients and Normal Controls.

For the ammonium sulfate precipitates obtained from 5 normal control and 3 cystic fibrosis plasma samples, desalting was performed on a Sephadex G-50 column using column size K 9/15. Incomplete separation of the protein and ammonium sulfate peaks can be seen in Figure 17. At a time when this problem was not appreciated, the partially desalted protein peak was pooled and chromatographed on DEAE-Sephadex. Arginine esterase activity was assayed using both TAME and BAEE as substrates and in the absence and presence of STI. An elution pattern for control plasma is shown in Figure 18. A large unadsorbed peak of arginine esterase emerged before the application of the gradient. This peak of enzyme activity was incompletely inhibited by STI. Negligible amounts of enzyme activity was detected after the application of an NaCl gradient.

Similar results were observed for the Sephadex G-50 eluate of the activated plasma from CF patients. An example of the elution profile of CF plasma is shown in

Figure 17: Desalting with Sephadex G-50 column (K9/15).

(Fractions nos. 6-13 were pooled and chromatographed on DEAE-Sephadex.)

Figure 18: DEAE-Sephadex chromatography of activated plasma from a control subject (F, age 37, plasma arginine esterase activity = 35.8 units)

• Assay without STI ○ Assay with STI

Figure 19. The peak of enzymatic activity was not absorbed on DEAE-Sephadex, and very little enzyme activity was detected after the application of the NaCl gradient.

The chromatographic technique was then improved using a Pharmacia column size of K 15/30 for Sephadex G-50. Complete separation of the protein peak from the ammonium sulfate peak was achieved as shown in Figure 20. Recovery of arginine esterase activity in the Sephadex G-50 eluate was ca 60%. The pooled eluate was then applied on a DEAE-Sephadex column and results of the normal control and cystic fibrosis plasma are shown in Figures 21 and 22 respectively.

Chloroform-ellagic acid treated plasma from normal individuals could be separated using DEAE Sephadex into two fractions by their arginine esterase activities (Figure 21). The first peak (I) which comprised approximately 40% of the total activity emerged immediately after the application of the NaCl gradient. Peak II emerged at a higher ionic strength (ca 0.15 M NaCl) and constituted ca. 60% of the arginine esterase activity. The peak fractions were also assayed in the presence of STI. Peak I was found to be completely inhibited by STI

Figure 19: DEAE-Sephadex chromatography of activated plasma from a patient with cystic fibrosis (F, age 16, plasma arginine esterase activity = 32.6 units)

● Assay without STI

○ Assay with STI

Figure 20: Desalting with Sephadex G-50 column (K15/30)
(Fractions nos. 10-16 were pooled and chromatographed
on DEAE-Sephadex.)

Figure 21: DEAE-Sephadex chromatography of desalted plasma sample from a control subject (F, age 20, plasma arginine esterase activity = 77.4 units)

● Assay without STI

○ Assay with STI

while peak II was almost totally resistant to inhibition by STI. This pattern of distribution of activities was consistently observed in all plasma samples from controls.

An example of the DEAE-Sephadex column chromatography for cystic fibrosis plasma is presented in Figure 22. A similar elution pattern was obtained as for normal plasma. The major peak (II) emerged at an ionic strength of 0.15 M NaCl and was almost totally resistant to inhibition by STI. The minor peak (I) emerged at the beginning of the NaCl gradient and was almost completely inhibited by STI.

For both normal and CF plasma, chromatography on DEAE-Sephadex resulted in three protein peaks as measured by A280 (Figures 21 and 22). The first unadsorbed peak emerged with the void volume. After the beginning of the NaCl gradient, the second peak emerged, followed by a large third peak whose ascending limb contained most of the arginine esterase activity.

G. Isoelectrofocusing of Sephadex-G50 Eluates

Analysis of Sephadex G-50 eluates by polyacrylamide-gel IEF in the Brinkman thin-layer IEF double

Figure 22: DEAE-Sephadex chromatography of desalted plasma from a patient with cystic fibrosis (F, age 16, plasma arginine esterase activity = 42.2units).

● Assay without STI

○ Assay with STI

chamber was rather unsuccessful. Upon completion of focusing, the gel slab was immersed in arginine esterase staining solution A and incubated at 37°C in the dark for 1-2 hours. No reaction occurred no matter how long the gel slab was kept in the staining solution. Solution B was then prepared by increasing the concentrations of all the components of the staining solution. When a focused gel slab was incubated at 37°C in staining solution B, four distinct bands of apparent arginine esterase activity appeared. The cathodic end of the gel was darkly stained as shown in Figure 24. Protein staining by Coomassie brilliant blue G resulted in 9 bands (Figure 23).

Since the arginine esterase bands spread all across the gel, an experiment was performed which consisted of focusing a gel slab without the addition of any plasma samples except hemoglobin (used as the indicator). A similar pattern was obtained to that shown in Figure 24. Four distinct bands of so-called "arginine esterase activity" apparently artefacts, were observed.

Further work on isoelectrofocusing was carried out using vertical gel tubes in a Buchler Polyanalyst. Sephadex G-50 eluates of activated plasma from 5 normal

Figure 23: Photograph of arginine esterase separated by polyacrylamide gel-isoelectrofocusing.

N = Normal plasma

Figure 24: Photograph of arginine esterase staining in a control gel slab.

controls and 5 CF patients were analyzed. Arginine esterase staining was carried out using both solutions A and B. No enzyme activity was detected in the gels for incubation with solution A. Figure 25 shows the results obtained with solution B. A diagrammatic representation of the photograph is shown in Figure 26. In the blank gel with the application of no protein sample, 4 bands appeared. All gels exhibited intense staining at the upper (cathodic) end. The gels with the application of Sephadex G-50 eluates revealed 9-11 bands of arginine esterase activity. The pattern of activity for the bands differed for all 10 samples studied. There was no consistent pattern observed for normal control samples. Hence, it was difficult to detect any difference in the enzyme activity patterns of the CF plasmas and the controls.

Figure 25: Photograph of arginine esterase separated by disc gel electrophoresis using Buchler Polyanalyst.

B = Blank

N = Control plasma

CF = Cystic fibrosis plasma

Figure 26: Diagrammatic representation of Figure 25.

B = Blank

N = Normal

CF = Cystic fibrosis

IV. DISCUSSION

The present study using a micro analytical technique confirms the observation (114) of Rao and co-workers that there is a deficiency of STI-inhibited arginine esterase activity in plasma of patients with cystic fibrosis. The advantage of employing a micro-assay is to enable investigators to work on arginine esterase activity without requiring unacceptably large volumes of blood from pediatric patients. The writer obtained identical values for arginine esterase activity with the micro-method as with the macro-method used by Rao et al when assaying plasma arginine esterase.

In most families, parents (obligate heterozygotes) have levels of enzyme activity intermediate between that of their children and control subjects. The difference in the mean plasma arginine esterase activity between the control subjects and patients with cystic fibrosis is statistically significant. The mean enzyme activity in the plasma of the heterozygotes is also significantly different from that of the normal controls. However, we have been unable to detect the significant difference between the mean values of patients and carriers reported by Rao et al (114).

There is no significant correlation of the arginine esterase levels with sex or age of the control subjects, CF patients or heterozygotes studied. The values of STI-inhibited arginine esterase activity for patients with cystic fibrosis could not be related to the severity of the disease as assessed by Shwachman scoring.

The writer was unable to reproduce the work of Rao and Nadler (111) on DEAE-Sephadex column chromatography and polyacrylamide gel electrofocusing. Rao and Nadler reported that the arginine esterase activity could be resolved into two fractions by DEAE-Sephadex column chromatography as shown in Figure 3. The bulk of the arginine esterase activity is eluted at approximately 0.02M NaCl and is completely inhibited by STI. A smaller peak is eluted at about 0.18M NaCl and contains arginine esterase activity which is almost totally resistant to inhibition by STI. In plasma of CF patients, the first, major activity peak was reduced to approximately 30% of the corresponding fraction in control plasma. In contrast, the second peak of arginine esterase activity did not show any significant quantitative differences between control and CF plasma samples.

In my studies, ion exchange chromatography using DEAE-Sephadex was carried out for chloroform-ellagic acid treated plasma samples obtained from 8 patients with CF and 8 control subjects. The activated samples were precipitated with 55% ammonium sulfate. The precipitates were then desalted on a column (K 15/30) of Sephadex G-50. Chromatography of the Sephadex G-50 eluates was performed on DEAE-Sephadex columns. Arginine esterase activity was resolved into two component fractions. The minor peak I emerged at the beginning of the gradient (0.02M NaCl) while the major peak II was eluted at an NaCl concentration of 0.15M. Similar elution patterns were obtained in both cases of control and CF plasma, though a reduction in size of the two activity peaks was observed in CF plasma. No isoenzyme deficiency was detected using anion-exchange chromatography. Hence, the deficiency of arginine esterase activity in the CF plasma was apparently entirely quantitative but not qualitative.

Initial experimentation of DEAE-Sephadex column chromatography was performed on plasma samples desalted on a Sephadex G-50 column (K 9/15). Arginine esterase activity was eluted with the void volume before

the application of the NaCl gradient. Next DEAE-cellulose chromatography was tried. Similar results were observed for plasma samples obtained from 3 control subjects and 3 CF patients. Almost all of the enzyme activity emerged before the appearance of the NaCl gradient. Therefore, it seemed logical to try CM-cellulose column chromatography. Results of this cation exchange chromatography gave a strange pH profile. The pH of the eluate dropped from 6.0 to 5.0 as the arginine esterase peak emerged, and rose when all the protein was eluted from the column. At this point, the problem of incomplete desalting by the Sephadex G-50 column became apparent. A larger column of Sephadex G-50 (with volume increased four fold) was used and this time the desalting ability of this column was checked by measuring the ammonium sulfate concentration in the eluted fractions. DEAE-Sephadex column chromatography was then repeated and yielded the results discussed above.

My findings for completely desalted protein fractions of plasma subjected to DEAE-Sephadex chromatography do not agree with those of Rao and Nadler. Their DEAE-Sephadex chromatography gave elution profiles which seemed to be rather similar to the elution

patterns obtained in my work when the protein samples were incompletely desalted. It is tempting to speculate that one could account for the results of Rao and Nadler by incomplete desalting. This would be impossible to prove.

Electrofocusing on polyacrylamide gels and subsequent staining for arginine esterase activity revealed 9-11 bands in plasma samples from controls and CF patients. Since four bands were artifacts, about 5-7 bands represented the arginine esterase activity. The activity pattern was rather variable in all the control samples studied; therefore, it was difficult to detect any difference in the arginine esterase activity patterns of plasma samples from the CF patients and the controls. According to Rao and Nadler (111), six activity bands could be detected in control samples, whereas five bands were detected in CF samples. However, the writer was unable to confirm their observations.

In this investigation, the plasma arginine esterase activity has been determined using a micro-method. The mean of STI-inhibited activity is lowest for CF patients while the mean for normal controls is the highest. The mean of STI-inhibited activity for the heterozygotes is midway between the values of the

patients and the normal individuals. The deficiency of arginine esterase activity is statistically significant for both cystic fibrosis patients and heterozygotes. However, no qualitative difference could be demonstrated by ion-exchange chromatography on DEAE-Sephadex or isoelectric focusing on polyacrylamide gels.

Next, I will discuss arginine esterases in general before returning to the relevance of this work in the whole field. Arginine esterases are a group of enzymes exhibiting proteolytic activity involved in the hydrolysis of peptide bonds derived from the carboxyl groups of arginine. Trypsin, chymotrypsin, kallikrein, thrombin, plasmin, and carboxypeptidase B, are all present in plasma and possess arginine esterase activity. All these enzymes will be examined to determine which of the various arginine esterases is most likely to be involved in cystic fibrosis and in this investigation.

The possibility that trypsin is the arginine esterase deficient in cystic fibrosis is unlikely because Rao et al were unable to assay the arginine esterase activity using more specific substrates such as α -N-benzoyl-L-arginine amide (BAA) or α -N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (113, 114). Also, plasma trypsin activity should be completely inhibited by soybean

trypsin inhibitor (134). In this work, the arginine esterase that is deficient in cystic fibrosis is only partially inhibited by STI. Hence, the arginine esterase studied in this investigation is not trypsin.

Chymotrypsin, an endopeptidase, also acts on amides and esters of susceptible amino acids. It preferentially catalyzes the hydrolysis of bonds of leucyl, methionyl, asparaginyll and glutamyl residues. Since TAME is not hydrolyzed by chymotrypsin (134), the possibility of the arginine esterase measured in this work being chymotrypsin is extremely unlikely.

In the present study, arginine esterase activity was measured after activation with ellagic acid (4, 4', 5, 5', 6, 6' -hexahydroxy-diphenic acid 2,6: 2'6'-delactone). Ratnoff and Crum reported that ellagic acid activates Hageman factor at concentrations as low as 10^{-8} M (116). The chemical nature of its action seems to be related to the presence of o-dihydroxyl groups in the ellagic acid molecule. Once activated, Hageman factor is able to trigger four proteolytic enzyme systems in plasma, the coagulation, fibrinolytic, complement and kinin-forming pathways (Figure 27) (21). Among the enzymes directly or indirectly activated by

Figure 27: Pathways of activation of plasma proteolytic enzymes (21).

'activated' Hageman factor, thrombin, plasmin, carboxypeptidase B and kallikrein exhibit arginine esterase activity and they are all theoretically possible candidates to represent the arginine esterase activity measured in my studies and those of others discussed in this section.

Thrombin catalyzes the transformation of fibrinogen to fibrin. It is generated from its inactive zymogen, prothrombin, and is not itself present in normal plasma. Its formation requires calcium and thromboplastin (146). Glover et al have worked on thrombin and reported that thrombin could be inactivated by 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) which complexes with thrombin and alkylates a crucial histidine residue in the active site of thrombin (62). Rao and Nadler suggested that the arginine esterase that they measured using TAME as a substrate did not represent thrombin because of the inability of TLCK to inactivate arginine esterase activity (112). The present study has not re-examined this.

Plasmin also exists in plasma as an inactive precursor, plasminogen. Plasminogen, can be converted to plasmin by the direct action of thrombin or indirectly by

activated Hageman factor (Figure 27). One of the major biological roles of plasmin is to digest fibrinogen or fibrin to fragments known as fibrin degradation products (21). Groskopf et al have shown that TLCK could inhibit the proteolytic activity of plasmin by a stoichiometric irreversible reaction at the active site of plasmin (64). Hence, the inability of TLCK to inhibit the arginine esterase activity demonstrated by Rao and Nadler (112) also excludes the possibility that the arginine esterase measured by the hydrolysis of TAME may represent plasmin. Again, this has not been verified in this present study.

Carboxypeptidase B is formed from an inactive zymogen precursor, procarboxypeptidase by the action of trypsin. Carboxypeptidase B can destroy plasma bradykinin (this is discussed in the "Introduction" q.v.) by removing the C-terminal arginine (21). Carboxypeptidase B exhibits anaphylatoxin (C3a) inhibitor activity (30). Hence, serum carboxypeptidase B inactivates the complement derived anaphylatoxins C3a and C5a, as well as bradykinin. Carboxypeptidase B presumably serves as the major regulatory enzyme of circulating vasoactive peptides. Conover et al reported that in normal serum

treated with a carboxypeptidase inhibitor, anti-ciliary activity develops. This biologic property of CF serum is abolished by treatment with carboxypeptidase B (27). They postulated that a genetic deficiency of serum carboxypeptidase in CF patients may account for the expression of a factor in serum from cystic fibrosis homozygotes and heterozygotes which induces ciliary dyskinesia. Unfortunately, Lieberman has shown recently that carboxypeptidase B-like activity is normal in CF serum (89). This study has no information on this point.

Early studies by Rao et al suggested that the arginine esterase activity measured by them might represent plasma kallikrein (114). Kallikrein is a plasma proteolytic and esterolytic enzyme that acts on specific β -globulin substrates called kininogens to release bradykinin (Figure 2). The esterolytic ability of kallikrein can be inhibited by soybean trypsin inhibitor as well as by α_2 -macroglobulin (21, 80). Colman et al provided evidence that the arginine esterase activity in plasma after the addition of kaolin or ellagic acid was probably identical with plasma kallikrein (22). The evidence was as follows: (i) purification data and similarities in esterase activity between

purified kallikrein and kaolin-activated esterase; (ii) evidence that the activity cannot be ascribed to other known plasma or tissue arginine esterases; (iii) the requirement of Hageman factor for the activation of arginine esterase activity; and (iv) similarities in the behaviour of the plasma enzyme and purified kallikrein toward inhibitors (22). Colman and co-workers also compared the kaolin-activated plasma arginine esterase with purified plasma preparations of human thrombin, human plasmin, bovine trypsin and urinary kallikrein as indicated in Table 10 (22). Very little difference was noted in the substrate ratios between the kaolin-activated arginine esterase activity and the purified kallikrein preparation. Hence, the arginine esterase activity measured in this work seems very likely to represent plasma kallikrein activity.

Rao and associates next reported a deficiency of arginine esterase activity in the plasma of CF patients (114) and this time called it a kallikrein deficiency without equivocation. Their findings have been confirmed by my present study, as well as by Wilson and Fudenberg (148), using TAME as a substrate. However, Lieberman (91) could not reproduce the work of

Table 10: Hydrolysis of various arginine and lysine esters by plasma and tissue esterases.

TAME = Tosyl - arginine methyl ester
 BAME = Benzoyl - arginine methyl ester
 AAME = Acetyl - arginine methyl ester
 TLME = Tosyl - lysine methyl ester
 ALME = Acetyl - lysine methyl ester

Enzyme	Ratio: $\frac{\text{Substrate}}{\text{TAME}}$			
	BAME	AAME	TLME	ALME
Kaolin activated arginine esterase	0.89	0.66	0.22	0.15
Thrombin	1.00	1.30	1.70	0.40
Plasmin	1.01	0.49	1.48	2.35
Trypsin	0.18	0.51	1.00	0.73
Kallikrein	0.81	0.07	0.25	0.23

Rao et al. He found that the level of arginine-esterase activity in CF plasma was normal when he used BAEE as a substrate. Lieberman and Littenberg even reported an increased kallikrein content of saliva from patients with CF using biological assays (92). In other works, Talamo et al could not find any 'severe' abnormality of the plasma kallikrein-kinin system in cystic fibrosis using biochemical and immunochemical methods (140). There is obviously confusion in this field but it still seems likely that the arginine esterase activity measured in my study and in those of Rao and Nadler is kallikrein.

After the experimental work in this thesis was completed, ideas concerning the pathogenesis of cystic fibrosis began to center around the role of α_2 -macroglobulin as an inhibitor of arginine esterase activity. This is coming about because Wilson et al showed earlier that sera from CF patients and carriers were deficient in a protein of the β -globulin fraction and speculated that the deficiency might be responsible for the accumulation of CF factor(s) in serum (147, 150). Later, using multiple chromatographic techniques, gel filtration, and starch block electrophoresis, Wilson and Fudenberg documented the absence of a protein fraction with a pI

of 5.5 in plasma from CF patients (148). They produced evidence that this was a protein normally derived from α_2 -macroglobulin as a result of interaction of α_2 -macroglobulin with arginine esterase proteins. They found a normal concentration of intact Alpha $_2$ -macroglobulin (α_2 M) in cystic fibrosis plasma but a slightly reduced binding capacity of the α_2 M from CF patients for trypsin and suggested that the deficient protein may be a proteolytic derivative of α_2 M (148).

α_2 -macroglobulin is a plasma inhibitor which appears to participate in the regulation of several circulating enzyme systems, since it forms complexes with plasmin (72), thrombin (36) and kallikrein (71). Its interaction with the hemostatic inflammatory and complement pathways is shown in Figure 28. In addition, α_2 M has been found to bind trypsin, chymotrypsin, cathepsins and other endopeptidases (6, 72,). Harpel has shown that the subunit structure of α_2 M is altered by interaction with the proteolytic enzymes it inhibits (72). α_2 M normally consists of four subunit chains of molecular weight approximately 185,000 each but upon complex formation with trypsin, plasmin, thrombin, kallikrein and possibly other plasma proteases, it is proteolytically

cleaved at the same region in each subunit chain, resulting in the production of a single protein with a molecular weight approximately half that of the subunit chain (85,000). (72).

Interaction of arginine esterase with α_2 M may involve proteolytic cleavage at a single arginine or lysine residue in α_2 M, located within a peptide loop formed by intrachain disulfide bridging (72). A covalent bond is then formed between the enzyme and α_2 M, involving an acyl enzyme or tetrahedral intermediate, in which a serine residue at the active site of the enzyme forms an ester bond with the carboxyl of an arginine or lysine residue at the active site of α_2 M. In this reaction, the active site of the enzyme is blocked, and an enzyme - α_2 macroglobulin complex is formed (72, 148). This complex retains its esterolytic activity towards low molecular weight substrates but not proteolytic activity towards protein. The other product of the reaction is a smaller protein with a molecular weight of approximately 85,000 as mentioned earlier.

Wilson and Fudenberg reported a decreased level of the proteolytic fragments from α_2 M in plasma from CF homozygotes and obligate heterozygote carriers (148).

Possible mechanisms suggested by Wilson and Fudenberg are the following:

- (a) Plasma proteases in general may not be activated to normal levels because of an abnormality in Hageman factor or in the activation of Hageman factor. Before activation of Hageman factor by glass or ellagic acid, almost all plasma arginine esterase activity is in an inactive or proenzyme form and will not interact with α_2 M (72, 79). At this time the proteolytic derivatives of α_2 M cannot be observed by isoelectrofocusing of whole plasma (148). When Hageman factor is activated, it can initiate the conversion of the proteases to active forms capable of complexing to α_2 M (77). They can at this time also interact with the systems that generate kinin and anaphylatoxin (Figure 28) (79, 115).
- (b) A specific protease may be abnormal structurally or remain partially in its proenzyme form (e.g. Kallikrein)
- (c) There may be a genetically determined abnormality in the structure of α_2 M, decreasing its binding affinity for all plasma proteases.
- (d) The active site by which α_2 M binds to proteases

Figure 28: Diagram illustrating the points of interaction of α_2 -macroglobulin with the hemostatic, inflammatory and complement pathways. (Modified from Wilson and Fudenberg(148))

may be blocked by an inhibitor.

- (e) The active site of all the proteases by which they bind to α_2 M may be blocked, inhibiting their ability to interact with α_2 M (148).

Possibilities (c) and (d) seem rather attractive. A structural abnormality in, or a blockage of, the binding site of α_2 M could decrease the inhibition of kallikrein, plasmin, and other plasma proteases.

α_2 M is thought to be the major inhibitor of plasmin (79). Plasmin, if not effectively inhibited, could participate in increased activation of kallikrein and thus increase the production of kinins, (Figures 2, 27).

This series of events should at first glance cause an increase in kallikrein activity. However, in my investigation, a decrease in arginine esterase was observed. According to Wilson and Fudenberg, this could be explained by the fact that the increased production of plasmin might be enough to inhibit Hageman factor formation (148). Unfortunately, no evidence can be found to support this hypothesis that increased plasmin would inhibit Hageman factor activation. This in turn would inhibit prekallikrein and hence kallikrein.

A correlation between decreased arginine

esterase activity and a decrease observed in the α_2 M fragment bands by isoelectric focusing was demonstrated (148). Wilson and Fudenberg suggested that this concomitant decrease in the concentration of α_2 M fragments and arginine esterase activity or kallikrein activity in CF might be explained by assuming (i) hyperconversion of prekallikrein to kallikrein, brought about by plasmin, with subsequent inhibition of kallikrein by inhibitors present in the plasma; (ii) feedback inhibition of Hageman factor activation by uninhibited plasmin; or (iii) a concurrent defect in kallikrein, or another arginine esterase, but not plasmin (148).

Additional support for the role of α_2 -macroglobulin in the pathogenesis of arginine esterase deficiency comes from the work by Shapira, Rao, Wessel and Nadler (129). Using immunological methods, they demonstrated that the arginine esterase activity present in normal plasma and deficient in cystic fibrosis patients was bound to α_2 M- like molecule. Pooled plasma from normal individuals was activated by chloroform-ellagic acid treatment and precipitated by ammonium sulfate at 50% saturation. Since the arginine esterase activity could be inhibited by STI, the plasma precipitate was

purified by affinity chromatography using STI covalently bound to Sepharose 4B. This purified protein fraction was resolved by isoelectric focusing on polyacrylamide gels. Proteins with most of the arginine esterase activity had a pI range of 5.5. This fraction was the one which is decreased in the plasma of patients with CF. Immuno-electrophoresis with activated plasma samples from controls and patients with CF was performed using the antiserum. In controls, four precipitin arcs with arginine esterase activity were observed whereas only three were seen in plasma from CF patients. One arc with enzymatic activity was found to be antigenically an α_2 -macroglobulin. On immuno-electrophoresis, this α_2 M precipitin band corresponded to the band absent in plasma of CF patients. The presumed implication of the above work is that the arginine esterase band deficient in cystic fibrosis plasma contained α_2 M determinants.

α_2 M levels measured immunologically were similar in plasma from controls and CF patients. The work of Shapira et al is consistent with the hypothesis that in cystic fibrosis there is an absence of an α_2 M - arginine esterase complex and that this reflects a molecular defect in either of the two components. This

is the same hypothesis as that suggested by Wilson and Fudenberg (148). More recent studies by Shapira et al have shown that the α_2 M molecule is altered in patients with cystic fibrosis and obligate heterozygotes (128). This work consisted of an investigation of the binding capacity of isotopically labelled papain and isotopically labelled bovine trypsin to α_2 M. They showed that the amount of binding in normal controls is greater than that of CF patients (127). Binding in obligate heterozygotes is intermediary between the normal and CF levels. They conclude that the primary defect in cystic fibrosis is likely to reside in the α_2 -macroglobulin molecule.

Deficiency in plasma arginine esterase activity reported in this study would then be a secondary effect caused by a decreased binding affinity of α_2 M from CF patients for the enzyme.

A decreased binding affinity of α_2 M to arginine esterase can lead to a deficiency of STI-inhibited arginine esterase activity due to the following hypotheses proposed by the writer:

- (i) Positive feedback of arginine esterase (or kallikrein) synthesis may be diminished by the lack of binding of the enzyme to α_2 M.

- (ii) Decreased binding of α_2 M to arginine esterase facilitates the interaction of arginine esterase with other plasma inhibitor by a different mechanism. Unlike the α_2 M-arginine esterase binding which retains the enzyme activity, the interaction of arginine esterase with other inhibitors may result in the formation of an enzyme-inhibitor complex with no arginine esterase activity. Therefore, total and STI-inhibited arginine esterase activities decrease in cystic fibrosis as demonstrated by the present investigation.

In the annual meeting of the Cystic Fibrosis Club in April, 1977, Shapira reported the results of his most recent work on the interaction of α_2 -macroglobulin with trypsin (151). He suggested the following sequence of events for the interaction.

- (i) α_2 M plus trypsin forms an α_2 M-trypsin complex in less than 30 seconds. This complex has no activity to the high molecular weight substrates such as casein and no susceptibility to inhibition by high molecular weight inhibitors such as STI.
- (ii) Over a period of 30-60 minutes, this α_2 M-trypsin

complex undergoes a conformational change in which it regains some activity to high molecular weight substrates and some susceptibility to inhibition by high molecular weight inhibitors.

(iii) This altered complex then decomposes slowly to α_2M fragments and trypsin.

In my work, the bulk of arginine esterase activity as measured by the enzymological assay is inhibited by STI, in both cases of control and cystic fibrosis plasma. If the above mechanism of interaction between α_2M and trypsin is true and can be applied to arginine esterase, the writer would speculate that all three steps would take place in cystic fibrosis patients and normal controls. However, Shapira and Nadler suggested that the second step in the proposed mechanism does not occur in cystic fibrosis (151). This cannot be true because Nadler's earlier work in arginine esterase showed that about 70% the total arginine esterase activity of both control and cystic fibrosis plasma is inhibited by STI. These findings are similar to the data of the present study. Therefore, the second step in Shapira's suggested mechanism should occur in cystic fibrosis.

Work on column chromatography suggests that there is no deficiency of an isoenzyme of arginine esterase. This is consistent with a primary defect elsewhere which leads to a secondary deficiency of arginine esterase activity in cystic fibrosis. My chromatography findings are different from those of Rao and Nadler in the following aspects:

- (i) In this study, two peaks of arginine esterase activity are observed with the major peak eluted at an NaCl concentration of 0.15 M. In Rao and Nadler's work, two peaks of enzyme activity are demonstrated with the major peak eluted at approximately 0.02 M NaCl.
- (ii) The major activity peak in my study is not inhibited by STI. The major peak of arginine esterase activity in the work of Rao and Nadler is inhibited by STI.

The discrepancy of my observations and those of Rao and Nadler may be accounted for by the following assumptions:

- (i) Incomplete desalting of Rao and Nadler's column chromatography work might cause the bulk of arginine esterase activity to be eluted at the beginning of

the NaCl gradient.

- (ii) The interactions of α_2 M and arginine esterase may be time dependent. Longer time used in the manipulation of the column chromatography experiments would enable the interactions of α_2 M and arginine esterase to take place in a longer period of time before protein precipitation. Since α_2 M and STI both react with arginine esterases through the same mechanisms (72) longer time allowed for the interactions of α_2 M and arginine esterase would make the molecule more resistant to STI. This may give rise to the different degrees of inhibition of arginine esterase activity by STI in the chromatography results of Rao and Nadler, and in this study.
- (iii) If Shapira's suggested mechanism of the interaction of α_2 M with trypsin can be applied in general to other proteases including arginine esterase, then the time taken for the ammonium sulphate precipitation step is very critical. Presumably, the interaction between α_2 M and arginine esterase stops once precipitation occurs. If the timing of Shapira's proposed mechanism is

correct, the precipitation step in my column work would take place in the second step when the α_2 M-enzyme complex begins to regain some susceptibility to inhibition by STI. Hence, in my DEAE-chromatography data, the bulk of arginine esterase activity, is resistant to STI.

The biological role of the α_2 M-arginine esterase complex is not yet clear. Certainly, further research is required to explore the relationship of arginine esterase deficiency to α_2 -macroglobulin and to elucidate the involvement of α_2 M in the pathophysiology of cystic fibrosis. However, it may well be that an abnormality in α_2 M-arginine esterase interactions could explain the presence of CF factor(s) in cystic fibrosis.

V. CONCLUSION

The results of this investigation have shown that there is a significant reduction in the level of arginine esterase activity in the plasma of patients with cystic fibrosis relative to that of control subjects. Although as a group, obligate heterozygotes have arginine esterase activities higher than those of patients, the detection of individual heterozygous carriers is not possible.

Arginine esterase activity in chloroform-ellagic acid treated plasma from CF patients and control subjects has been resolved into two component fractions by ion exchange chromatography on DEAE-Sephadex. No qualitative difference is observed in the elution profiles of arginine esterase activity in control and CF plasma. This suggests that there is no iso-enzyme deficiency of arginine esterase in cystic fibrosis.

REFERENCES

1. Anderson, D.H.: Cystic fibrosis of the pancreas and its relation to celiac disease. *Am. J. Dis. Child.*, 56: 344, 1938.
2. Anderson, D.H.: Pathology of cystic fibrosis. *Ann. N.Y. Acad. Sci.*, 93: 500. 1962.
3. Applegarth, D.A., A.G.F. Davidson, E.M. Haworth, E.E. Quist, R. Zuk, B.D. Roufogalis and D.G. Clark: Cystic fibrosis: The effect of medium from cultured cystic fibrosis fibroblasts on ATPase activity. *Clin. Chim. Acta*, 74: 183, 1977.
4. Balfe, J.W., C. Cole and L.G. Welt: Red-cell transport defect in patients with cystic fibrosis and in their parents. *Science*, 162: 689, 1968.
5. Barnett, D.R. and B.H. Bowman: Cystic fibrosis ciliary inhibitor. *Pediatr. Res.*, 8: 687, 1974.
6. Barrett, A.J. and P.M. Starkey: The interaction of α_2 -macroglobulin with proteinases. *Biochem. J.*, 133: 709, 1973.
7. Bastman, J., and B.H. Landing: Morphology of the sweat apparatus in cystic fibrosis. *Am. U. Clin. Path.* 45: 455, 1966.
8. Beier, F.R., A.D. Renzetti, Jr., M. Mitchell and S. Watanabe: Pulmonary pathophysiology in cystic fibrosis. *Am. Rev. Resp. Dis.*, 94: 430, 1966.
9. Beratis, N.G., J.H. Conover and E.J. Conod, R.J. Bonforte and K. Hirschhorn: Studies on ciliary dyskinesia factor in cystic fibrosis. *Pediatr. Res.*, 7: 958, 1973.
10. Besley, G.T., A.D. Patrick and A.P. Norman: Inhibition of the motility of gill cilia of Dreissensia by plasma of cystic fibrosis patients and their parents. *J. Med. Genet.*, 6: 228, 1969.

11. Bodian, M. (ed.): Fibrocystic Disease of the pancreas: A congenital Disorder of Mucus Production-Mucosis. p. 86. Heinemann, London, 1952.
12. Bowman, E.H., L.H. Lockhart and M.L. McCombs: Cyster ciliary inhibition by cystic fibrosis factor. Science, 164: 325, 1969.
13. Bowman, E.H., M.L. McCombs and L.H. Lockhart: Cystic fibrosis: Characterization of the inhibitor to ciliary action in oyster gills. Science, 167: 871, 1970.
14. Carter, C.O.: In Fibrocystic Disease of the Pancreas. Edited by M. Bodian, p. 50. Grune & Stratton, New York, 1953.
15. Candill, M., I. Schafer and R. Stjernholm: Sulphate incorporation of leucocytes from patients with cystic fibrosis. Lancet, I: 32, 1974.
16. Chernick, W.S., G.J. Barbero and F.M. Perkins: Studies on submaxillary saliva in cystic fibrosis: J. Pediatr., 59: 890, 1961.
17. Chernick, W.S., H.J. Eichel and G.J. Barbero: Submaxillary salivary enzymes as measure of glandular activity in cystic fibrosis. J. Pediatr. 65: 694, 1964.
18. Chou, L. and H. Nadler: Absence of a serum factor in patients with cystic fibrosis. Pediatr. Res. 2: 312, 1975.
19. Clarke, J.T., E. Elian and H. Shwachman: Components of sweat. Am. J. Dis. Child. 101: 490, 1961.
20. Cole, C.H. and J.H. Dirks: Changes in erythrocyte membrane ATPase in patients with cystic fibrosis of the pancreas. Pediatr. Res., 6: 616, 1972.
21. Colman, R.W.: Formation of human plasma kinin. New England J. Med., 291: 509, 1974.

22. Colman, R.W., L. Mattler and S. Sherry: Studies on the prekallikrein (kallikreinogen)-kallikrein enzyme system of human plasma. *J. Clin. Invest.*, 48: 11, 1969.
23. Conod, E.J., J.H. Conover and K. Hirschhorn: Demonstration of human leukocyte degranulation induced by sera from homozygotes and heterozygotes for cystic fibrosis. *Pediatr. Res.*, 9: 724, 1975.
24. Conover, J.H., R.J. Bonforte, P. Hathaway, S. Paciuc, E.J. Conod, K. Hirschhorn and F.B. Kopel: Studies on ciliary dyskinesia factor in cystic fibrosis. I. Bioassay and heterozygote detection in serum. *Pediatr. Res.*, 7: 220, 1973.
25. Conover, J.H. and E.J. Conod: Complement in cystic fibrosis. *Lancet I*: 47, 1975.
26. Conover, J.H., E.J. Conod, and K. Hirschhorn: Ciliary-dyskinesia factor in immunological and pulmonary disease. *Lancet I*: 1194, 1973.
27. Conover, J.H., E.J. Conod and K. Hirschhorn: Complement components in cystic fibrosis. *Lancet, II*: 1501, 1973.
28. Conover, J.H., E.J. Conod and K. Hirschhorn: On the Nature of the defect in cystic fibrosis. *Texas Rep. Biol. Med.*, 34: 45, 1976.
29. Conover, J.H., E.J. Conod and K. Hirschhorn: Serum hexosaminidase levels in cystic fibrosis. *Lancet I*: 1122, 1973.
30. Conover, J.H., E.J. Conod and K. Hirschhorn: Studies on ciliary dyskinesia factor in cystic fibrosis. *IV Life Sci.*, 14: 253, 1974.
31. Constantopoulos, G., A.S. Dekaban, A. Lapey and P.A. di-Sant'Agnesse: Urinary acid mucopolysaccharides in cystic fibrosis. *J. Pediatr.*, 78: 806, 1971.
32. Corbin, N.C., T.E. Hugli and H.J. Muller-Eberhard: Serum carboxypeptidase B: A spectrophotometric assay using protamine as substrate. *Analyt. Biochem.* 73: 41, 1976.

33. Danes, B.S. and A.G. Bearn: Cystic fibrosis: An improved method for studying white blood-cells in culture. *Lancet* II: 439, 1969.
34. Danes, B.S. and A.G. Bearn: Cystic fibrosis of the pancreas. A study in cell culture. *J. Exp. Med.*, 129: 775, 1969.
35. Danes, B.S., K.M. Foley, S.D. Dillon and A.G. Bearn: Genetic study of cystic fibrosis of the pancreas using white blood cell cultures. *Nature* (London), 222: 685, 1969.
36. Danes, B.S., S. Litwin, T. Hutteroth, H. Cleve, A.G. Bearn: Characterization of cystic fibrosis factor and its interaction with human immunoglobulin. *J. Exp. Med.*, 137: 1538, 1973.
37. Danks, D.M., J. Allan and C.M. Anderson: A genetic study of fibrocystic disease of the pancreas. *Ann. Hum. Genet.*, 28: 323, 1965.
38. Developments in cystic fibrosis research (Editorial). *Lancet*, II: 307, 1973.
39. Dische, A., P.A. di Sant'Agnese, C. Pallavicini and J. Youlos: Composition of mucoprotein fractions from duodenal fluid of patients with cystic fibrosis of the pancreas and from controls. *Pediatrics*, 24: 74, 1959.
40. di Sant'Agnese, P.A.: Pulmonary manifestations of fibrocystic disease of pancreas. *Dis. of Chest.*, 27: 654, 1955.
41. di Sant'Agnese, P.A., Darling, R.C., Perera, G.A. and Shea, E.: Abnormal electrolyte composition of sweat in cystic fibrosis of the pancreas. *Pediatrics*, 12: 549, 1953.
42. di Sant'Agnese, P.A. and P.B. Dairs: Research in cystic fibrosis. *New Engl. J. Med.*, 295: 481, 534, 597, 1976.

43. di Sant'Agnese, P.A., Z. Dische and A. Danilchenko: Physicochemical differences of mucoproteins in duodenal fluid of patients with cystic fibrosis of the pancreas and controls: Clinical aspects. *Pediatrics*, 19: 252, 1957.
44. di Sant'Agnese, P.A., and Posell, G.F.: Eccrine sweat defect in cystic fibrosis of pancreas (mucoviscidosis). *Ann. New York Acad. Sc.* 93: 555, 1962.
45. di Sant'Agnese, P.A. and Talamo, R.C.: Pathogenesis and physiopathology of cystic fibrosis of the pancreas. *New Engl. J. Med.* 277: 1287, 1343, and 1399, 1967.
46. Doershuk, C.G., Chairman: NCFRF Data Registry Conference. Gap Conference Reports, National Cystic Fibrosis Research Foundation, October, 1971.
47. Doggett, R.G., G.M. Harrison, R.N. Stillwell and W.S. Wallis: An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis of the pancreas. *J. Pediatr.*, 68: 215, 1966.
48. Donnison, A.B., H. Shwachman and R.E. Gross: A review of 164 children with meconium ileus seen at the Children's Hospital Medical Centre, Boston. *Pediatrics*, 37: 833, 1966.
49. Durand, P., C. Borrone and G. Della Cella: Fucosidosis. *J. Pediatr.* 75: 665, 1969.
50. Emrich, H.M., E. Stoll, B. Friolet, J.P. Columbo, R. Richterich and E. Rossi: Sweat composition in relation to rate of sweating in patients with cystic fibrosis of the pancreas. *Pediatr. Res.* 2: 464, 1968.
51. Farber, S.: Some organic digestive disturbances in early life. *J. Mich. Med. Soc.*, 44: 587, 1945.
52. Feig, S.A., G.B. Segel, K.A. Kern, A.B. Osher and R.H. Schwartz: Erythrocyte transport function in cystic fibrosis. *Pediatr. Res.*, 8: 594, 1974.

53. Fox, R.J. and S.M. Hilton,: Bradykinin formation in human skin as a factor in heat vasodilatation. *J. Physiol., Lond.* 142: 219, 1958.
54. Fujimoto, Y., H. Moriya, K. Yamaguchi and C. Moriwaki: Detection of arginine esterase of various kallikrein preparations on gellified electrophoretic media. *J. Biochem.*, 71: 751, 1972.
55. Furtado, M.R.F. and M.M. Machado: Effects of bradykinin on the movement of water and sodium in some isolated living membranes. *Acta. Physiol. Latinoamer.* 16: 63, 1966.
56. Gage, G.W., R.L. Dobson and R. Waller: Sweat Gland function in cystic fibrosis. *J. Clin. Invest.*, 45: 1373, 1966.
57. Ghadimi, H., M. Stern and H. Shwachman: Study of free amino acids in sweat from patients with cystic fibrosis. *Am. J. Dis. Child.* 99: 333, 1960.
58. Gibson, L.E. and R.E. Cooke: A test for concentration of electrolytes in sweat in cystic fibrosis of the pancreas utilizing pilocarpine by iontophoresis. *Pediatrics*, 23: 545, 1959.
59. Gibson, L.E. and P.A. di Sant'Agnese: Studies of salt excretion in sweat. *J. Pediatr.*, 62: 855, 1963.
60. Gibson, L.E., W.J. Matthews, Jr., P.T. Minihan and J.A. Patti: Relating, mucus, calcium and sweat in a new concept of cystic fibrosis. *Pediatrics*, 48: 695, 1971.
61. Girey, G.J.D., R.C. Talamo and R.W. Colman: The kinetics of the release of bradykinin by kallikrein in normal human plasma. *J. Lab. Clin. Med.*, 80: 496, 1972.
62. Glover, G. and E. Shaw: The purification of thrombin and isolation of a peptide containing the active center histidine. *J. Biol. Chem.*, 246: 4594, 1971.
63. Goldring, R.M.: Pulmonary hypertension and cor pulmonale in cystic fibrosis of the pancreas. *J. Pediatr.*, 65: 501, 1964.

64. Groskopf, W.R., B. Hsieh, L. Summaria and K.C. Robbins: Studies on the active center of human plasmin. *J. Biol. Chem.*, 244: 359, 1969.
65. Gugler, E., J.C. Pallavicini, H. Swerklow and P.A. di Sant'Agnese: Role of calcium in submaxillary saliva of patients with cystic fibrosis. *J. Pediatr.*, 71: 585, 1967.
66. Guide to Diagnosis and Management of Cystic Fibrosis. Prepared by the Professional Education Committee, National Cystic Fibrosis Research Foundation, New York, 1963.
67. Hadorn, B., P.G. Johansen and C.M. Anderson: Pancreozymin secretin of exocrine pancreatic function in cystic fibrosis and the significance of the results for the pathogenesis of the disease. *Aust. Pediatr. J.*, 4: 1, 1968.
68. Hadorn, B., P.G. Johansen and C.M. Anderson: Pancreozymin secretin test of exocrine pancreatic function in cystic fibrosis and the significance of the result for the pathogenesis of the disease. *Canad. Med. Ass. J.*, 16: 377, 1968.
69. Hadorn, B., G. Zoppl, D.H. Shmerling, A. Prader, I. McIntyre and C.M. Anderson: Quantitative assessment of exocrine pancreatic function in infants and children. *J. Pediatr.*, 73: 39, 1968.
70. Hann, S., D.S. Holsclaw and H. Shin: Complement components in cystic fibrosis. *Lancet*, II: 520, 1974.
71. Harpel, P.C., Human plasma alpha 2-macroglobulin, an inhibitor of plasma kallikrein. *J. Exp. Med.*, 132: 329, 1970.
72. Harpel, P.C.: Studies on human plasma α_2 -macroglobulin-enzyme interactions. *J. Exp. Med.*, 138: 508, 1973.
73. Hilton, S.M.: Further experiments on the role of plasma kinins as mediators of functional vasodilatation in glandular tissues: in: Hypotensive peptides, pp. 281-188 (Springer-Verlag, New York, 1966).

74. Hirschhorn, H.: Genetic Studies in disease. In: J. A. Mangos and R.C. Talamo: Fundamental Problems of Cystic Fibrosis and Related Disease, p. 11. (Intercontinental Medical Book Corporation, New York, 1973).
75. Horton, C., W. Cole and H. Bader: Depressed (Ca^{++})-transport ATPase in cystic fibrosis erythrocytes Biochem. Biophys. Res. Commun., 40: 505, 1970.
76. Iacocca, V.F., M.S. Sibinga and G.J. Barbero: Respiratory tract bacteriology in cystic fibrosis. Am. J. Dis. Child., 106: 315, 1963.
77. Jones, J.M., J.M. Creeth and R.A. Kerwick: Thiol reduction of human α_2 -macroglobulin. The subunit structure. Biochem. J., 127: 187, 1972.
78. Kaiser, D., E. Drack and E. Rossi: Effect of cystic fibrosis sweat on sodium reabsorption by the normal sweat gland. Lancet, 1: 1003, 1970.
79. Kaplan, A.P.: Trends in microvascular research: The Hageman factor dependent pathways of human plasma. Microvasc. Res., 8: 97, 1974.
80. Kassell, B.: Trypsin and chymotrypsin inhibitors from soybeans. Methods in Enzymology, 19: 853, 1970.
81. Kramm, E.R., M.M. Crane, M.G. Sirken and M.L. Brown: A cystic fibrosis pilot survey in three New England states. Am. J. Public Health. 52: 2041, 1962.
82. Kulczycki, L.L., K.I.E. Macleod and H. Shwachman: A survey of school children for cystic fibrosis. Am. J. Hum. Genet. 12: 416, 1960.
83. Kuo, P.T. and N.N. Huang: The effect of medium chain triglyceride upon fat absorption and plasma lipid and depot fat of children with cystic fibrosis of the pancreas. J. Clin. Invest., 44: 1924, 1965.

84. Kwart, H. and V.E. Shashoua: Section of physics and chemistry structure and constitution of mucus. Tr. New York Acad. Sc., 19: 595, 1957.
85. Lamblin, E., P. Degand, P. Roussel, R. Havez, E. Hartemann and M. Fillat: Les glycopeptides du mucus bronchique fibrillaire dans la mucoviscidose, Clin. Chim. Acta, 36: 329, 1972.
86. Lanchantin, G.F., M.L. Plesset, J.A. Friedman and D.W. Hart: Dissociation of esterolytic and clotting activities of thrombin by trypsin-binding macroglobulin. Proc. Soc. Exp. Biol. Med., 121: 444, 1966.
87. Lapey, A. and J.D. Gardner: Abnormal erythrocyte sodium transport in cystic fibrosis. J. Pediatr. Res., 4: 478, 1970.
88. Lev, R. and S.S. Spicer: Histochemical comparison of human epithelial mucins in normal and in hypersecretory states including pancreatic cystic fibrosis. Am. J. Path. 46: 23, 1965.
89. Lieberman, J.: Carboxypeptidase B-like activity and C3 in cystic fibrosis. In: Cystic Fibrosis Club Abstracts, p. 44 (Cystic Fibrosis Foundation, Atlanta, 1975).
90. Lieberman, J.: Complement components in cystic fibrosis. Lancet, I: 1230, 1974.
91. Lieberman, J.: Plasma arginine esterase activity in cystic fibrosis. Am. Rev. Resp. Dis., 109: 399, 1974.
92. Lieberman, J. and G.D. Littenberg: Increased Kallibrein content of saliva from patients with cystic fibrosis of the pancreas. Pediatr. Res., 3: 571, 1969.
93. Lobeck, C.C.: Cystic fibrosis. In: The Metabolic Basis of Inherited Disease. 3rd Edition Ed. by J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson. New York, McGraw-Hill, 1972.

94. Lobeck, C.C.: Discussion in Research on Pathogenesis of Cystic Fibrosis, edited by P.A. di Sant'Agnese, p. 107. Wickersham Printing Co., Lancaster, Pa. 1966.
95. Lobeck, C.C. and N.R. McSherry: The ionic composition of pilocarpine induced sweat in relation to gland output during aging and in cystic fibrosis. Fourth Int. Conf. Cystic Fibrosis of the Pancreas (Mucoviscidosis). Mod. Probl. Pediatr. 10: 41, 1967.
96. Lowe, C.V., C.D. May and S.C. Reed: Fibrosis of the Pancreas in infants and children. Am. J. Dis. Child., 78: 349, 1949.
97. Mandel, I.D., A. Kutschev, C.R. Denning, R.H. Thompson, Jr. and E.V. Zegarelli: Salivary studies in cystic fibrosis. Am. J. Dis. Child. 113: 431, 1967.
98. Mangos, J.: Cystic fibrosis factor. Gap Conference Reports, National Cystic Fibrosis Research Foundation, February, 1971, p. 6.
99. Mangos, J.A. and N.R. McSherry: Sodium transport: Inhibitory factor in sweat of patients with cystic fibrosis. Science, 158: 135, 1967.
100. Mangos, J.A., N.R. McSherry, and P.J. Benke: A sodium transport inhibitory factor in the saliva of patients with cystic fibrosis of the pancreas. Pediatr. Res. 1: 436, 1967.
101. Martinez-Tello, F.J., D.G. Braun and W.A. Blanc: Immunoglobulin production in bronchial mucosa and bronchial lymph nodes, particularly in cystic fibrosis of the pancreas. J. Immunol., 101: 989, 1968.
102. McCombs, M.L.: Research in cystic fibrosis: A review. Tex. Rep. Biol. Med. 31: 615, 1973.
103. McEvoy, F.A., R.J. Davies, M.C. Goodchild and C.M. Anderson: Erythrocyte membrane properties in cystic fibrosis. Clin. Chim. Acta, 54: 195, 1974.

104. Melmon, K.L. and M.J. Cline: Editorial-Kinins.
Am. J. Med., 43: 153, 1967.
105. Merritt, A.S., B.L. Hanna, C.W. Todd, Jr. and T.L. Myers: Incidence and mode of inheritance of cystic fibrosis. J. Lab. Clin. Med. 60: 998, 1962.
106. Molenaar, J., M. Muller, C. Engelfreit and K. Pondman. Changes in antigenic properties of human C3 upon activation and conversion by trypsin. J. Immun. 192: 1444, 1974.
107. Moss, A.J., W.H. Harper, R.R. Dooley, J.R. Murray and J.R. Mack: Cor pulmonale in cystic fibrosis of the pancreas. J. Pediatr., 67: 797, 1965.
108. Munger, B.L., S.W. Brusilow and R.E. Cooke: Electron microscopic study of eccrine sweat glands in patients with cystic fibrosis of pancreas. J. Pediatr. 59: 497, 1961.
109. Newhouse, M., J. Sanchis, M. Dolovich, C. Rossman and W. Wilson: Mucociliary clearance in children with cystic fibrosis. In: Fundamental Problems in Cystic Fibrosis and Related Diseases, J.A. Mangos and R.C. Talamo, eds. Intercontinental Medicine Book Corp., New York, p. 319, 1973.
110. Potter, J.L., L.W. Matthews, J. Lemm and S. Spector: Human pulmonary secretions in health and disease. Ann. N.Y. Acad. Sci., 106: 692, 1963.
111. Rao, G.J.S. and H.L. Nadler: Arginine esterase in cystic fibrosis of the pancreas. Pediatr. Res., 8: 684, 1974.
112. Rao, G.J.S. and H.L. Nadler: Deficiency of arginine esterase in cystic fibrosis of the pancreas: Demonstration of the proteolytic nature of the activity. Pediatr. Res., 9: 739, 1975.
113. Rao, G.J.S. and H.L. Nadler: Deficiency of trypsin-like activity in saliva of patients with cystic fibrosis. J. Pediatr., 80: 573, 1972.

114. Rao, G.J.S., L.A. Posner and H.L. Nadler:
Deficiency of kallikrein activity in
plasma of patients with cystic
fibrosis. *Science*, 177: 610, 1972.
115. Ratnoff, D.D.: Some relationships among homo-
stasis, fibrinolytic phenomena, immunity,
and the inflammatory response. *Advan.*
Immunol., 10: 145, 1969.
116. Ratnoff, O.D. and J.D. Crum: Activation of
Hageman factor by solutions of ellagic
acid. *J. Lab. Clin. Med.*, 63: 359,
1964.
117. Reemtsma, K., P.A. di Sant'Agnese, J.R. Malm,
and H.G. Barker: Cystic fibrosis of
pancreas. Intestine absorption of fat
and fatty acid labelled with I^{131} .
Pediatrics, 22: 525, 1958.
118. Rick, W.: Untersuchung zur exokrinen Funktion
des Pankreas bei Cystischer Pankreas-
fibrose. *Med. Welt*, 42: 2158, 1963.
119. Robertson, J.A., B. Chernick, D.J. Segal and
E.E. McCoy: Increased uptake of ^{35}S -
heparin by lymphocytes from patients with
cystic fibrosis. *Lancet*, I: 1256,
1974.
120. Roeifs, R.E., G.E. Gibbs and G.D. Griffin:
The composition of rectal mucus in
cystic fibrosis. *Am. J. Dis. Child.*
113: 419, 1967.
121. Rossman, C., J. Dolovich, M. Dolovich, W.
Wilson and M. Newhouse. Effect of CF
serum on nasal mucociliary clearance.
Pediatr. Res. 8: 469, 1975.
122. Royce, S.W.: Cardiac and pulmonary complication
in fibrocystic disease of the pancreas.
In: Fibrocystic Disease of the Pancreas:
Report 18th Ross Pediatric Research
Conference, Ross Lab., Columbus, Ohio,
1956.

123. Sanchis, J., M. Dolovich, C. Rossman, W. Wilson and M. Newhouse: Pulmonary mucociliary clearance in cystic fibrosis. *N. Engl. J. Med.*, 288: 651, 1973.
124. Schmoyer, I.R. and F.A. Baglia: Cystic fibrosis. Effect of media from cultured cystic fibrosis fibroblasts on ATPase activity. *Biochem. Biophys. Res. Commun.* 58: 1066, 1974.
125. Schulz, I.J.: Micropuncture studies of the sweat formation in cystic fibrosis patients. *J. Clin. Invest.*, 48: 1470, 1969.
126. Schulz, I., K.J. Ullrich, E. Fromter, H. Holzgreve, A. Frick and J. Hegel: Mikropunktion und elektrische Potentialmessung an Schweissdrüsen des Menschen. *Pfluegers Arch.*, 284: 360, 1965.
127. Shapira, E., Y. Ben-Yoseph and H.L. Nadler: Decreased formation of α_2 -macroglobulin protease complexes in plasma of patients with cystic fibrosis. *Biochem. Biophys. Res. Commun.* 71: 864, 1976.
128. Shapira, E., Y. Ben-Yoseph, H.U. Wessel and H.L. Nadler: Decreased trypsin binding by α_2 -macroglobulin from cystic fibrosis patients and obligate heterozygotes. *Lancet*, in press (Letter to the Editor).
129. Shapira, E., G.J.S. Rao, H.U. Wessel and H.L. Nadler: Absence of an α_2 -macroglobulin-protease complex in cystic fibrosis. *Pediatr. Res.*, 10: 812, 1976.
130. Shwachman, H. and L.L. Kulczycki: Long-term study of one hundred five patients with cystic fibrosis. *Am. J. Dis. Child.*, 96: 6, 1958.
131. Shwachman, H., L.L. Kulczycki and K. Shaw: Studies in cystic fibrosis: a report on sixty-five patients over 17 years of age. *Pediatrics*, 36: 689, 1965.

132. Shwachman, H. and A. Mahmoodian: Pilocarpine iontophoresis sweat testing results of seven year experience. 4th International Conference on Cystic Fibrosis of the Pancreas (mucoviscidosis). Mod. Probl. Pediat., 10: 153, 1967.
133. Sibinga, M.S. and G.J. Barbero: Studies in the physiology of sweating in cystic fibrosis. Arch. Dis. Child., 36: 537, 1961.
134. Siegelman, A.M., A.S. Carlson and T. Robertson: Investigation of serum trypsin and related substances. Arch. Biochem. Biophys., 97: 159, 1962.
135. Slegers, J.F.G.: The Secretion and Reabsorption of Salt and Water in the Sweat Gland. Drukkerij Gebr. Janssen N.J., Nijmegen, 1966.
136. Spock, A., H.M.C. Heick, H. Cress and W.S. Logan: Abnormal serum factor in patients with cystic fibrosis of the pancreas. Pediatr. Res., 1: 173, 1967.
137. Sproul, A., and N. Huang: Growth patterns in children with cystic fibrosis. J. Pediatr. 65: 664, 1964.
138. Steinberg, A.G. and D.C. Brown: On the incidence of cystic fibrosis of the pancreas. Am. J. Hum. Genet. 12: 416, 1960.
139. Svennerholm, L.: Discussion of Villev, C.A. Evidence of enzymatic lesion. In Transactions International Research Conference on Cystic Fibrosis. Edited by R. McIntosh. Baltimore, Maryland, French-Bray, 1960, page 27.
140. Talamo, R.C., R.W. Colman and A. Milunsky: The plasma kallikrein-kinin system in cystic fibrosis. Pediatr. Res., 6: 430/170(1972).
141. Vaze, D., K.M. Kutty, R.K. Chandra and S.I. Hycheviah: Glycoproteins in the saliva and platelets of cystic fibrosis patients. Abstracts of medical sessions. VII International Cystic Fibrosis Congress, 1976, p. 6.

142. Verterberg, L. and G. Nise: Urinary proteins studied by use of isoelectric focusing. I. Tubular malfunction in association with exposure to cadmium. Clin. Chem. 19: 1179, 1973.
143. Verterberg, O. and H. Svensson: Isoelectric fractionation, analysis, and characterization of ampholytes in natural pH gradients. Acta Chem. Scand., 20: 820, 1966.
144. Waring, W.W., C.H. Brunt and B.C. Hilman: Mucoid impaction of bronchi in cystic fibrosis. Pediatrics, 39: 166, 1967.
145. Warwick, W.J. and R.E. Pogue: The prognosis of children with cystic fibrosis based on reasoned approaches to therapy: past, present, and future. J. Asthma Res., 5: 277, 1968.
146. White, A., P. Handler and E.L. Smith. Principles of Biochemistry. 4th Edition. McGraw-Hill Book Co., New York. 1968. pp. 724-734.
147. Wilson, G.B.: Studies on the cystic fibrosis serum factor and on the nature of the ciliary beat of Colpidium striatum. Ph. D. Thesis. University of California, Los Angeles. Dis. Abst. Intern., 35: 479, 1974.
148. Wilson, G.B. and H.H. Fudenberg: Studies on cystic fibrosis using isoelectric focusing II. Pediat. Res., 10: 87, 1976.
149. Wilson, G.B. and T.L. Jahn: Decreased rate of cytolysis of Colpidium striatum by cystic fibrosis serum. I. Life Sci., 15: 551, 1974.
150. Wilson, G.B., T.L. Jahn and J.R. Fonseca: Demonstration of serum protein differences in cystic fibrosis by isoelectric focusing in thin-layer polyacrylamide gels. Clin. Chim. Acta, 49: 79, 1973.

151. Shapira, E. Lecture on "Interaction of α_2 -macro-globulin and trypsin". 18th Annual Meeting of Cystic Fibrosis Foundation, San Francisco, April, 1977.

APPENDIX I

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PLASMA ARGININE ESTERASE ACTIVITY IN CYSTIC FIBROSIS OF THE PANCREAS

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Summary

Using a micro-method for the determination of plasma arginine esterase activity, we have investigated the values for soybean trypsin inhibitor (STI)-inhibited arginine esterase activity in patients with cystic fibrosis, obligate heterozygotes and age matched control individuals. The mean of STI-inhibited activity is lowest for cystic fibrosis patients while the mean for normal controls is the highest. The mean of STI-inhibited activity for the heterozygotes is midway between the values of the patients and the normal individuals. The deficiency of arginine esterase activity was statistically significant for both cystic fibrosis patients and heterozygotes.

Introduction

A deficiency in plasma arginine esterase activity in patients with cystic fibrosis of the pancreas was reported in 1972 [1]. This deficiency was measured by using as substrate tosylarginine methyl ester (TAME). The work was extended in 1974, using as a substrate α -N-benzoyl-L-arginine ethyl ester (BAEE) [2]. In another paper in 1974 [3] this work was reinvestigated. BAEE was used as substrate and the deficiency of arginine esterase activity was not confirmed. We have modified the original method of Rao et al. [1], to allow for determination of arginine esterase activity in micro samples of blood and here report the results of our measurements in patients with cystic fibrosis. We used TAME as substrate and our results substantially confirm those of Rao et al.

A recent report has demonstrated deficient proteolytic cleavage of α_2 -macroglobulin in cystic fibrosis plasma [4]. Since α_2 -macroglobulin appears to be involved in control of the arginine esterase system [5], it seems to be relevant to

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report our confirmation of the deficiency of plasma arginine esterase activity in patients with cystic fibrosis at this time.

Materials and methods

Blood samples were obtained from 19 patients with cystic fibrosis, 19 obligate heterozygotes, 34 normal, healthy adults and 16 hospitalized control children who were age matched for the cystic fibrosis patients. Cystic fibrosis patients were assessed for the severity of their disease, using the Shwachman Scoring System [6].

Arginine esterase activity in the plasma samples was measured using techniques modified from those of Rao et al. [1], with most volumes scaled down to approximately one-fifth. All samples were handled in polypropylene containers. 1.8 ml blood was mixed with 0.2 ml 3.8% sodium citrate. The plasma was separated by centrifugation at 3000 r.p.m. for 15 min at 4°C and used immediately or stored at -20°C. An equal amount of cold chloroform was added to the plasma and mixed using an electric rotator at approximately 10 r.p.m. for 5 min at 4°C. The mixture was then centrifuged at 2000 r.p.m. for 30 min at 4°C, after which the plasma was separated, and incubated with an equal vol-

Fig. 1. Investigations of the parameters of the micro-assay for plasma arginine esterase activity. (The arrows indicate the values used in the micro-assay).

ume of 0.1 mM ellagic acid (Aldrich Chemical Co. Inc.) suspended in 0.10 M phosphate buffer, pH 7.6, containing NaCl at a concentration of 0.15 M, at 25°C, for 15 min. 50 μ l of the activated plasma was then added to a tube containing 150 μ l of 0.10 M phosphate buffer pH 7.6 with 0.15 M NaCl and 0.015 M TAME. The reaction mixture was incubated at 37°C for 15 min. The reaction was terminated by the addition of 200 μ l of 10% trichloroacetic acid, and the mixture centrifuged at 2000 r.p.m. for 10 min at 4°C. Methanol in the supernatant was assayed according to Siegelman et al. [7] modified by using one-fifth of the reported volumes of the samples and reagents.

Micro-assay for arginine esterase activity was also performed in the presence of soybean trypsin inhibitor (STI) (Grand Island Biological Co). 10.0 mg of STI was dissolved in 1.0 ml of normal saline and 4 μ l of the STI solution was added to 0.2 ml of the chloroform treated plasma to give a final concentration 200 μ g/ml. The enzyme activity was expressed as STI-inhibited arginine esterase activity which was the difference between the values obtained by assaying in the presence and absence of STI.

The micro-assay technique was validated by assessing the effects of different concentrations of ellagic acid and STI. The effects of varying incubation times for ellagic acid and chloroform on arginine esterase activity were studied. The macro-method of Rao et al. [1] for arginine esterase activity was repeated for 26 plasma samples which had already been assayed using our micro-assay. A calibration plot was constructed to provide a correlation coefficient between the two assays. Results of the micro-assays on the various control subjects, patients with cystic fibrosis and obligate heterozygotes were assessed using the Student "t" test. The Gaussian distribution of STI inhibited arginine esterase activity in each population was confirmed by both the Kolmogorov-Smirnov test and the chi-square test.

TABLE I

DIFFERENCES OF STI-INHIBITED ARGININE ESTERASE ACTIVITIES IN THE PLASMA OF CONTROL SUBJECTS, PATIENTS WITH CYSTIC FIBROSIS (C.F.) AND OBLIGATE HETEROZYGOTES. THE P VALUES WERE OBTAINED BY USING THE STUDENT "t" TEST.

Subject	Age (years)	No.	STI-inhibited arginine esterase activity (μ mol of TAME hydrolyzed/h/ml of plasma)		
			Mean $\pm$ S.D.	Range of actual values	Probability (P)
C.F. children	4-15	11	27.6 $\pm$ 8.2	13.5-42.2	<0.003 *
Control children	1-15	16	44.8 $\pm$ 18.2	12.2-82.2	
C.F. adults	16-26	8	34.5 $\pm$ 12.7	18.9-52.5	<0.028 *
Control adults	16-26	21	47.6 $\pm$ 12.9	26.8-71.6	
Obligate heterozygotes	32-42	19	37.1 $\pm$ 15.0	6.4-65.3	<0.002 *
Control adults	27-46	13	53.4 $\pm$ 12.6	30.0-81.2	
C.F. adults	16-26	8	34.5 $\pm$ 12.7	18.9-52.5	<0.65 **
Obligate heterozygotes	32-42	19	37.1 $\pm$ 15.0	6.4-65.3	

* Difference significant.

** Difference not significant.

Fig. 2. Distribution of STI inhibited arginine esterase values in the populations studied.

Results

The results of the investigations of the method parameters are shown in Fig. 1. The comparison of the actual results of the micro-method with the macro-method, using a linear regression plot showed a correlation coefficient of 0.947. The values of STI inhibited arginine esterase activity in plasma for the various populations are shown in Table I and Fig. 2. The difference in the mean plasma arginine esterase activity between the control subjects and patients with cystic fibrosis was statistically significant. The mean enzyme activity in the plasma of the heterozygotes was also significantly different from that of the normal controls. There was no significant difference between the mean values of patients and carriers. The actual values of STI inhibited arginine esterase activity for patients with cystic fibrosis could not be related to the severity of the disease as assessed by Shwachman scoring.

Discussion

It can be seen that our results confirm the original work of Rao et al. [1], showing deficient arginine esterase activity in cystic fibrosis. It seems noteworthy that not only is the deficiency in arginine esterase activity statistically significant for both patients and heterozygotes, but the mean heterozygote activity is midway between normal control and patient values.

Our findings take on greater significance in view of the demonstration of deficient proteolytic cleavage of α_2 -macroglobulin in cystic fibrosis plasma [4] and the suggestion that α_2 -macroglobulin is involved in the control of plasma arginine esterase activity. Although much more work remains to be done on the

relationships between α_2 -macroglobulin and arginine esterase activities, the likelihood of a functional correlation between these two areas must be seriously considered and evaluated with respect to the pathogenesis of cystic fibrosis.

The micro-method which we have described for the estimation of arginine esterase activity should aid other investigations in confirming and extending these results without requiring unacceptably large volumes of blood from pediatric patients.

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References

- 1 Rao, G.J.S., Posner, L.A. and Nadler, H.L. (1972) *Science* 177, 610
- 2 Rao, G.J.S. and Nadler, H.L. (1974) *Pediatr. Res.* 8, 684
- 3 Lieberman, J. (1974) *Am. Rev. Resp. Dis.* 109, 399
- 4 Wilson, G.B. and Fudenberg, H.H. (1976) *Pediatr. Res.* 10, 87
- 5 Harpel, P.C. (1973) *J. Exp. Med.* 138, 508
- 6 Shwachman, H. and Kulczycki, L.L. (1958) *Am. J. Dis. Child.* 96, 6
- 7 Siegelman, A.M., Carlson, A.S. and Robertson, T. (1962) *Arch. Biochem. Biophys.* 97, 159

APPENDIX II: Shwachman scoring system of clinical evaluation of patients with cystic fibrosis (130).

GRADING	POINTS	GENERAL ACTIVITY	PHYSICAL EXAMINATION	NUTRITION	X-RAY FINDINGS
Excellent (88-100)	25	Full normal activity; plays ball, goes to school regularly, etc.	Normal; no cough; pulse and respirations normal; clear lungs; good posture	Maintains weight and height at above 25th percentile; well-formed stools, almost normal; good muscle mass and tone	Clear lung fields
Good (71-85)	20	Lacks endurance and tires at end of day; good school attendance	Resting pulse and respirations normal; rare coughing or clearing of throat; no clubbing; clear lungs; minimal emphysema	Weight and height at approximately 15th to 20th percentile; stools slightly abnormal; fair muscle tone and mass	Minimal accentuation of bronchovascular markings; early emphysema
Mild (55-70)	15	May rest voluntarily during the day; tires easily after exertion; fair school attendance	Occasional cough, perhaps in morning upon rising; respirations slightly elevated; mild emphysema; coarse breath sounds; rarely localized rales; early clubbing	Weight and height above 3rd percentile; stools usually abnormal, large and poorly formed; very little, if any abdominal distension; poor muscle tone with reduced muscle mass	Mild emphysema with patchy atelectasis; increased bronchovascular markings
Moderate (41-55)	10	Home teacher; dyspneic after short walk; rests a great deal	Frequent cough, usually productive; chest retraction; moderate emphysema; may have chest deformity; rales usually present; clubbing 2 to 3+	Weight and height below 3rd percentile; poorly formed, bulky, fatty, offensive stools; flabby muscles and reduced mass; abdominal distension, mild to moderate	Moderate emphysema; widespread areas of atelectasis with super-imposed areas of infection; minimal bronchiectasis
Severe (40 or below)	5	Orthopneic, confined to bed or chair	Severe coughing spells; tachypnea with tachycardia and extensive pulmonary changes; may show signs of right-sided cardiac failure; clubbing 3 to 4+	Malnutrition marked; large protuberant abdomen; rectal prolapse; large, foul, frequent, fatty movements	Extensive changes with pulmonary obstructive phenomena and infection; lobar atelectasis and bronchiectasis