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**SODIUM TRANSPORT  
IN CHEMICALLY MODIFIED ERYTHROCYTES  
FROM NORMAL SUBJECTS AND  
CARRIERS OF THE CYSTIC FIBROSIS GENE**

By  
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Thesis submitted to the School of Graduate Studies  
in partial fulfillment of the requirements for  
the Degree of Master of Science in Biochemistry

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## DEDICATION

This thesis is dedicated to my husband, Larry and to my children, Kristin and David, whose constant support and encouragement sustained the extra effort necessary to complete this work and to my parents whose dedication to my education will always be a catalyst for its continuation. It is further dedicated to all of those parents and children who cope daily with the reality of cystic fibrosis.

"Most of us serve our ideals by fits and starts. The person who makes a success of living is the one who sees his goal steadily and aims for it unswervingly."

Cecil B. DeMille

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## SUMMARY

The initial experiments were designed to adapt the procedure developed by Garay and Meyer (1979) for the measurement of total net sodium efflux and total net potassium influx in human erythrocytes to our clinical laboratory setting. The gradients for sodium and potassium were first reversed by treating the erythrocytes with parachloromercuribenzenesulfonic acid (PCMBS). Recovery of the gradients by the resealed cells in a  $\text{Na}^+/\text{K}^+$  Ringers media was then monitored by timed measurements of intracellular sodium and potassium concentration using flame photometry. In the first set of experiments the ion transport inhibitors ouabain and furosemide were added to the system to operationally define the  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport system. The procedure was then used to compare membrane sodium and potassium transport in erythrocytes from control subjects and from carriers of the cystic fibrosis gene.

Rates of net sodium efflux and net potassium influx were determined as the slope of the lines resulting from linear regression analysis of hourly measurements of intracellular sodium and potassium concentration. The values for the controls and CF heterozygotes were analyzed statistically using Student's *t*-test.

For clarity, the experimental work is divided into three series of paired experiments. In Series I, total net sodium efflux and total net potassium influx are measured in the presence and absence of the above inhibitors. In Series II and Series III, these same cation fluxes are measured only in the absence of inhibitors under two modifications of the same basic procedure. In Series II, the concentration of PCMBS was reduced from 0.1mM to 0.01mM in the

loading phase and the concentration of cysteine in the sealing phase was increased from 2.0mM to 4.0mM. These modifications were designed to reduce damage to the erythrocyte membrane caused by PCMBS treatment and to improve reconstruction of the membrane by more complete reversal of the PCMBS effect using cysteine in the sealing stage. These changes were maintained in Series III along with addition of more substrate to the sealing media to improve repletion of the cells. This was done to support better recovery of competent erythrocyte membranes and membrane transport systems as well as to stimulate higher net sodium efflux and net potassium influx. Investigative procedures were carried out to clarify the nature of the cation loading procedure and the net flux procedure. Results from these procedures were used to determine which modifications to pursue.

Evidence is presented to the effect that treatment of the erythrocytes with PCMBS results in marked damage to the cell membranes. Successful recovery of competent cell membranes and membrane transport systems depends both on extent of cell damage in the loading phase and conditions of reconstruction and repletion in the sealing phase. The balance between these experimental conditions as well as the construction of the recovery media in the recovery phase determines which transport systems will predominate in that phase.

The results do not directly demonstrate significant differences in erythrocyte membrane sodium transport between CF heterozygotes and controls. The data suggests, however, that further investigation of the ouabain-insensitive sodium transport component might demonstrate such differences. Consequently, further research directed toward assessment of the ouabain-insensitive, furosemide-sensitive  $\text{Na}^+, \text{K}^+$  co-transport system is

recommended. Attempts to assess this component in a physiologically constructed media in this study using flame photometry as the basis of measurement were unsuccessful. It is concluded that at the present time, measurement of this ion transport component must be done in specially constructed media designed to stress the steady state to expose movements of a magnitude sufficient to be measured by flame photometry.

It is difficult to extrapolate in vitro observations to in vivo actions of a biological system even when conditions in vitro are maintained as closely as possible to physiological. Although the modifications made in the solutions used deviate markedly from physiological conditions, the procedure developed by Dagher and Garay (1980) may be the answer for a more definitive isolation of the sodium transport abnormality in cystic fibrosis.

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## INTRODUCTION

The homeostasis of metabolic processes is complicated, delicate, fascinating, and essential to the successful maintenance of life. Perturbation of this balance results in compromised ability of the organism to cope with the normal fluctuations, physical or chemical, in its environment. The ultimate result is disease, leading at least to reduction in the quality of life, if not, as in many cases, to a shorter life span. An extensive number of diseases described today are characterized by familial predisposition to their development. Investigations directed toward the identification of the basic biochemical abnormality resulting in disequilibrium have long captured a large portion of biochemical research. In some cases the clinical symptoms of the disease indicate a clear direction for the research. In other diseases, the approach is not so apparent.

Subtle changes in a single gene product can lead to a cascade of events downstream from the lesion, compromising a number of major pathways and resulting in widespread organ system involvement. Under these circumstances, often no clear relationship appears to exist between the variety of secondary clinical symptoms and a common biochemical lesion. Cystic fibrosis (CF) is an example of one such disease. Although it is currently diagnosed at an early age in the homozygote based on a characteristic increase in sweat chloride, the basic biochemical lesion remains unidentified. Because of this and because clinical symptoms are not manifest in the heterozygote in an intermediate manner distinct from non-carriers, there is no diagnostic marker to identify the carrier.

Genetically, cystic fibrosis presents an autosomal recessive pattern of inheritance, being expressed in the homozygous state only. In one of every 1500 to 2000 live births in caucasians the infant is affected with CF. (Mangos, 1978). The gene frequency of cystic fibrosis is 5%. It is the most common disease in the caucasian population with an established Mendelian recessive mode of inheritance.

The two characteristic clinical symptoms invariably associated with the disease are the abnormally thick, viscous secretions produced by the exocrine glands and unusually high concentrations of certain inorganic ions in the secretions, most notably, sodium, chloride, and in some secretions, calcium. The clinical severity of the disease varies with the extent of organ system involvement: Commonly implicated are the secretions in the lungs, pancreas, intestine, liver, salivary glands and sweat glands. In most cases all of these organs are involved, but some cases may involve only digestive processes or respiratory processes. Almost invariably, however, the secretions of the salivary and sweat glands contain abnormally high concentrations of sodium and chloride.

As a result of increased viscosity, the secretions tend to obstruct or completely block the ducts from the exocrine glands. Blockage of the bronchi in the lungs leads to atelectasis and respiratory insufficiency. Infection easily develops in the blocked air passages resulting in recurrent or chronic pulmonary complications. Successful therapy against these symptoms is difficult and 90% of deaths in CF may be attributed to pulmonary complications of obstruction and infection. (Snodgrass, 1970). When pancreatic secretions are blocked, digestion is perturbed and malnutrition ensues with subsequent growth retardation. Mucus secreting glands of the GI tract may be involved as well,

and obstruction in the small biliary channels in the liver can result in portal hypertension. (Oppenheimer and Esterly, 1975; Stern et al. 1976).

In 1953, Darling et al. observed a higher incidence of heat prostration and accompanying dehydration in children with cystic fibrosis compared to unaffected children. Since the amount of sweat produced by the two groups did not differ, they suspected an abnormally high loss of electrolytes. Further investigation revealed that the concentration of chloride in the sweat from CF patients was more than three times greater than in normal controls. Extensive studies then confirmed this as a consistent finding in patients with CF regardless of the extent of organ system involvement. Diagnostic research, therefore, centered on methods to detect and quantitate the electrolyte levels easily and reliably, culminating in the development of the classic sweat test, which still today is the basic diagnostic test for cystic fibrosis. (Gibson and Cooke, 1959). The test does not detect the heterozygote, however, as only the homozygote manifests sweat electrolyte levels markedly different from normal controls.

The search for the basic biochemical lesion in cystic fibrosis is complicated by confusion over classification of clinical symptoms as primary or secondary. Attempts to formulate a unifying hypothesis to explain all of the symptoms have so far met with defeat. Research continues, however, and presently encompasses five basic areas. These include: 1) the analysis of the contents of CF secretions, 2) the characterization of abnormal circulating factors detected in the serum, sweat, and saliva of patients with CF, 3) the investigation of the presence or absence of proteolytic enzymes and their possible involvement in membrane structure, modification of secretory products and metabolism of circulating factors that might affect ion transport or the secretory process, 4) the analysis of stimulus-secretion coupling between

4

the nervous system and the secretory process, and 5) investigation of possible direct abnormalities in ion transport mechanisms.

A major objective of these studies in addition to the identification of the primary lesion, is the development of a diagnostic test for the carrier and for the detection of affected infants in utero. Thus, most research studies assess the status of the abnormality defined in the project in both homozygotes and heterozygotes when possible. There are many instances in which significant differences can be demonstrated between homozygotes and normal controls, but so far, identified abnormalities are either not expressed in the carrier, or exist in a broad range of values that overlap the normal controls and are thus, not useful as a reliable marker. Consequently, the carrier is identified only after the fact as an obligate heterozygote based on parental responsibility for the birth of an infant with CF. Clearly, the development of a diagnostic test for the carrier and for ante-natal testing would be advantageous in genetic counseling and in the assessment of siblings of known cystic fibrosis patients.

## LITERATURE REVIEW

### ANALYSIS OF THE CONTENTS OF SECRETIONS

Although the volume of secretions from the exocrine glands of patients with cystic fibrosis is generally normal, investigation of the nature of the contents has revealed differences. Water content is reduced in some secretions, but is not a generalized finding, and in sweat and parotid saliva the content is normal. (Di Sant'Agnese and Talmo, 1967). Elevated levels of calcium in submaxillary saliva and duodenal fluid have been noted as well as increased sulfate content of glycoproteins. (Di Sant'Agnese and Talmo, 1967; Di Sant'Agnese and Davis, 1976; Gibson et al. 1971). Added calcium causes normal mucus to become hyperpermeable to water and these studies suggest that the increased levels of calcium in some secretions in CF result in the same phenomenon, leading to increased loss of water and small electrolytes. A recent study reports decreased permeability to water in red cells from cystic fibrosis patients. (Galey et al. 1980). Others have demonstrated the presence of calcium-protein complexes in submaxillary saliva. (Boat et al. 1974). The protein involved apparently is normal in both CF patients and controls implicating the increased levels of calcium as causative in the precipitation of the calcium-protein complex with consequent turbidity. (Di Sant'Agnese and Davis, 1976).

Because glycoproteins and glycosaminoglycans are normal mucus constituents, the possibility of abnormalities in their metabolism has been considered. As previously noted, increased incorporation of sulfate into glycoproteins and glycosaminoglycans has been observed, but its relation to the progression of

the disease is not clear. No consistent defect in the metabolism of these two constituents has been seen. (Di Sant'Agnese and Talmo, 1967; Di Sant'Agnese and Davis, 1976). Although previous studies assessing the ratio of fucose to sialic acid in carbohydrate moieties of glycoproteins were inconclusive, recent work by Ben-Yoseph reports decreased sialylation and increased fucosylation in CF secretory glycoproteins. (Ben-Yoseph, 1981). Enzyme levels in the process were found normal, but the author suggests that alpha<sub>2</sub> macroglobulin in CF patients is a poor acceptor of sialic acid.

Lysosomal enzymes are involved in the metabolism of glycoproteins, consequently, some research has centered on assessment of the properties of these enzymes in CF. Hosli and Vogt (1979) reported altered heat stability of alpha-D-mannosidase in both CF plasma and fibroblast culture medium. However, recent work expanding this analysis fails to show a difference between CF patients and controls. (Maler et al. 1980; Butterworth, 1980). Using isoelectric focusing, Butterworth resolved the enzyme into a neutral and an acid component and demonstrated that the neutral component is heat labile. Based on percent enzyme inactivation on exposure to 50°C. temperature, he showed that the neutral component predominated in fibroblast extracellular alpha-D-mannosidase. Consequently, the proportion of the components in the enzyme from any one source has direct bearing on observed heat inactivation. He found no difference between controls and CF patients when accounting for this variable. The investigation of aberrant fucosylation, was extended by Maler et al. who reported a two to three fold reduction in alpha fucosidase activity in cultured lymphoblasts from CF patients. Noting that there was no evidence for an intracellular accumulation of glycolipids or glycoproteins, characteristic of some lysosomal storage diseases, the authors suggest that fucose containing compounds are cleaved at a reduced rate. They

point out that in fucosidosis where decreased alpha-L-fucosidase is even more marked, elevated sweat chlorides are sometimes also present. This is one reported source of false positives in the diagnostic sweat test. (Durand et al. 1969; Maler et al. 1980, 1981). Qualitative properties of the enzyme,  $K_m$  and isoelectric properties appear normal even though activity is reduced. Mixing experiments demonstrated no inhibitor to the normal enzyme in CF cells. The authors propose this abnormal enzyme is more useful as a clue to the basic lesion rather than a diagnostic marker, as it may not have the same expression in all cell types.

## THE CONCEPT OF CF "FACTORS"

The work of Spock et al. in 1967 and Mangos and colleagues in 1967 and 1968 introduced the concept of factors present in CF secretions, notably saliva, sweat or serum, that were responsible for the clinical manifestations of CF. (Table 1) Further investigation demonstrated that CF saliva or sweat, could produce symptoms similar to CF when introduced into normal salivary or sweat glands. (Kaiser et al. 1971; Mangos et al. 1967).

Spock and co-workers described a factor in CF serum that caused assymetrical movements of the cilia of isolated rabbit tracheal epithelial cells. (Spock et al. 1967). Column separation resulted in elution of the factor in the macroglobulin fraction between gamma globulin and albumin. Upon concentration of the serum euglobulin fraction, the factor could be demonstrated in obligate heterozygotes as well. Characteristically, it was heat labile and non-dialyzable. The factor was labeled the ciliary dyskinesia factor (CDF).

Modifying the assay using oyster gill cilia, other work confirmed this action of CF serum and its characteristics. (Bowman et al. 1969, 1970). These workers determined molecular weights of 75,000 to 180,000 in one instance and 125,000 to 200,000 a second time using sephadex column separation. Further purification with DEAE cellulose isolated the majority of dyskinesic activity in the gamma G fraction. Also, Bowman and colleagues demonstrated that the polybasic electrolytes shown by Mangos to inhibit sodium transport in rat parotid glands, also produced inhibition of the ciliary beat. (Mangos and McSherry, 1968).

The presence of the CDF was also demonstrated in cell free media from cultured leukocytes and lymphoid cells of patients with CF. (Conover et al.

**TABLE I**  
**ABNORMALITIES IN BIOLOGIC FLUIDS IN CYSTIC FIBROSIS (SO-CALLED "CF FACTORS")**

Anomaly Affecting	Properties		IgG	Inactivated By		Present In		
	Heat Labile	Net Charge		Bound	Needed for Activity	Arginine Esterase	Heparin	Homozygotes
Ciliary Activity Rabbit Trachea	Yes	+	Yes	Yes	Yes		Serum, Saliva Culture Medium	Yes
Oyster Gill	Yes	+	Yes	No	No	Yes	Serum, Saliva Culture Medium	Yes
Sodium Reabsorption	Yes	+				Yes	Saliva, Sweat Serum	Yes (?)
Short Circuit Current (Rat Intestinal Across Mucosa)	Yes	+					Serum	Yes
Isoelectric Focusing		+	Yes				Serum	Yes

Adapted from Di Sant'Agnese (1976)  
 New Eng J. Med., Part II, Page 537

1973; Wilson and Bahm, 1980). Conover's studies showed that anti-human IgG inhibited the activity. He suggested that the factor was a normal cell product bound to IgG and that dyskinesic activity resulted from deficiency of production or release of a factor that inhibits the CDF. He reported no clear distinction between CF patients and heterozygotes.

Culturing peripheral blood leukocytes from CF patients and heterozygotes, Wilson observed the production of three different ciliary dyskinesia factors. Using the rabbit ciliary bioassay, he noted that different types of leukocytes, namely T, B, and null lymphocytes displayed varied requirements for cellular cooperation to successfully produce the CDF. (Wilson and Bahm, 1980). He also presented evidence that the dyskinesia factor with a molecular weight of 9,000 was related to complement factor C3a as antisera specific for this component neutralized the CDF activity.

A relationship between ciliary dyskinesia and the enzyme amylase, isoenzymes of which are produced by the salivary glands and by the pancreas, has also been noted. (Doggett et al. 1973). Comparing the serum and heparinized plasma from patients with CF, they noted that the CDF activity of the serum was not present in heparinized plasma from the same source. The inhibition of activity was transient, returning to the plasma after 48 hours. Immunoelectrophoresis of the heparin precipitate showed amylase specificity and addition of the amylase inhibitor, chloranil, removed CDF activity as did treatment with rabbit monospecific antisera to amylase. Finally, they demonstrated that addition of purified amylase to normal sera makes the sera cilostatic and purified parotid amylase from CF patients caused cilostasis in oyster cilia. Noting that serum pancreatic and salivary amylase levels correlate with their output from the respective exocrine glands in CF patients, Gillard et al. recently assessed serum amylase isoenzymes and

confirmed that elevated salivary amylase is a primary factor in CF and not secondary to pancreatic insufficiency. Although heterozygotes demonstrated increased levels of the pancreatic isoenzyme and total amylase, their ranges overlapped with normal controls, rendering measurement of this parameter as a diagnostic marker for the carrier unreliable. (Gillard et al. 1980).

Another aspect of factor research in which abnormal levels or distribution of calcium may play a part arises from observations of the action of the calcium ionophore A23187. It has been shown that A23187 can produce ciliary dyskinesia and goblet cell mucus secretion similar to the action of the CDF in cystic fibrosis serum. (Bogart et al. 1977, 1978). These studies reveal that CF serum requires calcium to produce the dyskinesia effect and chelation of the calcium neutralizes the effect of both CF serum and the ionophore. Since the action of the ionophore is to increase membrane permeability to divalent cations, especially calcium, the authors suggest that the ciliary dyskinesia factor may cause the same change in membrane permeability to calcium resulting in possible loss of intracellular communication and an effect on mucus secretion.

As substantial as the foregoing evidence is for the existence of the CDF in CF secretions, controversy remains and the restriction of the activity to CF serum as well as the objectivity of the cilia assay techniques have been questioned. Using an assay developed in sea mussels, Sanderson and Sleight observed that disruption of the cilia was not particular to CF serum but also occurred with other human and animal serum. (Sanderson and Sleight, 1981). Tegner et al. applying a newly developed standardized method of photoelectric recording of mucociliary activity found no difference in the ciliary movements in rabbit trachea upon exposure to CF sera or control sera or cell culture

medium. (Tegner et al. 1981). With electron microscopy they noted normal ultrastructure and orientation of the cilia for both groups.

Applying isoelectric focusing to cystic fibrosis serum, Wilson in 1979 demonstrated the presence of a particular protein associated with the alpha<sub>2</sub> macroglobulin fraction in homozygotes and heterozygotes with a significant difference between the two groups. (Wilson, 1979). He proposed an abnormality in the alpha<sub>2</sub> macroglobulin produced by persons with the CF gene as the primary defect resulting in abnormal regulation of activity of specific serine or thiol carboxyproteases with consequent increased amounts of cystic fibrosis protein and other biologically active substances in the serum. Although another independent study done at that time confirmed the validity of Wilson's procedure, a more recent evaluation of the procedure does not support his contention that it is a reliable diagnostic marker for detection of heterozygotes. (Tully et al. 1979; Hallinan et al. 1981). The latter study notes that the banding pattern is faint, that heterogeneity among positive samples increases the subjectivity of the scoring procedure, and that there is an increasing percentage of positive results among normals with increased sample volume as well as with different reagents. The authors suggest that differences between normals, heterozygotes, and homozygotes may be in the concentration rather than in distinct levels and thus, detection and assessment by electrophoresis is not appropriate.

In addition to the ciliary dyskinesia factor and the abnormal protein just discussed, another activity of CF serum, saliva, and sweat that has dominated much of cystic fibrosis factor research is that of inhibition of sodium reabsorption. Using retrograde perfusion of rat parotid glands, Mangos demonstrated that saliva of cystic fibrosis patients inhibited sodium transport in the gland. (Mangos et al. 1967). In a subsequent study he assessed the

effect of perfusion with basic polyelectrolytes on the sodium and chloride content of normal saliva. (Mangos and McSherry, 1968). They observed that exposure of the luminal side of the parotid duct to these substances resulted in significant inhibition of sodium reabsorption. Since adding heparin, an anionic molecule, to the preparations abolished the effect, they suggested that the sodium transport inhibitory factor might be a strongly basic macromolecule. The presence and activity of this factor was confirmed again in Taylor's studies that demonstrated the factor in the secretions of the separate salivary glands with the exception of the parotid gland. (Taylor et al. 1974).

Using micropuncture techniques, Kaiser et al. in 1971, demonstrated the transferable nature of the factor by perfusing normal sweat glands with sweat from CF patients and noting that the sodium content of the sweat produced by the normal gland increased. Application of the sweat to the contraluminal side of the gland produced no effect, leading to the conclusion that the sodium transport inhibitory factor was derived from the CF sweat gland and present in that secretion. (Kaiser et al. 1971).

Other studies based on the transport of nutrients across the intestine by the sodium gradient have added mixed support to the inhibitory effect of CF serum and plasma. Brown et al. (1971) reported that CF plasma inhibited the transport of the glucose analogue, arbutin, across rat jejunal tissue in vitro. They observed good separation between CF homozygotes and controls and a two-fold greater inhibition by homozygotes compared to heterozygotes in keeping with the autosomal recessive mode of inheritance. The demonstration of this activity in the heterozygote was considered evidence for a primary rather than a secondary defect in membrane transport in CF. Taussig and Gardner (1972) reported no difference in sodium transport across human erythrocyte membranes after exposure to CF saliva or plasma or in the uptake

of  $^{14}\text{C}$ -alanine or  $^{14}\text{C}$ -3-O methyl glucose by rat jejunum. In contrast, another study observed decreased uptake of amino acids in rat intestine after exposure to CF saliva or serum. (Morin et al. 1973). Reduction of short circuit current across rat jejunal tissue after exposure of the tissue to CF serum has also been observed. (Araki et al. 1975). Like Spock et al. (1967), this latter study was able to demonstrate the effect with serum from heterozygotes after concentration of the serum.

Two other areas of research related to CF factor studies include assessment of RNase isoenzymes and a recently observed lectin-like activity in CF serum. Past research has indicated a variation in salivary and pancreatic RNase values in homozygotes and heterozygotes. (Bardon et al. 1976). Noting the physicochemical similarities of alkaline isoelectric point and low molecular weight, between protein RNase and the postulated CF factor, Thomas et al. measured serum RNase activity in CF patients. RNase activities were increased in both CF patients and heterozygotes compared to controls, but ranges were broad and much overlap of values resulted between the three groups. (Bardon et al. 1976; Bardon and Shugar, 1980; Thomas et al. 1981). Another activity in CF serum recently identified is that of a lectin-like substance that agglutinates mouse erythrocytes. The activity is present in serum from heterozygotes as well. However, technical problems and inconclusive clinical studies make its use as a diagnostic marker controversial at the present time. (Lieberman et al. 1979, 1981).

In light of the work by Mangos with polybasic polyelectrolytes and the cationic nature of the CDF noted by Bowman, who also showed that these substances produced the dyskinesia effect, it is not surprising that studies have been directed toward assessment of polyamines in cystic fibrosis. Further stimulation for these studies originated from the observation that methionine

deficiency is not well tolerated by CF patients and from studies assessing RNA methylation in CF fibroblasts and lymphocytes. (Di Sant'Agnese and Talmo, 1967; Farrell and Lundgren, 1976). An increased spermidine to spermine ratio for both homozygotes and heterozygotes has also been noted, however, another study reported no difference in polyamines for cystic fibrosis patients. (Lundgren et al. 1975; McEvoy and Hartley, 1975). In sugar transport studies in rat enterocytes, one group reported that the inhibition of sugar transport by CF serum was reversed after preincubation of the plasma with diamine oxidase and increased by addition of spermidine. (Arvanitakis et al. 1973). Most recently, Baylin et al. noted that polyamine levels are higher in homozygotes and heterozygotes than in normal controls and don't decrease with age as they do in normal controls. (Baylin et al. 1980).

## PROTEOLYTIC ENZYME STUDIES

Considerable interest in the presence or absence of normal or abnormal proteases originated from the observations of Rao and Nadler (1972). They noted significant reductions in salivary trypsin-like activity of CF patients vs. siblings or parents and between parents and controls, but broad ranges made identification of heterozygotes on this basis unreliable. Further investigation by others demonstrated the absence in CF serum of a protease with arginine esterase specificity along with reduced to absent alpha<sub>2</sub> macroglobulin protease complexes in plasma of patients with cystic fibrosis. (Shapira et al. 1976). These studies suggest that failure of the alpha<sub>2</sub> macroglobulin to form the protease complex leads to the lack of an enzyme with arginine esterase specificity. This precludes modification of the alpha<sub>2</sub> macroglobulin resulting in the circulating polybasic, polypeptide factors postulated by others.

Recent work centered on development of an assay technique designed to detect the deficient plasma protease activity shows considerable promise for the detection of homozygotes and heterozygotes as well as for ante-natal diagnosis. (Rao et al. 1972; Rao and Nadler, 1974, 1975; Rao et al. 1978; Walsh and Nadler, 1979; Walsh-Platt et al. 1978, 1979). The assay is based on the titration of the active site of trypsin-like proteases with 4-methylumbelliferylguanidinobenzoate (MUGB) and their results show reduced MUGB-reactive proteases in skin fibroblasts as well as in plasma of patients with cystic fibrosis vs. obligate heterozygotes or controls. (Walsh-Platt et al. 1977).

## CYCLIC AMP STUDIES

Exocrine gland secretions are under direct nervous and hormonal control, thus, investigation of the coupling of external stimuli from the central nervous system to the production and secretion of glandular products is another area of current research. Since internal cyclic AMP response plays an integral part in transmission of these messages, studies measuring levels of cAMP as well as enzymes associated with that system in various tissues after beta adrenergic stimulation, have formed a basis for this type of investigation. Considering the data implicating abnormal distribution and levels of calcium in the production of the CF secretions, along with the known relationship between calcium and the cyclic AMP response, this avenue of research becomes even more compelling. Additionally, studies in the animal model, the reserpinized rat, that demonstrate development in the animal of pathological changes characteristic of CF after treatment with reserpine and/or isoproterenol, add support to the possibility of an abnormality at some point along the pathway of stimulus-secretion coupling. (Martinez et al. 1975; Wood and Martinez, 1977).


It was realized early that cAMP was implicated in the secretory response. (Robison et al. 1971). Fibroblasts were found to contain the substances involved in cAMP metabolism as well as surface receptors for the cyclic AMP response. (Franklin et al. 1975; Haslam and Goldstein, 1974; Kelly and Butcher, 1974; Manganiello and Breslow, 1974; Rao et al. 1971). Consequently, investigators began to use this tissue to evaluate this response in cystic fibrosis. Initially it was shown that fibroblasts from cystic fibrosis patients secrete the ciliary dyskinesia factor. (Danes and Bearn, 1972). (Beratis et al. 1973; Bowman et al. 1973). In 1976, Buchwald observed two to five times greater levels of internal cAMP in skin fibroblasts of patients with

CF after isoproterenol stimulation. The effect was specific to beta adrenergic agonists as it did not occur following prostaglandin  $E_1$  stimulation. No difference in phosphodiesterase activity between CF and controls was seen and this has been confirmed by others. (Epstein et al. 1978). The suggestion was that if hormonal stimulation caused prolonged elevated levels of cAMP, this might result in abnormal secretory products. (Buchwald, 1976).

In contrast, studies by Davis et al. (1978) in leukocytes from CF patients demonstrated reduced response of cAMP following isoproterenol stimulation. Heterozygote values were intermediate to homozygotes and controls. In basal cAMP levels or in  $PGE_1$  stimulated cAMP, the author observed no differences among the three groups. Davis suggested that since the number of beta receptors on CF cells does not appear reduced, possibly a circulating factor in CF affects desensitization of beta receptors.

Adenylate cyclase activity was also lower in leukocytes from CF patients. (Davis and Hill, 1979). Since they noted the difference in broken cell preparations as well, they ruled out the problem as reduced substrate or cofactor availability in whole cells. They suggested the possibility of faulty coupling between beta receptors and adenylate cyclase. (Davis and Laundon, 1980).

Clearly, the results in tissue cultures with fibroblasts and leukocytes are conflicting and inconclusive. This was initially attributed to differences in technical preparations and experimental conditions for culture. (Davis et al., 1978). Additionally, difficulties were observed in reproducibility of cyclic AMP levels in repeated measurements within strains as well as comparative interpretation of levels between strains whose basal levels differ. (Buchwald and Riordan, 1980).



In a study designed to assess effects of culture conditions on the cAMP response levels in fibroblasts, Davis et al. point out that the maximum stimulation ratios for isoproterenol and PGE<sub>1</sub> differ based on age after subculture and show broad variation between cell lines. (Davis et al., 1980). They also noted that disruption of the cells from the culture medium altered the stimulation ratio for both isoproterenol and PGE<sub>1</sub>. Daily variability in cAMP content of cell lines makes its measurement less than reliable for comparing cell lines. Others have also reported that cell lines cloned from the same culture can demonstrate wide variation in cAMP content and isoproterenol response. (Gilman and Minna, 1973). The author further points out that in tissue culture, more than one cell type may be present in different platings, metabolic status and damage after trypsin treatment vary among cells, and small time differences between subcultures may be a significant variable. On the other hand, a recent study based on isoproterenol or PGE<sub>1</sub> stimulated cAMP response in CF fibroblasts shows no significant differences from control groups regardless of the growth phase of the cells. (Kurz and Perkins, 1981).

Therefore, due to demonstrated inconsistencies in cell culture studies based on cAMP response as well as technical problems in experimental procedure, it appears that the fibroblast system is not reliable for differentiating CF patients and heterozygotes from controls. Leukocytes, however, may present a possible reliable tissue model for evaluating cAMP in CF. Future refinements and standardization in techniques may make reliable assessment possible.

## STUDIES ON MECHANISMS OF ION TRANSPORT

Based on the consistent finding of increased sodium and chloride in sweat and saliva in CF patients as well as the fact that the primary secretory product in the coils of these glands in CF patients is normal in ion content, it seems clear that the normal reabsorption of sodium and chloride along the ducts of these glands does not occur. Flow rate and volume of secretions, both of which influence the ion content of the final product are also normal. (Darling, 1953; Davis and Di Sant'Agnese, 1980; Di Sant'Agnese and Taimo, 1967). Consequently, the possibility of an abnormality in membrane sodium transport mechanisms has been considered as a contributing factor, if not the primary pathology, in cystic fibrosis.

Coincident with the observations of Darling et al. in 1953 and the development of the sweat test in 1959, the development of techniques for the investigation of membrane sodium transport per se, and the characterization of sodium transport systems were underway. Especially notable was the work by I. M. Glynn using the cardiac glycoside ouabain to characterize  $\text{Na}^+$  and  $\text{K}^+$  movements in human red cells. (Glynn, 1957). Subsequent extensive studies described the nature of what is today considered the major mechanism for sodium transport against its gradient across cell membranes the ouabain-sensitive, ATP-dependent  $\text{Na}^+, \text{K}^+$ - Pump. (Garrahan and Glynn, 1967; Post and Jolly, 1957; Post, Merritt et al., 1960).

Little attention was given to ouabain-insensitive sodium movements until 1966 when Hoffman and Kregnow first described the transport of sodium out of the human red cell against its gradient in the presence of ouabain. This process was to that time thought to be accomplished only by the ouabain-sensitive pump. The diuretic, ethacrynic acid, was found to specifically inhibit

this portion of ouabain-insensitive sodium efflux and was subsequently used to operationally define it for the next four years, after which it was replaced with the diuretic furosemide which demonstrated methodological advantages over ethacrynic acid. A number of studies followed elucidating the characteristics of this transport component culminating with definitive studies in the human erythrocyte by Wiley and Cooper describing it as carrier-mediated facilitated diffusion. (Dunn, 1970, 1973; Lubowitz and Whittam, 1969; Sachs, 1971; Wiley and Cooper, 1974). After the description of these different sodium transport components along with the development of the inhibitor techniques for their operational definition in normal cells, these techniques were applied to the assessment of membrane sodium transport in CF cells.

In 1968, Balfe, Cole, and Welt first reported both decreased ouabain-sensitive and ouabain-insensitive sodium transport in erythrocytes from CF patients. They also noted decreased ATPase activity as well as decreased sodium leak from the cells. In obligate heterozygotes, however, the only decrease was in the ouabain-insensitive component, but it compared in magnitude to the decrease in that component in the homozygotes and they suggested it as a marker for the carrier of the CF gene. On the other hand, in 1970, Lapey and Gardner could confirm the decreased ouabain-insensitive sodium efflux only in CF males and post-pubertal females. Additional studies confirmed again the decrease in ouabain-insensitive sodium transport, but not the decrease in the ouabain-sensitive component. (Cole and Dirks, 1972); Grinwald and Segal, 1978). Finally, a recent study reported that the  $\text{Na}^+, \text{K}^+$ -pump in CF fibroblasts shows a decreased affinity for external  $\text{K}^+$  compared to control fibroblasts. No difference was seen in the dependence of the pump on internal  $\text{Na}^+$ . (Reznik et al., 1981).

A number of studies assessed membrane ATPase activities in CF erythrocytes as well as the effects of CF saliva or plasma on erythrocyte membrane ATPase activity in CF and control cells. Besley and Patrick observed no difference in ATPase activity in CF red cells or in the proportion of that activity attributable to either  $Mg^{++}$  ATPase or  $Na^{+},K^{+}$ -ATPase compared to normal controls. (Besley and Patrick, 1969). However, plasma from cystic fibrosis patients inhibited total ATPase,  $Mg^{++}$  ATPase and  $Na^{+},K^{+}$ -ATPase in erythrocyte ghosts from normal controls and to a lesser extent in CF erythrocyte ghosts. In contrast, CF red cells incubated in control plasma demonstrated a reduced loss of the total ATPase activity compared to their loss after incubation in CF plasma. Their data further demonstrated a reversal of the stimulatory effect of magnesium and the inhibitory effect of calcium on the  $Na^{+},K^{+}$ -ATPase. This led them to suggest that the sodium transport inhibitory factor might interfere with the combining sites for these divalent cations. Since the inhibitory effect of the CF plasma persisted through the erythrocyte ghost preparation, it appeared that there was a modification of the membrane by the factor resulting in irreversible loss of enzyme activity. Additional studies have confirmed normal levels of ouabain-sensitive  $Na^{+},K^{+}$ -ATPase in CF red cells. (Cole and Dirks, 1972; Feig et al., 1974; Hadden et al., 1973; Horton et al., 1970; Katz, 1978). (See Table 2)

In a few instances, assessment of  $Mg^{++}$ -ATPase and  $Ca^{++}$ -ATPase have been done as well. Reporting decreased  $Ca^{++}$ -ATPase activity, Horton et al. (1970) observed a correlation between the extent of reduced activity and the clinical severity of disease. In contrast, McEvoy et al. (1974) reported a decreased  $K_m$  for  $Ca^{++}$ -ATPase, but could not demonstrate a correlation with clinical score. More recently, Katz reported a decrease in  $Mg^{++}$ -dependent  $Ca^{++}$ -ATPase. (Katz, 1978). Other workers noted no difference in this

TABLE 2

**SUMMARY OF ION TRANSPORT STUDIES  
IN CYSTIC FIBROSIS**

Study	Na <sup>+</sup> ,K <sup>+</sup> ATPase	Mg <sup>++</sup> ATPase	Ca <sup>++</sup> ATPase	Total ATPase
Balfe, Cole & Welt (1968)	↓ ↓ OI			
Besley & Patrick (1969)	ND	ND	ND	ND
Horton et al. (1970)	ND	ND	↓ observed correlation with clinical score	
Cole & Dirks (1972)	↓ OI OS=ND			
Hadden et al. (1973)	ND		ND	
McEvoy et al. (1974)		ND	Lower Km no correlation with clinical score	
Feig et al. (1974)	ND in OS or OI			
Grinwald & Segal (1978)	↓ OI Na <sup>+</sup> efflux			
Katz (1978)	ND	ND	↓ Mg <sup>++</sup> dependent Ca <sup>++</sup> ATPase	
Will & Taylor (1980)	amiloride sensitive Na <sup>+</sup> transport			
Reznik et al. (1981)	↑ Km for K <sup>+</sup>			

**LEGEND**

ND = no difference  
OS = ouabain sensitive  
OI = ouabain insensitive

enzyme activity in CF erythrocytes. (Besley and Patrick, 1969; Hadden et al., 1973). Although the reports are inconsistent concerning  $\text{Na}^+, \text{K}^+$ -ATPase and  $\text{Ca}^{++}$ -ATPase, there seems to be general agreement that there is no difference in  $\text{Mg}^{++}$ -ATPase activity in CF erythrocytes. (Besley and Patrick, 1969; Horton et al., 1970; Katz, 1978; McEvoy et al., 1974). Other work concerning the effects of CF saliva or plasma on sodium transport has produced inconsistent results. Taussig and Gardner in 1972 found no effect of CF plasma or saliva on erythrocyte sodium transport. Duffy and Shwarz (1972), however, observed a stimulation of sodium transport upon exposure of the red cells to CF secretions. In contrast, Grinwald and Segal (1978) presented evidence that both CF and control saliva increased erythrocyte sodium efflux.

Another recent study of interest identifies an abnormality in ion transport in CF by measurement of the potential difference across the respiratory epithelia. (Knowles et al. 1981). These authors report an increased potential difference across the nasal epithelia of CF patients compared to controls. Application of amiloride, an inhibitor of active sodium transport, results in a greater reduction of the potential difference in the CF patients. They suggest that this reflects increased absorption of sodium and water in CF respiratory passages resulting in dehydration of the secretions. Measurements in heterozygotes were not significantly different from controls. However, the increased potential difference was present in CF infants free of clinically apparent disease and in an older female with borderline sweat chloride. Thus, although the measurement is not useful as a diagnostic marker for the carrier, it may be applicable in diagnosis of CF in infants or older persons for whom sweat tests are sometimes unreliable.

Additional studies report increased resistance of CF fibroblasts to ouabain and dexamethasone. Problems in experimental technique and method-

ology that accompany the cyclic AMP studies in fibroblasts appear to plague these experiments also. Nevertheless, one group has done a considerable amount of work in this area that should be considered. In 1977, Epstein and Breslow demonstrated increased resistance to ouabain by CF fibroblasts cultured in potassium-free media. Although conflicting with previous reports, subsequent studies by the same group reported decreased number of ouabain binding sites on these cells in CF patients. (Breslow et al., 1977; Quissell and Pitot, 1974). Extending their work, Epstein and colleagues reported increased resistance to dexamethasone as well. (Epstein et al., 1977). However, this study has been challenged. (Kurz et al., 1979). Epstein and Breslow defend their position suggesting that failure to duplicate their test conditions is the reason others fail to reproduce their results. (Epstein and Breslow, 1980). They further point out that a blind study using their procedure successfully identified CF patients with 100% accuracy. (Breslow and Epstein, 1980). More recently, these authors report less ouabain-insensitive sodium influx in fibroblasts from CF patients and suggest this may prove valuable as a diagnostic marker for studying relatives of CF patients. (Breslow et al., 1981). Before measurement of either of these parameters in CF fibroblasts can be accepted as a diagnostic marker, however, others maintain that blind studies assessing them both in cell lines not previously tested must be performed. (Erbe, 1981).

### A MEMBRANE ABNORMALITY ?

Much research in all of these areas points to the possibility of a primary membrane disorder as the basic lesion in cystic fibrosis. Work in the early 1970's demonstrated no difference in plasma membrane fractions in CF cells. (Baig et al. 1975; Changus et al. 1975; Fletcher and Lin, 1973). A recent study using CF lymphoblasts again reports no difference in total phospholipids, in the individual phospholipid classes or their fatty acid composition. (Maler and Riordan, 1980). They also report no difference in total membrane lipid, carbohydrate content of the cell surface, fluidity, or the amount of fucose in the intact plasma membrane.

In contrast, abnormal patterns of plasma fatty acids have been reported and correlated with similar changes in erythrocyte membranes. (Campbell et al. 1976; McEvoy, 1975; Rogiers et al. 1980). The latter two studies report decreased linoleic, linolenic, and stearic acids along with increased palmitic, palmitoleic, and oleic acids. Campbell implicates the abnormal lipid pattern in impaired oxygen supply to the tissues. This is attributed to decreased dissociation of oxygen from hemoglobin when oleic acid predominates and to interference with erythrocyte membrane oxygen permeability. McEvoy noted no correlation between the abnormal lipid pattern and clinical severity of disease, but Rogiers reports a correlation with the severity of pulmonary disease. It is still controversial whether the abnormality arises from dietary malabsorption due to pancreatic insufficiency or is a primary defect in fatty acid metabolism. Rogiers did demonstrate the pattern in four cystic fibrosis patients without a pancreatic problem and suggests that both possibilities may be contributory to the pathogenesis of CF. In 1975, Wiley and Cooper demonstrated that cholesterol enrichment of human erythrocyte membranes

resulted in inhibition of the  $\text{Na}^+, \text{K}^+$ -co-transport system. (Wiley and Cooper, 1975). In addition, it has been established that general membrane fluidity influenced by the cholesterol/phospholipid ratio, alters membrane ATPase, hence, interference with ion transport mechanisms may be relative to altered lipids in the membrane. (Cooper, 1977).

## THE THESIS

Although the attempts to elucidate the possible abnormality in sodium transport in cystic fibrosis have produced no definitive results, abnormalities have been identified. One must bear in mind that the majority of the studies done to date were accomplished simultaneously with the development and refinement of methodology for assessing the membrane transport components for sodium. Consequently, it is not surprising that reports were inconsistent, as investigators were only then familiarizing themselves with these techniques as well as with the characteristics of the systems involved.

The high sodium and chloride concentrations in CF sweat and saliva persist as the most obvious clinical marker for diagnosis. So obvious is the abnormality in sodium handling in CF, that until its molecular basis is elucidated, research in this area must continue. The "state of the art" in the past may not have been sufficiently refined to measure the more subtle systems now being delineated. As new methods develop, it is prudent to reassess areas of research that were previously nonproductive, especially if peripheral investigations continue to signal the existence of an abnormality. Such is the case for the reassessment of sodium transport in cystic fibrosis. The recent development by Garay et al. of a new method to measure net sodium and potassium fluxes across the human erythrocyte membrane presented the opportunity for a new approach to the investigation of sodium transport components in cystic fibrosis. (Garay and Meyer, 1979).

This procedure is based on measurement of net sodium efflux and net potassium influx in sodium-loaded, potassium-depleted human erythrocytes, prepared according to the method of Garrahan and Rega (1967). Garay and

co-workers have applied their method to the assessment of these parameters in patients with essential hypertension. Clinical studies demonstrate that these patients manifest decreased net  $\text{Na}^+$  efflux and increased net  $\text{K}^+$  influx with consequent decreased  $\text{Na}^+/\text{K}^+$  net flux ratios. In addition, they have demonstrated this erythrocyte sodium transport abnormality in some normotensive offspring with at least one essential hypertensive parent indicating a genetic basis for the transmission of the abnormality. Further evidence that the abnormality is genetic rather than a secondary consequence of the hypertension is the fact that it is absent in persons with secondary hypertension.

With one major exception, that being the apparent direction of sodium movement, the similarities between identified abnormalities in essential hypertension and cystic fibrosis are intriguing. First, in essential hypertension, patients tend to retain sodium, whereas in cystic fibrosis the patients lose sodium. In both diseases there is evidence for an abnormality in a circulating sodium transport inhibitory factor. (de Wardener and MacGregor, 1980; Kaiser et al. 1971; Mangos et al. 1967). In cystic fibrosis, increased amounts of the factor preclude reabsorption of sodium from exocrine secretions resulting in sodium loss. Contrastingly, in essential hypertension, reduced amounts of the factor permit excessive reabsorption of sodium by the kidney leading to sodium retention. Abnormalities in the levels and distribution of calcium as well as sodium are reported in both diseases and theories relating the two in the pathological progression of the respective diseases have been suggested (Blaustein, 1976, 1977; Sorscher and Breslow, 1982). In both, sodium transport abnormalities have been reported in both the ouabain-sensitive and ouabain-insensitive components of sodium transport. In essential hypertension the ouabain-insensitive sodium transport abnormality appears to

be reflected in the measurement of net sodium efflux according to Garay's procedure, since subsequent investigation of this component in the essential hypertensives and their offspring who manifested low net flux ratios isolated the defect in the ouabain-insensitive,  $\text{Na}^+$ , $\text{K}^+$ -co-transport component. (Dagher and Garay, 1980; Garay, Dagher et al., 1980). This seemingly has more closely defined the sodium transport defect in essential hypertension. Although inconclusive, previous studies in sodium transport in CF have identified abnormal ouabain-insensitive sodium transport. Consequently, it is possible that this procedure might also more clearly define the sodium transport defect in cystic fibrosis.

Based on the foregoing discussion, one can easily see the enticing prospect of applying this procedure to the reassessment of sodium transport in erythrocytes from cystic fibrosis heterozygotes and homozygotes. Consequently, hoping to contribute further to the present knowledge accumulated toward elucidation of the basic biochemical lesion in CF, as well as to the development of a clinical diagnostic marker for the identification of carriers of the CF gene, this study was designed to evaluate the procedure developed by Garay in the clinical setting and subsequently, to apply the established procedure to the investigation of sodium transport in erythrocytes from persons carrying the CF gene.

## PROCEDURE FOR MEASUREMENT OF CATION TRANSPORT IN HUMAN ERYTHROCYTES

### Alteration of Ion Gradients

The procedure developed by Garay and Meyer (1979) assesses net sodium and potassium fluxes in human erythrocytes based on measurement of intracellular sodium and potassium concentrations at hourly intervals. The cells are initially loaded with sodium and depleted of potassium by treatment with parachloromercuribenzenesulfonic acid (PCMBS) using a modification of the procedure developed by Garrahan and Rega (1967). When incubated in a physiological  $\text{Na}^+/\text{K}^+$  Ringers medium, the cells recover their normal ion gradients by moving sodium out of the cells and potassium into the cells via the membrane transport systems for the two ions.

PCMBS has been shown to increase the permeability of the plasma membrane to cations. (Sutherland et al., 1967; Garrahan and Rega, 1967; Will and Hopfer, 1979). The effect is restricted mainly to smaller cations such as  $\text{Na}^+$  and  $\text{K}^+$  and does not affect anion permeability. The increase in permeability can be as much as 100 fold. (Knauf and Rothstein, 1971 I, 1971 II). Increase in permeability to choline is very slight, suggesting that the agent does not open non-specific channels. (Sutherland et al. 1967). The mechanism of action of PCMBS is interaction with membrane sulfhydryl (-SH) groups. Rapid recovery of normal cell membrane permeability is subsequently accomplished by incubation of the PCMBS treated cells with cysteine in the procedure by Garrahan and can be accomplished with other penetrating thiols as well. (Sutherland et al. 1967).

The substance, PCMBS, is an organic mercurial having the high affinity for sulfhydryl groups characteristic of mercury. Early work assessing the

interaction of mercury with human erythrocytes began the determination of the location of sulfhydryl groups affecting cation permeability. (Weed et al., 1962). These authors demonstrated that changes in permeability began when mercury had bound to all of the stromal -SH groups and to approximately 25% of the total cellular -SH groups. Further binding to the remaining 75% of total cell -SH groups resulted in no appreciable increase in permeability. Reaction with glutathione did not occur until nearly 70% of the total -SH groups were occupied. They concluded from this that the mechanism by which mercury increased permeability did not involve reaction with glutathione. Further studies comparing the reactions of the organic mercurials chloromerodrin, parachloromercuribenzoate (PCMB), and PCMBS on glucose transport in erythrocyte membranes demonstrated that PCMBS inhibited glucose transport but did not significantly penetrate the membrane in doing so in contrast to the more lipid soluble PCMB and chloromerodrin. (Van Steveninck et al., 1965). This suggested that the action of PCMBS, at least in this instance, was with surface -SH groups.

Further studies suggested, however, that the PCMBS affected cation permeability only after slow penetration into the membrane and that sulfhydryl groups basic to control of cation permeability were located on the inside of the membrane in an aqueous compartment. (Sutherland et al., 1967; Knauf and Rothstein, 1971 I, 1971 II). These latter authors proposed two ion permeability barriers involving proteins in the plasma membrane, one superficial barrier containing amino groups and controlling anion flow and another internal barrier containing sulfhydryl groups and controlling cation permeability. The study suggested that the affinity of PCMBS for sulfhydryl groups forms the basis for its action on cation permeability. The time course of its effect results from its relative lipid insolubility and the position of cation

controlling -SH groups in an aqueous compartment in the lipid environment of the membrane.

Based on the measurement of erythrocyte  $K^+$  loss after PCMBS treatment, Sutherland et al. in 1967 determined the effect of temperature on PCMBS binding, assessed hemolysis occurring in conjunction with PCMBS treatment, and the effect of PCMBS treatment on cell volume. They demonstrated that there was very little change in cell volume, only a 10% increase, after PCMBS treatment as long as NaCl was present in the media. In contrast, treatment in association with choline rather than NaCl resulted in marked decrease in cell volume leading to the suggestion that the loss of cell  $K^+$  was offset by a greater cell gain of  $Na^+$  during the treatment, resulting in slightly increased cell volume.

Temperature studies demonstrated a different time course of the total reaction as well as differences in the occurrence of hemolysis of cells treated with the same concentration of PCMBS. In cells incubated at 25° C, the rate of  $K^+$  loss rose slowly and reached a rapid constant rate followed by a return to normal rates of  $K^+$  loss after 20 hours. The rate of  $K^+$  loss at a given concentration of PCMBS was reduced as temperature was lowered to 2° C, but the recovery to normal permeability after 20 hours didn't occur as it did at 25° C. Consequently, total  $K^+$  loss was greater at lower temperatures. It was suggested that the recovery phase was due in part to reduction of free PCMBS in the medium over the time period. This was thought caused by increased PCMBS binding substances such as glutathione and hemoglobin, leaking from the cells with progressive hemolysis and then competing for the available free PCMBS. At both temperatures, hemolysis increased directly with PCMBS concentration and, although ultimate hemolysis was greater at lower temperatures, its initiation was delayed under these circumstances. Consequently,

less of the intracellular PCMBS binding substances were released over the 20 hours at 20° C compared to 25° C and competition for free PCMBS was delayed, recovery of normal K<sup>+</sup> loss was delayed and maximum K<sup>+</sup> depletion occurred in 20 hours at 20° C. As a result of these events, by 20 hours, PCMBS binding and K<sup>+</sup> loss leveled off in cells incubated at 25° C, whereas these parameters were still increasing in the cells incubated at 20° C with no reduction in free PCMBS evident for up to 30 hours. Renewal of the PCMBS solution after the recovery period at 20 hours was shown to induce an additional, but lower, response of K<sup>+</sup> loss in cells incubated at 25° C. The increased cation permeability did not appear to be a result of the initial rapid binding of the agent which was a feature common to both temperatures, since K<sup>+</sup> leakage did not occur until 120 minutes after the initial rapid uptake.

Immediate return to normal cation permeability resulted with the addition of cysteine and other penetrating thiols, in contrast to slower recovery effected by addition of sulfhydryl containing proteins. Since cysteine had a more rapid effect than the protein and unlike protein, can freely penetrate the erythrocyte membrane, they concluded that the permeability sites were located on the inside of a diffusion barrier and were, therefore, more accessible to the cysteine than the protein. This added further support to the theory that the sulfhydryl groups involved in control of cation permeability were not on the outer surface of the membrane.

The preceding discussion makes the basis for the cation loading procedure developed by Garrahan and Rega quite clear. Addition of the PCMBS to a suspension of erythrocytes in a 150 mM NaCl medium results in potassium loss and sodium gain with minimal change in cell volume. The results are maximal at 20 hours using low temperatures, in this case 4° C. Rapid reversal of the PCMBS action is affected by removal of the loading

medium from the cells followed by addition of a sealing medium containing cysteine. The final product is a population of sodium loaded, potassium depleted erythrocytes with restored normal membrane cation permeability.

It is well established that mammalian cells maintain ion gradients across the cell membrane with respect to sodium and potassium. Extracellular sodium concentration is higher than intracellular sodium and intracellular potassium is higher than extracellular potassium. Membrane transport processes are responsible for the maintenance of these gradients. Diffusion of the ions down their electro-chemical gradients would result in dissipation of the gradients were this not offset by active transport of the ions against their gradients primarily by the ATP-dependent  $\text{Na}^+$ ,  $\text{K}^+$ - pump.

If membrane permeability is increased concomitantly with inhibition of cation pumping, intracellular sodium will increase and potassium will decrease as the downhill diffusion of the ions predominates. Since PCMBs causes an increase in cation permeability as well as inhibition of the  $\text{Na}^+$ ,  $\text{K}^+$ - ATPase, cells treated with this agent reverse their normal intracellular concentrations of sodium and potassium. (Skou and Hilberg, 1965; Fahn et al., 1966).

Providing subsequent treatment of the cells results in recovery of competent membrane transport systems and normal permeability, once reconstructed and exposed to a physiological extracellular environment, the cells recover their normal ion gradients via the membrane transport systems. Assessment of abnormalities in membrane transport systems might logically be conducted by monitoring the progression of gradient recovery as reflected in ion movements. Although precise determination of rate constants requires careful maintenance of steady state conditions and isotopic flux determinations, if the magnitude of ion movements is large enough over a reasonable time period, non-steady state net movements of ions can be measured and used

to estimate rates reasonably well. This is accomplished, for example, by measuring total change in intracellular sodium and potassium concentration at hourly intervals as the cells recapture their sodium and potassium gradients under optimal physiological conditions. The latter method forms the basis for the net flux procedure developed by Garay and is the method applied in this study. (Garay and Meyer, 1979).

Reversal of the sodium and potassium gradients across the cell membrane by PCMBs allows the membrane transport systems to be studied at maximal velocities. Net movements reflect the sum of bidirectional fluxes of the ions. In the steady state these bidirectional fluxes are geared to maintain the ions at a constant concentration in the cell. Hence, methods designed to detect net movements based on timed measurements of changes in intracellular ion concentrations are not applicable. Under stressed conditions, however, the transport systems' efforts to move the ions in one direction to recover the proper gradients will overshadow opposing movements. The net movement of each ion in one direction will then be large enough to be measured by timed changes in intracellular ion concentration. Thus, the use of isotopic determinations can be replaced by flame photometry. This results in a procedure that is less technically demanding as well as less expensive and thus more appropriate to the clinical setting as well as to possible application in screening procedures. In addition, the collection of 20-30 ml of heparinized venous blood, as is required for obtaining the erythrocytes, is minimally invasive and low risk to the subject as well as technically simple, affording another feature attractive to the clinical setting.

## Erythrocyte Membrane Sodium and Potassium Transport - The Use of Inhibitors

**Ouabain and the Na<sup>+</sup>, K<sup>+</sup>-Pump.** In 1956, Glynn presented evidence to support the coupled active transport of sodium and potassium across the human erythrocyte membrane. (Glynn, 1956). Increasing external potassium resulted in an increase in both potassium influx and sodium efflux. Subsequent removal of glucose from the medium reduced both Na<sup>+</sup> efflux and K<sup>+</sup> influx by the same amount. The residual sodium efflux that was observed in K<sup>+</sup>-free media was not glucose dependent and increasing external potassium had no effect on Na<sup>+</sup> efflux in the absence of glucose. More convincing evidence to support active coupled transport of sodium and potassium was presented by Post and Jolly (1957). Their studies demonstrated that the system required the presence of external potassium and internal sodium for activation. In addition, they presented evidence that the coupling ratio involved the efflux of three sodium ions in exchange for the influx of two potassium ions.

Based on earlier studies that demonstrated an inhibitory effect of cardiac glycosides on the potassium influx and sodium efflux in glucose repleted cold-stored human red cells, Glynn presented a study designed to clarify the mechanism of the ion transport inhibitory action of these substances in human erythrocytes. (Glynn, 1957). His study suggested that the inhibitory effects were due to direct action by the glycoside on the carrier rather than by interference with an energy supply, since the glycosides were still effective at reducing glucose-independent sodium efflux that occurred into K<sup>+</sup>-free media. This latter component of sodium efflux was subsequently shown to be Na<sup>+</sup>-Na<sup>+</sup> exchange accomplished by the Na<sup>+</sup>, K<sup>+</sup>-pump in K<sup>+</sup>-free media. (Garrahan and Glynn, 1967 a, b, c). The premise that glycoside-inhibited sodium and potassium movements represented transport of sodium

and potassium by this active transport system formed the basis of subsequent ion transport studies designed to elucidate the characteristics of this transport system.

Further work by Post and colleagues established a direct relationship between an enzyme, ATPase, isolated from erythrocyte membranes and the active transport system then referred to as the  $\text{Na}^+$ ,  $\text{K}^+$ -pump. (Post et al. 1960). Both the enzyme and the transport system were located in the cell membrane, required ATP, and were dependent on the simultaneous presence of sodium and potassium for activity. The half-maximal concentrations of sodium and potassium required for activation of the enzyme and the  $\text{Na}^+$ ,  $\text{K}^+$ -pump were comparable and the concentration of the glycoside ouabain required for half-maximal inhibition of the enzyme and the  $\text{Na}^+$ ,  $\text{K}^+$ -pump were also similar.

An extensive study by Garrahan and Glynn (1967) presented evidence supporting a  $\text{Na}^+$ - $\text{Na}^+$  exchange attributable to the  $\text{Na}^+$ ,  $\text{K}^+$ -pump transport protein in  $\text{K}^+$ -free media (Garrahan and Glynn, 1967a). This exchange was ouabain sensitive and did not occur in the absence of ATP. As external potassium increased,  $\text{Na}^+$ - $\text{Na}^+$  exchange progressively decreased and  $\text{Na}^+$ - $\text{K}^+$  exchange increased. (Garrahan and Glynn, 1967c). Garrahan and Glynn also verified the  $\text{Na}^+:\text{K}^+$  3:2 coupling ratio for the pump transport of these ions against their gradients and demonstrated a third mode of action for this transport protein, that of reversal with formation of ATP as opposed to hydrolysis of ATP that occurred in the forward mode. (Garrahan and Glynn, 1967d, 1967e). A more recent review by Glynn and Karlish (1975) lists five modes of action accomplished by the ouabain-sensitive  $\text{Na}^+$ ,  $\text{K}^+$ -pump. These include  $\text{Na}^+$ - $\text{K}^+$  exchange, a reversed mode,  $\text{Na}^+$ - $\text{Na}^+$  exchange,  $\text{K}^+$ - $\text{K}^+$  exchange and unaccompanied  $\text{Na}^+$  efflux.

**Furosemide and the Na<sup>+</sup>, K<sup>+</sup>-Co-Transport System.** The previously described studies have convincingly demonstrated the inhibitory specificity of the cardiac glycoside ouabain for membrane transport of sodium and potassium by the Na<sup>+</sup>, K<sup>+</sup>-pump. Therefore, the use of ion transport inhibitors to operationally define membrane transport systems in erythrocytes is well established. Traditionally, the ouabain insensitive movements of these ions have been summarily attributed to passive diffusion or "leak" down their concentration gradients. However, research over the last decade has shown this concept to be invalid. A recent article lists five additional transport pathways for these two ions in addition to the Na<sup>+</sup>, K<sup>+</sup>-pump and the diffusion component. (Tosteson, 1981). Each of these five pathways is active in the presence of ouabain and is defined by its own specific inhibitor.

The ouabain-insensitive, furosemide-sensitive Na<sup>+</sup>, K<sup>+</sup>-co-transport system is of particular interest to sodium transport research in cystic fibrosis. Abnormalities in membrane transport of sodium and chloride are characteristic of this disease as evidenced by the consistently high sodium and chloride concentrations in the exocrine secretions from affected persons. Several recent studies demonstrate a tight relationship between the ouabain-insensitive, furosemide-sensitive transport of sodium and potassium and movements of chloride. (Chipperfield, 1980; Geck et al. 1978, 1980; Schmidt and McManus, 1977 I, 1977 III). Taken with the fact that past research in sodium transport in cystic fibrosis has documented differences between normal controls, cystic fibrosis patients, and cystic fibrosis heterozygotes in the ouabain insensitive component of sodium transport, investigation of this co-transport system in cystic fibrosis is indicated. (See page 23, Table 2).

Although not identified as such at the time, the initial studies of this transport component began with the work of Hoffman and Kregnow in 1966.

They used inhibition by the diuretic ethacrynic acid to define a portion of sodium efflux in human erythrocytes that transported sodium against its concentration gradient in the presence of ouabain. Up to that time, the ouabain-sensitive  $\text{Na}^+$ ,  $\text{K}^+$ -pump was the only transport system credited with the ability to move sodium and potassium against their gradients.

Later research in human erythrocytes centering on this transport component demonstrated that furosemide, a sulfonamide diuretic, inhibited the same portion of ouabain-insensitive sodium efflux as did ethacrynic acid. (Dunn, 1970, 1973). Furosemide displayed methodological advantages in that it did not cause as much hemolysis of the cells nor as much membrane leakage as did ethacrynic acid implying less membrane damage. Consequently, furosemide has been used as the selective inhibitor to characterize this transport component in the majority of studies done in the last decade. (Sachs, 1971; Schmidt and McManus, 1977 I-III; Wiley and Cooper, 1974).

From these and other studies, the concept has emerged of a transport system for sodium and potassium defined as carrier-mediated facilitated diffusion. (Wiley and Cooper, 1974). The system involves the coupled transport of the two ions in the same direction across the membrane, each facilitating the movement of the other. Consequently, the downhill movement of one ion appears coupled to the uphill movement of the other against its gradient. Additionally, the downhill movement of one ion appears facilitated by the presence of the co-ion. The system does not require hydrolysis of ATP to accomplish the ion movements. (Hoffman and Kregnow, 1966; Dunn, 1973). This is to be contrasted with the exchange of internal sodium for external potassium by the ATP dependent  $\text{Na}^+$ ,  $\text{K}^+$ -pump.

Assessing the ouabain-insensitive, furosemide sensitive influx of sodium and potassium in human erythrocytes, Wiley and Cooper (1974) demonstrated

that the influx of each ion was a saturable function of its external concentration in the presence of the co-ion. Work in avian erythrocytes confirmed these characteristics of the co-transport system and implicated the system in the physiological role of cell volume control. (Kregnow, 1971, 1973; Schmidt and McManus, 1977 I-III). These studies in avian red cells further demonstrated that the volume response, and therefore, this transport system, was responsive to beta adrenergic stimulation with norepinephrine and to cyclic AMP stimulation as well as to changes in the osmolarity of the external media. (Kregnow et al. 1976). This latter characteristic might help to relate abnormalities in stimulation-secretion coupling via cyclic-AMP in CF tissues and abnormalities in membrane transport systems in CF tissues that respond to hormonal stimulation, such as the cells in exocrine glands.

Although the definitive studies of this transport component apply furosemide as the selective inhibitor, the precise target of its inhibitory action remains controversial. As a diuretic, its primary target in the kidney is the inhibition of active chloride transport across the luminal membrane of tubular epithelial cells in the thick ascending Loop of Henle, with secondary inhibition of sodium transport. (Burg et al. 1973; Burg and Stoner, 1976). Increased sodium and chloride in the tubule provide osmotically active solute that precludes reabsorption of water from the tubule in the distal nephron with subsequent diuresis.

Several studies in human erythrocytes as well as other tissues have also shown a relationship between furosemide inhibition of sodium transport and chloride transmembrane movements. (Geck et al. 1978; Nicoll, 1978; Reinach et al. 1977). Furosemide inhibits chloride-chloride self-exchange across the erythrocyte membrane. (Brazy and Gunn, 1976). Other recent work suggests that chloride must be present in the external medium to demonstrate

furosemide inhibition of the  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport system in erythrocytes. (Chipperfield, 1980; Geck et al. 1980; Schmidt and McManus, 1977 III). Evidence also suggests that furosemide inhibits chloride-dependent passive potassium transport in human erythrocytes. A  $\text{K}^+$ ,  $\text{Cl}^-$ -co-transport system involved in the regulation of cell volume has been proposed. (Dunham et al. 1980). This latter observation is compatible with the avian red cell studies that implicate the system in cell volume control and demonstrate a linear relationship between the ouabain-insensitive movements of sodium, potassium, and chloride and the movement of water into and out of the cell. (Schmidt and McManus, 1977 I). These authors suggest, however, that the action of furosemide on the volume response in avian red cells is not specific and it is unclear which flux components it actually inhibits. Finally, in a number of instances, furosemide has been used as a research tool based on its ability to inhibit anion movements. (Lucci and Warnock, 1979; Nicoll, 1978; Suki et al. 1980).

Interference with either the production of energy or the delivery of energy to the transport proteins has also been suggested as a basis of furosemide inhibition. Hook and Williamson (1965) reported furosemide inhibition of the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in mammalian kidney. Others observed differences in its effect on the  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase in medullary as opposed to papillary renal tissue. (Czackes, 1977). In a study comparing various effects of ethacrynic acid and furosemide in the stria vascularis, no significant furosemide inhibition of the  $\text{Na}^+$ ,  $\text{K}^+$  ATPase was observed. (Kusakari et al. 1978). In human erythrocytes, Dunn demonstrated that furosemide inhibited the ATPase activity at higher concentrations. (Dunn, 1973). At concentrations insufficient to cause ATPase inhibition, however, the drug still inhibited the ouabain insensitive co-transport sodium efflux component. Furo-

semide has been shown to inhibit oxygen consumption produced by adding glucose or pyruvate to kidney slices. (Klahr et al. 1971). Other studies as well have demonstrated inhibition of oxygen consumption and glycolysis. (Cunarro, 1978; Eveloff et al. 1981; Klahr et al. 1973; Reinach et al. 1977).

Interference with the adenylate cyclase-cyclic AMP messenger system is another mode of inhibitory action suggested for furosemide. (Kusakari et al. 1978). These authors point out that in several systems cAMP facilitates water and electrolyte transport. Furosemide has been shown to displace cAMP from its binding sites in a manner that correlates with changes in water and electrolyte transport. (Ferguson, 1966; Ferguson and Twite, 1974, 1975). Thus, perturbation of the second messenger system might reflect furosemide action.

This latter suggestion is particularly interesting in relation to sodium transport in cystic fibrosis. Abnormalities in electrolyte transport in cystic fibrosis are well established and, although inconclusive at present, abnormalities in cAMP response in cystic fibrosis tissues are also reported. (See page 17). As previously mentioned, the ouabain-insensitive, furosemide-sensitive  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport system responds to cyclic AMP. (Kregnow, 1977). Both the sodium transport inhibitory factor found in cystic fibrosis saliva and serum and furosemide are effective only on the luminal side of the epithelial cell membrane. (Burg and Stoner, 1976; Grantham et al. 1978; Kaiser et al. 1971; Lassiter, 1975; Will et al. 1980). Finally, one study demonstrated that furosemide affected chloride movements in both stimulated and unstimulated salivary flow and inhibited a part of the secretory process without abolishing secretion. (Crook et al. 1976). In cystic fibrosis, the final secretory products are abnormal in ion content, but secretion itself continues in salivary and sweat glands. Considered along with the abnormal calcium

concentration in some cystic fibrosis secretions and the relationship between calcium and the second messenger function of cAMP, this possible mode of furosemide action presents as a possible "tool" for evaluating these abnormalities in cystic fibrosis.

In the final analysis, the precise target of furosemide inhibition of sodium transport in the human erythrocyte remains uncertain. It is quite conceivable that its action is multifarious. It has been shown to inhibit 10-15% of the active fluxes of sodium and potassium occurring in the absence of ouabain. (Wiley and Cooper, 1974). These authors further demonstrated that furosemide inhibited a greater percentage, approximately 50%, of sodium efflux that occurred in the presence of ouabain. Sachs (1971) demonstrated that furosemide inhibited more sodium efflux in human red cells in potassium free media without ouabain than with ouabain. Hence, it appears to inhibit the  $\text{Na}^+$ - $\text{Na}^+$  exchange mode mediated by the pump in  $\text{K}^+$ -free media as well as to inhibit non-pump mediated sodium efflux. Thus, furosemide apparently affects both active and ouabain-insensitive sodium transport components with predominant effects on the ouabain-insensitive component.

Despite the controversy over the exact mode of inhibition by furosemide of ouabain-insensitive sodium and potassium movements in human erythrocytes, furosemide remains a proven inhibitor of the ouabain-insensitive saturable flux components of these two ions defined as the  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport system. Consequently, it is applied to that purpose in the procedure developed by Garay for assessment of the  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport system. (Garay et al. 1980; Dagher and Garay, 1980).

## MATERIALS AND METHODS

### Preparation and Incubation of Erythrocytes

**Specimen collection and processing.** Venous blood was collected in heparinized tubes and centrifuged for 10 minutes at 600xg. (Damon IEC Centrifuge, model HN-S, Damon/IEC Division, Needham Hts, Mass.). The plasma and buffy coat were removed by aspiration and the cells were subsequently washed 4 times with 10 volumes of 150mM NaCl. After removal of the last wash by aspiration, the cells were packed by centrifugation for an additional 10 minutes at 600xg and the remaining supernatant was removed. These procedures were done at room temperature.

**Cation loading phase.** Sodium loading and potassium depletion of the red blood cells (RBCs) were accomplished using the procedure of Garrahan and Rega, (1967) based on alteration of red cell membrane permeability by PCMBS with subsequent reversal of increased permeability using cysteine. Thus, following the final packing of the RBCs, a sodium loading medium was added to the cells to achieve a 5% hematocrit. The loading medium contained: (mM) PCMBS 0.1, NaCl 150, MgCl<sub>2</sub> 1, TRIS 5(pH 7.4, 25°C). The suspension was incubated at 4° C for 20 hours.

**Membrane sealing phase.** Following the 20 hour loading incubation, the suspension was centrifuged at room temperature for 10 minutes at 600xg and the supernatant was removed by aspiration. A sealing medium was immediately added to the cells adjusting the suspension to a hematocrit of approximately 10%. The suspension was incubated for 30 minutes with agitation in a 37° C water bath. The sealing medium contained: (mM) NaCl 150, MgCl<sub>2</sub> 1, TRIS 5(pH 7.4, 25°C), Glucose 11, and cysteine 2.

**Preparation for recovery phase.** Following the sealing incubation, the suspension was centrifuged for 10 minutes at 600xg and the supernatant was removed by aspiration. The cells were immediately washed 3 times with 10 volumes of cold choline chloride (4°C) (150mM crystalline choline chloride in 5mM TRIS, pH 7.4, 25°C). After removal of the final supernatant, 3-4 drops of the packed cells were removed to a small Eppendorf centrifuge tube, resuspended with 5-6 drops of cold choline chloride to achieve a hematocrit of about 20-25% and set aside for subsequent measurement of hematocrit and total intracellular sodium and potassium.

**Recovery phase.** The remainder of the cells were subjected to a four hour recovery incubation in a Na/K Ringers medium which contained: (mM) NaCl 145, KCl 5, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 2, Glucose 10, TRIS 2.5. This solution and the loading and sealing media were adjusted to pH 7.4, 25°C prior to addition to the cells. Into appropriately labeled 25ml Erlenmeyer flasks, 0.5 ml of cells were pipetted, followed by the addition of 9.5 ml of Na/K Ringers media to achieve a fluxing hematocrit of 5%. The cell suspensions were then immediately placed in the 37°C water bath and incubated with continuous agitation for four hours.

**Processing of Hourly Samples.** Each hour a 2.0ml aliquot was removed from each flask, placed in an Eppendorf 3200 centrifuge (Eppendorf Geratebau Netheler Hinz GMBH, Germany) tube and centrifuged at 12,000xg for 1 minute. The supernatant was removed by aspiration and the cells were resuspended in ice cold 150mM choline chloride and centrifuged at 12,000xg for 30 seconds. This washing was done three times. Upon aspiration of the last wash, the cells were resuspended in cold choline chloride and set aside for measurement of hematocrit and intracellular sodium and potassium.

**Preparation and Use of Inhibitors.** Ouabain and furosemide were used in some experiments to define the sodium and potassium movements attributable to the  $\text{Na}^+, \text{K}^+$ -pump and the  $\text{Na}^+, \text{K}^+$ -co-transport system. Stock furosemide was prepared in 0.1M  $\text{Na}_2\text{HPO}_4$  (pH 7.4, 25°C) at a concentration of 20mM. Stock ouabain was dissolved in the Na/K Ringers media at a final concentration of  $1.4 \times 10^{-1}$ mM. These stock solutions were prepared fresh on the day of use. When inhibitors were used, two additional flasks, each with 0.5 ml of the same subject's cells were prepared. Flask No. 1 contained no inhibitors, flask No. 2 received 0.1 ml of stock ouabain, and flask No. 3 received 0.5 ml of stock furosemide. Flasks No. 1 and No. 2 each received 0.5 ml of 0.1 M  $\text{Na}_2\text{HPO}_4$  (pH 7.4, 25°C) to control for that used to dissolve the furosemide. The final volume of the fluxing suspension was maintained at 10 ml. Consequently, the volume of Na/K Ringers added to each flask was adjusted to account for the above additions. The final concentration of ouabain was  $1.4 \times 10^{-3}$ mM and the final concentration of furosemide was 1 mM.

**Sample Measurements.** Packed cell volumes were measured using standard micro hematocrit procedure. Intracellular sodium and potassium measurements were made using a Corning 450 Flame Photometer (Corning Scientific Instruments, Medfield, Massachusetts). Dilutions for the photometer measurements were made using a Fisher automatic dilutor (Fisher Dilumat, Fisher Scientific Ltd., Ottawa, Ontario) with 15 meq lithium/liter diluent. A 1:100 dilution of a measured amount of the well mixed cell suspension resulted in lysis of the cells for measurement on the flame photometer. All measurements were done in duplicate.

Values for intracellular sodium and potassium were calculated by dividing photometer readings for each sample by the respective hematocrit

determination for that sample. Thus, values for each hour were recorded in milliequivalents per liter of cells.

**Sources of Special Reagents.** PCMBS (p-chloromercuribenzenesulfonic acid, monosodium salt), L-cysteine hydrochloride monohydrate, ouabain octahydrate (strophanthin-G), adenine (6-aminopurine) hydrochloride, inosine, and crystalline choline chloride were purchased from Sigma Chemical Company, St. Louis, Missouri. Furosemide was a gift from Hoechst, Canada Inc., Montreal, Quebec. All other salts were reagent grade.

**Preparation of Standards for Flame Photometry.** Standards were prepared from a commercial standard manufactured by Beckman Instruments, Inc., Fullerton, California. The standard was an aqueous sodium and potassium standard assayed at 100mM Na<sup>+</sup>/liter and 100mM K<sup>+</sup>/liter. Additional standards were prepared from the commercial standard by dilution with deionized water to read in the range of values expected for the samples. These standards were prepared fresh for each run of the procedure. Standards of 5 meq/l, 10 meq/l and 25 meq/l for both sodium and potassium were adequate to cover the range of readings for the samples.

**Test Subjects.** Controls were human volunteers taken from the laboratory and medical staff personnel of the Children's Hospital of Eastern Ontario. Cystic fibrosis heterozygotes were volunteer natural parents of patients attending the Cystic Fibrosis Clinic at the Children's Hospital. The project was reviewed by the Research Committee of the Children's Hospital of Eastern Ontario and judged ethically acceptable. All subjects gave written consent to participation in the program. (Figure 1).

**Statistical Analysis of Results.** The five values (hours 0-4) for intracellular sodium and potassium concentration in milliequivalents per liter of cells were evaluated using linear regression analysis. The slopes of the

FIGURE 1

CHILDREN'S HOSPITAL OF EASTERN ONTARIO

CONSENT TO SPECIAL

PROCEDURE

I hereby authorize Dr. \_\_\_\_\_ ("the doctor") or such other assistants as he may select to perform the following procedure.

(1) Removal of 20 ml of venous blood. This involves putting a needle in a vein and removing the blood. I understand that the sample is to be used in a study concerning cystic fibrosis and will be of no direct benefit. I also understand that any information will be kept confidential and published only without identifying me.

(2) Age

(3) Sex

(4) Family History (a) High blood pressure

(b) Cystic fibrosis

(5) Medications

Date: \_\_\_\_\_

Signature: \_\_\_\_\_

Relation to patient: \_\_\_\_\_

resulting lines were taken as the rate of efflux of sodium or influx of potassium in milliequivalents per liter of cells per hour. (Figure 2). Controls and cystic fibrosis heterozygotes were compared by applying the Student t-test to the sodium efflux and potassium influx rates, as determined above, for the two groups.

**Procedural Investigations.** Additional experiments were performed to investigate possible effects of alterations in methodology designed to achieve less cell membrane damage, better recovery of sodium transport components, and better consistency in results. Erythrocytes from two single sources were incubated in loading media of the same composition as before but with varying concentrations of PCMBs. In addition, each single loading suspension was sampled at 20, 22, 24, and 26 hours. Samples were removed from the loading suspension, centrifuged, and the supernatants were removed and saved for estimation of hemolysis. The cells were then processed through the sealing step following which they were washed three times in cold 150mM choline chloride as before and resuspended in choline chloride to a hematocrit of 20-25%. Hematocrit determinations and intracellular sodium and potassium measurements were done as previously described. Hemolysis in the loading media supernatants was estimated by measuring absorbance at 540nm against the loading medium blank using a Coulter Hemoglobinometer. (Coulter Electronics, Hialeah, Florida).

Sample value consistency over the holding period was evaluated by making measurements of intracellular sodium and potassium immediately after sample processing and then again at the end of the four hour holding period. This was done for the three concentrations of PCMBs as well as on an individual control and CF heterozygote in a paired experiment.

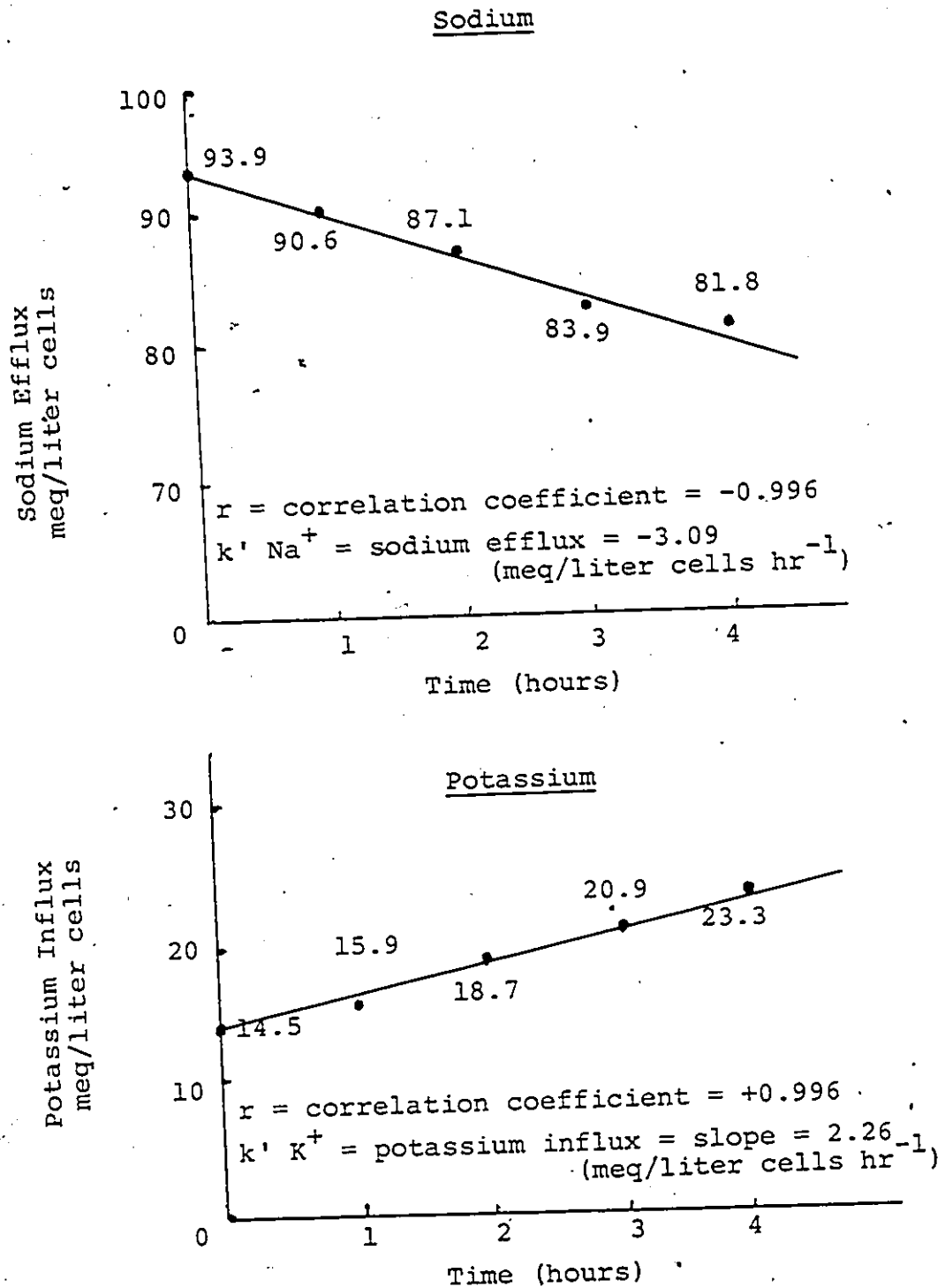
FIGURE 2

LINEAR REGRESSION ANALYSIS OF  
SODIUM EFFLUX AND POTASSIUM INFLUX  
OVER THE FOUR HOUR INCUBATION PERIOD

Values for intracellular sodium and potassium concentration in meq/liter of cells determined at hourly intervals during the recovery phase are plotted against time. Linear regression analysis of the concentration values results in determination of a correlation coefficient ( $r$ ) which indicates the linearity of the values with time. The slope of the line represents either net sodium efflux ( $k' \text{ Na}^+$ ) in which case the slope is negative, or net potassium influx ( $k' \text{ K}^+$ ), in which case the slope is positive. Values for net fluxes are in meq/liter cells  $\text{hr}^{-1}$ .

FIGURE 2

LINEAR REGRESSION ANALYSIS OF  
SODIUM EFFLUX AND POTASSIUM INFLUX  
OVER THE FOUR HOUR INCUBATION PERIOD



As repair of cell membranes progresses in the sealing phase, the possibility exists that transport systems might begin recovery of the gradients, especially since the substrate glucose is present in this medium. To investigate the possibility of this occurrence, a sealing phase was monitored. At ten minute intervals, 2ml aliquots were removed from a suspension of erythrocytes in the sealing medium. The cells were centrifuged at 12,000g for one minute in an Eppendorf 3200 centrifuge, washed three times in 150mM choline chloride as before and resuspended in the choline chloride to a hematocrit of 20%-25%. Ion movements were assessed by measurement of the intracellular sodium and potassium concentrations of each 10 minute sample using flame photometry as previously described.

## RESULTS

### Establishing the Procedure

Initially to assess the linearity of the net sodium efflux and net potassium influx reaction over the four hour incubation period, a number of net flux procedures were done using, in each case, samples of heparinized venous blood pooled from several donors. In these experiments the concentration of PCMBs in the loading phase was 0.1mM and the concentration of cysteine in the sealing phase was 2.0mM. No inhibitors were used in these tests. The cells were loaded, sealed, and incubated for ion gradient recovery as described in the Materials and Methods section (page 45). Results of net sodium and potassium fluxes based on these studies demonstrated good linearity over a four hour incubation period. Evaluated using linear regression analysis, the correlation coefficients for sodium efflux were, in most cases, 0.90 or greater and those for potassium influx were 0.95 or greater. This agreed with results reported by Garay and Meyer, (1979). The slopes of the lines, thus evaluated, were taken as representative of the net rate of sodium efflux or potassium influx. A negative slope indicated efflux or net loss of an ion species from the cells and a positive slope indicated influx or net gain of an ion species by the cells. It was expected that slopes for correlation of sodium values would be negative indicating sodium efflux from the cells and that slopes for potassium would be positive indicating potassium influx. Consequently, when occasional slopes for sodium were positive and those for potassium were negative, the results were interpreted as zero loss of sodium or zero gain of potassium. When correlation coefficients fell below 0.90 it was

clear that one or more points affected the linear correlation disproportionately and arbitrary elimination of one point or another could yield significantly different results. Therefore, based on these initial studies, when evaluating total fluxes and fluxes in the presence of inhibitors only tests with net sodium efflux correlation coefficients of 0.90 or greater in the absence of inhibitors were accepted.

### Series I

#### Net Flux (results using specimens from single donors)

In Series I, erythrocytes from single donors were incubated in a loading medium containing 0.1mM PCMBS and were resealed in a sealing medium containing 2.0mM cysteine. The recovery phase was accomplished in a  $\text{Na}^+/\text{K}^+$  Ringers medium. In these experiments, the inhibitors ouabain and furosemide were added to identical but separate cell suspensions from a single donor at the same time. Thus, recovery of the ion gradients by the cells of each donor was measured in the absence of inhibitors, in the presence of ouabain alone, and in the presence of furosemide alone.

**Values for net fluxes in the absence of inhibitors.** Values for net sodium efflux covered a wide range. For 20 determinations values varied from 1.47 to 5.51 meq/l cells  $\text{hr}^{-1}$  with a mean of  $3.30 \pm 1.04$  ( $\pm$  S.D.). In the case of net potassium influx values showed less variation. For 20 determinations the range was 0.95 to 2.50 meq/l cells  $\text{hr}^{-1}$  with a mean of  $1.66 \pm 0.52$ . (Table 3). These mean values are comparable to those reported by Garay in 1979.

**Values for net fluxes in the presence of ouabain or furosemide.** Ouabain in the concentration used ( $1.4 \times 10^{-3}$ mM) reduced sodium efflux to negligible levels and abolished potassium influx. In previous studies ouabain and furosemide in combination were used to evaluate ouabain-insensitive, furo-

TABLE 3

NET SODIUM EFFLUX AND POTASSIUM INFLUX  
WITH AND WITHOUT FUROSEMIDE FOR 20 CONTROL TESTS

Erythrocytes from 20 control subjects were incubated in a loading medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM TRIS and 0.1mM PCMBs. They were sealed in a medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM TRIS, 11mM glucose, and 2mM cysteine. Recovery of the gradients occurred in a Na<sup>+</sup>/K<sup>+</sup> Ringers medium containing 145mM NaCl, 1mM MgCl<sub>2</sub>, 2.5mM TRIS, 5mM KCl, 2mM CaCl<sub>2</sub> and 10mM glucose. Recovery occurred in the absence of furosemide or in the presence of furosemide. Statistical analysis was by Student's t-test.

TABLE 3

**NET SODIUM EFFLUX AND POTASSIUM INFLUX  
WITH AND WITHOUT FUROSEMIDE FOR 20 CONTROL TESTS**

	Sodium Efflux (meq/liter cells/hour)		Potassium Influx (meq/liter cells/hour)	
	Total	With Furosemide	Total	With Furosemide
1	-2.82*	-1.43	0.95	0.53
2	-3.43	-2.36	2.44	1.48
3	-1.94	-1.11	2.02	0.98
4	-1.47	-0.81	1.29	0.69
5	-4.60	-2.31	2.50	1.70
6	-3.63	-0.78	1.84	0.57
7	-2.61	+0.94	1.28	-0.02
8	-2.49	-1.31	1.52	0.53
9	-3.57	-1.93	1.67	1.23
10	-2.43	-1.19	1.59	0.88
11	-3.86	+0.16	0.97	0.79
12	-5.01	-2.93	2.08	1.41
13	-2.23	-2.10	1.14	0.86
14	-2.94	-1.47	1.89	1.59
15	-3.01	-1.99	1.94	1.11
16	-3.69	-0.44	0.98	0.66
17	-5.51	-1.58	2.23	1.42
18	-3.22	-2.12	1.05	0.86
19	-4.56	-4.52	2.42	1.14
20	-3.00	-1.73	1.32	1.08
$\bar{x}$	3.30	1.61	1.66	0.98
S.D.	1.04	1.04	0.52	0.43

\* (-) Indicates negative slope or loss of sodium (efflux) from the cells

Values are slopes of the lines via linear regression analysis of samples  
0-4 hours

semide-sensitive sodium and potassium movements. (Sachs, 1971; Dunn, 1973; Wiley and Cooper, 1974). Without the isolation of a consistent amount of ouabain insensitive ion movement, however, the combined use of the inhibitors was not feasible. Consequently, the use of furosemide alone was evaluated in this set of experiments.

Studies by Wiley and Cooper in 1974 demonstrated that furosemide alone inhibited approximately 10% to 15% of active total sodium efflux and potassium influx. The experiments here also demonstrated slight inhibition of active fluxes with furosemide. Variation in results was great, however, and in some cases values for sodium efflux with and without furosemide were nearly the same. (Table 3). These results appeared to indicate an unpredictable nature of furosemide action as well as wide variation in the amount of furosemide sensitive flux in this test system. As indicated by the mean and standard deviation values, there is inhibition of both fluxes by furosemide with greater reduction in sodium efflux than in potassium influx. The former is reduced by  $1.69 \text{ meq/l cells hr}^{-1}$  and the latter by  $0.68 \text{ meq/l cells hr}^{-1}$ . The range of values for potassium-influx in the presence of furosemide was narrower than that for sodium efflux in the presence of furosemide corresponding to the same patterns observed in the absence of inhibitors. It is clear that inhibition occurs in each case, but the amount varies considerably and no correlation is evident between amount of reduction in sodium efflux and corresponding potassium influx in individual cases.

#### Cystic Fibrosis Heterozygotes

During the same experiments for the 20 control tests, eight obligate heterozygotes for cystic fibrosis were paired with eight of the controls and determinations for each pair were made simultaneously. Measurements of net

sodium efflux and net potassium influx were carried out with and without the inhibitors ouabain and furosemide. Like the controls, no ouabain insensitive sodium efflux could be isolated consistently in the heterozygotes and the potassium influx was again abolished. Furosemide used alone showed the same variable pattern of inhibition in the heterozygotes as it did in the controls. Application of the t-test to the two populations revealed no significant difference. Mean sodium efflux in the presence of furosemide was  $1.61 \text{ meq/l cells hr}^{-1} \pm 1.04$  for the controls compared to  $1.26 \text{ meq/l cells hr}^{-1} \pm 0.59$  for the heterozygotes ( $p = 0.61$ ). (Figure 3). For potassium influx in the presence of furosemide the mean for the controls was  $0.98 \text{ meq/l cells hr}^{-1} \pm 0.43$  compared to  $1.10 \text{ meq/l cells hr}^{-1} \pm 0.63$  for the heterozygotes ( $p = 0.55$ ). (Figure 4).

No significant differences were observed between the two groups' values for net sodium efflux in the absence of inhibitors. This was observed when the 20 controls were compared to the 8 heterozygotes (Figure 3) or when the values were compared on a paired basis. (Table 4). However, there was a significant difference in the net potassium influx in the absence of inhibitors. (Figure 4). Using the t-test when 20 controls were compared to 8 heterozygotes, the mean  $K^+$  influx for the controls was  $1.66 \text{ meq/l cells hr}^{-1} \pm 0.52$  compared to  $2.30 \text{ meq/l cells hr}^{-1} \pm 0.81$  for the heterozygotes ( $p = 0.018$ ). Comparison of the paired experiments did not give as good a level of significance but did approach the 10% level of significance. Mean  $K^+$  influx for 8 controls was  $1.73 \text{ meq/l cells hr}^{-1} \pm 0.57$  compared to  $2.30 \text{ meq/l cells hr}^{-1} \pm 0.81$  for the heterozygotes ( $p = 0.121$ ). (Table 5).

Analysis of net Na/K flux ratios for the two groups again showed a significant difference when all data were compared by t-test. For the control group the mean ratio was  $2.13 \pm 0.80$  compared to  $1.39 \pm 0.71$  for the hetero-

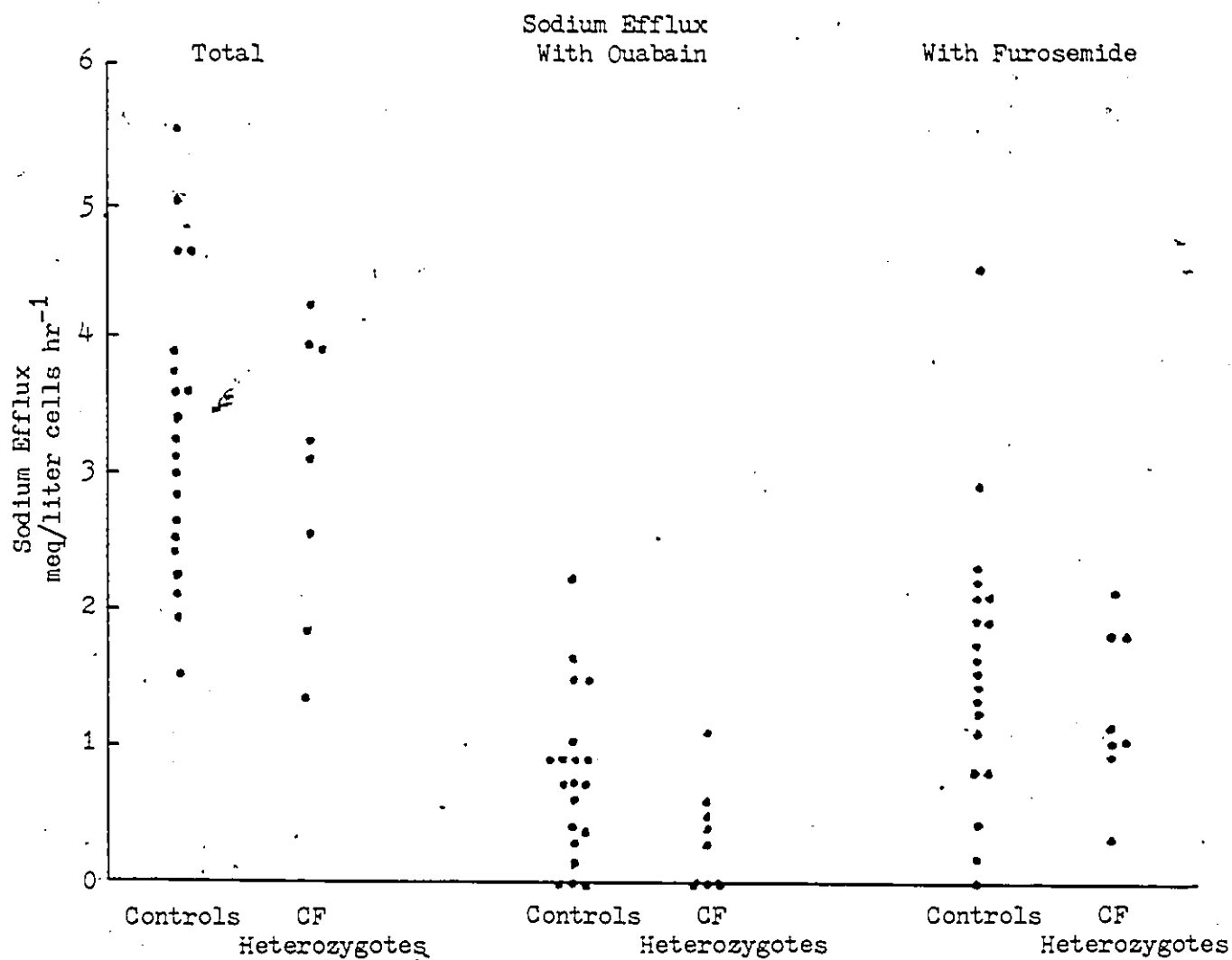
FIGURE 3

SODIUM EFFLUX WITH AND WITHOUT OUABAIN  
OR FUROSEMIDE FOR 20 CONTROL SUBJECTS  
AND 8 CF HETEROZYGOTES  
(SERIES I)

Erythrocytes from 20 control subjects and 8 CF heterozygotes were incubated in loading medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM TRIS and 0.1mM PCMBs. They were sealed in a medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM TRIS, 11mM glucose, and 2mM cysteine. Recovery of the gradients occurred in a Na<sup>+</sup>,K<sup>+</sup> Ringers medium containing 145mM NaCl, 5mM KCl, 1mM MgCl<sub>2</sub>, 2.5mM TRIS, 2mM CaCl<sub>2</sub>, and 10mM glucose. Recovery occurred in the absence of inhibitors (column 1), in the presence of ouabain (column 2), or in the presence of furosemide (column 3). Statistical analysis was by Student's t-test.

FIGURE 3

SODIUM EFFLUX WITH AND WITHOUT OUABAIN  
OR FUROSEMIDE FOR 20 CONTROL SUBJECTS  
AND 8 CF HETEROZYGOTES  
(SERIES I)



$\bar{x}$  3.30      2.97  
S.D. 1.04      1.04

P 0.535

NS

1.61      1.26  
1.04      0.59

0.611

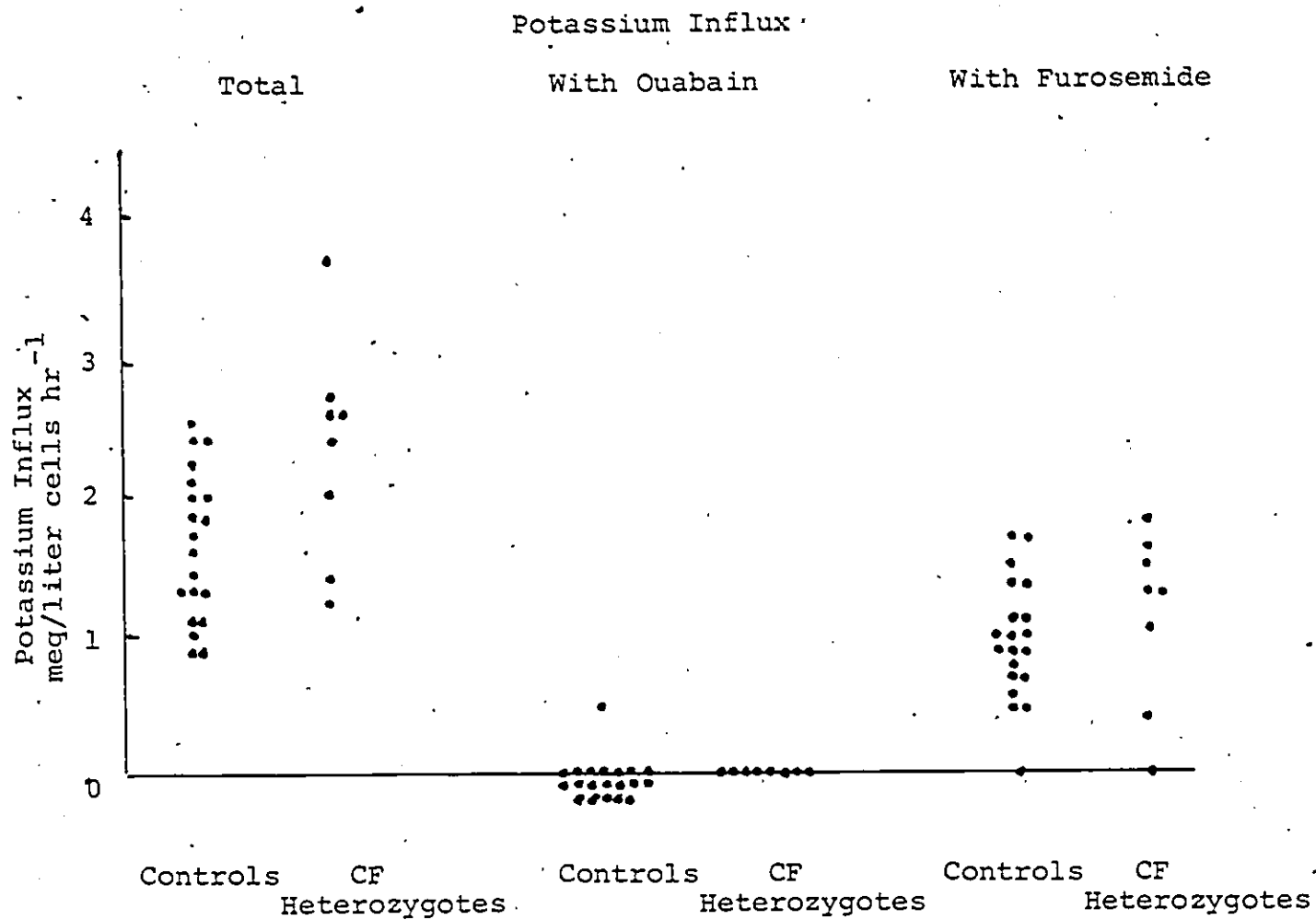
FIGURE 4

POTASSIUM INFLUX WITH AND WITHOUT OUABAIN  
OR FUROSEMIDE FOR 20 CONTROL SUBJECTS  
AND 8 CF HETEROZYGOTES  
(SERIES I)

Conditions of incubation and statistical analysis  
are identical to those in Figure 3.

FIGURE 4

POTASSIUM INFLUX WITH AND WITHOUT OUABAIN  
OR FUROSEMIDE FOR 20 CONTROL SUBJECTS  
AND 8 CF HETEROZYGOTES  
(SERIES I)



$\bar{x}$	1.66	2.30	0.98	1.10
S.D.	0.52	0.81	0.43	0.63
P		0.018		0.546

TABLE 4

ANALYSIS OF PAIRED EXPERIMENTS BY STUDENT'S T-TEST  
FOR Na<sup>+</sup> EFFLUX WITH AND WITHOUT FUROSEMIDE FOR  
8 CONTROL SUBJECTS AND 8 CF HETEROZYGOTES  
(SERIES I)

No.	Na <sup>+</sup> Efflux (Total) meq/liter cells/hour		Na <sup>+</sup> Efflux (with Furosemide) meq/liter cells/hour	
	Controls	Heterozygotes	Controls	Heterozygotes
1	-2.82*	-1.30	-1.43	-0.95
2	-3.43	-1.76	-2.36	-1.02
3	-1.94	-3.87	-1.11	-1.82
4	-1.47	-2.51	-0.81	-0.31
5	-4.60	-3.90	-2.31	-1.78
6	-3.63	-4.15	-0.78	-0.99
7	-2.61	-3.13	+0.94	-1.10
8	-2.49	-3.17	-1.31	-2.10
$\bar{x}$	-2.87	-2.97	$\bar{x}$ -1.26	-1.26
S.D.	0.99	1.04	S.D. 0.79	0.59
	p=0.841		p=0.987	

Incubation conditions are identical to those given in  
Table 3

\*(-) Indicates negative slope or loss of sodium (efflux) from  
the cells (meq/liter cells hr<sup>-1</sup>)

ANALYSIS OF PAIRED EXPERIMENTS BY STUDENT'S T-TEST  
FOR K<sup>+</sup> INFLUX WITH AND WITHOUT FUROSEMIDE FOR  
8 CONTROL SUBJECTS AND 8 CF HETEROZYGOTES  
(SERIES I)

No.	K <sup>+</sup> Influx (Total) meq/liter cells/hour		K <sup>+</sup> Influx (with Furosemide) meq/liter cells/hour	
	Controls	Heterozygotes	Controls	Heterozygotes
1	0.95	1.19	0.53	0.97
2	2.44	1.97	1.48	1.33
3	2.02	3.74	0.98	1.83
4	1.29	2.56	0.69	1.45
5	2.50	2.58	1.70	1.58
6	1.84	1.35	0.57	-0.07
7	1.28	2.35	-0.02	0.36
8	1.52	2.67	0.53	1.30
	$\bar{x}$ 1.73	2.30	$\bar{x}$ 0.81	1.10
	S.D. 0.57	0.81	S.D. 0.56	0.63

p=0.121

p=0.659

Incubation conditions are identical to those given in Table 3

zygotes ( $p = 0.030$ ). The same parameters applied to paired experiments gave a value of  $1.75 \pm 0.63$  for the controls with  $p = 0.30$ .

## Series II

Based on results from investigative procedures to be described later, in this series total net sodium efflux and potassium influx were measured in the erythrocytes after treatment in the loading phase with 0.01mM PCMBs and in the sealing phase with 4mM cysteine. All other salt concentrations in the loading, sealing, and recovery media were identical to those used in Series I. No inhibitors were used. The timing of the collection of the 0 hour specimen was also modified. Following the three choline chloride washes at the end of the sealing stage incubation, a small sample of cells was collected for later measurement of total sodium load and potassium loss as previously described. Whereas this initial sample was previously labeled the 0 hour sample, it was collected now only to document the total intracellular sodium gain and potassium loss achieved and was not included in the linear regression analysis. Instead, the Na/K Ringers solution was added to the 0.5ml of cells in the incubation flasks in the recovery phase as before, and these were placed in the 37° C water bath and agitated for five minutes to allow equilibration of the reaction. The first 0 hour sample was then collected and subsequent hourly samples were timed from that point. Results from this second set of paired experiments (Series II) are shown in Tables 6 and 7.

Sodium efflux increased for both groups. The mean  $\text{Na}^+$  efflux for the controls increased from 3.30 in Series I to 3.44 meq/liter cells  $\text{hr}^{-1}$  in Series II. For cystic fibrosis heterozygotes the increase in sodium efflux was even more marked from 2.97 in Series I to 3.89 meq/liter cells  $\text{hr}^{-1}$  in Series II. (Table 6). Potassium influx also increased for both groups. In the controls, the mean  $\text{K}^+$

TABLE 6

**SODIUM EFFLUX, LINEAR REGRESSION CORRELATION COEFFICIENTS FOR  $\text{Na}^+$  EFFLUX AND 0 HR. ( $\text{Na}^+_i$ ) FOR 14 CONTROL SUBJECTS AND 14 CF HETEROZYGOTES**

Erythrocytes from 14 control subjects and 14 CF heterozygotes were incubated in loading medium containing 150mM NaCl, 1mM  $\text{MgCl}_2$ , 5mM TRIS, and 0.01mM PCMBs. They were sealed in a sealing medium containing 150mM NaCl, 1mM  $\text{MgCl}_2$ , 5mM TRIS, 11mM glucose and 4mM cysteine. Recovery of the gradients occurred in a  $\text{Na}^+/\text{K}^+$  Ringers medium containing 145mM NaCl, 5mM KCl, 1mM  $\text{MgCl}_2$ , 2mM  $\text{CaCl}_2$ , 2.5mM TRIS and 10mM glucose. Statistical analysis was by Student's t-test.

$r_{\text{Na}^+}$  = linear regression  
correlation coefficient

$(\text{Na}^+_i)_{0 \text{ hr.}}$  = initial  
intracellular  
sodium concentration  
(meq/liter cells)

$k'_{\text{Na}^+}$  = sodium efflux  
(meq/liter cells  $\text{hr}^{-1}$ )

SODIUM EFFLUX, LINEAR REGRESSION CORRELATION  
COEFFICIENTS FOR Na<sup>+</sup> EFFLUX AND 0 HR. (Na<sub>i</sub><sup>+</sup>) FOR  
14 CONTROL SUBJECTS AND 14 CF HETEROZYGOTES

r Na <sup>+</sup>	Controls		r Na <sup>+</sup>	CF Heterozygotes	
	(Na <sub>i</sub> <sup>+</sup> ) 0 Hr.	k' Na <sup>+</sup>		(Na <sub>i</sub> <sup>+</sup> ) 0 Hr.	k' Na <sup>+</sup>
-0.98	71.5	-3.29	-0.99	69.7	-4.68
-0.98	80.6	-3.90	-0.96	60.7	-3.52
-0.99	93.9	-3.09	-0.99	92.1	-5.46
-0.99	94.7	-3.88	-0.98	98.4	-4.93
-0.99	70.4	-3.71	-0.98	80.4	-4.15
-0.99	84.2	-4.20	-0.96	73.7	-3.79
-0.98	86.0	-2.93	-0.98	87.9	-4.32
-0.93	84.8	-3.43	-0.96	88.2	-3.09
-0.99	67.6	-3.15	-0.98	78.1	-4.83
-0.95	83.8	-3.73	-0.93	91.7	-3.20
-0.96	80.3	-2.81	-0.98	74.0	-2.51
-0.97	57.6	-2.35	-0.99	77.1	-3.99
-0.99	67.6	-4.25	-0.96	66.4	-2.56
-0.97	64.2	-3.37	-0.99	56.5	-3.44
$\bar{x}$		3.44			3.89
S.D.		0.55			0.90
P			0.113		

influx increased from 1.66 to 2.65 meq/liter cells hr<sup>-1</sup> and in the cystic fibrosis heterozygotes this influx increased from 2.30 to 2.93 meq/liter cells hr<sup>-1</sup>. (Table 7).

As an indication of cell membrane reaction to PCMBS, total sodium loading and potassium depletion values for the two populations were compared in Series I and II. (Table 8). In Series I total sodium loading values were slightly higher in the controls than in the heterozygotes. Potassium depletion values were nearly the same. Total sodium loading and potassium depletion values were reduced in Series II in both groups. There was no difference in average total sodium loading or potassium depletion values between the two groups in this series.

The mean sodium efflux in Series II was greater in the cystic fibrosis heterozygotes than in the controls, 3.89 versus 3.44 meq/liter cells hr<sup>-1</sup>. (Table 6). This was a reversal of the situation observed in Series I in which mean sodium efflux in the absence of inhibitors was lower in the heterozygotes compared to the controls, 2.97 versus 3.30 meq/liter cells hr<sup>-1</sup>. (Figure 3). In Series I, there was no significant difference in the sodium efflux between the two groups when the t-test was applied. (Figure 3). By comparison, in Series II a significant difference between controls and heterozygotes was evident when two restrictions were placed on the data. Using all values, the evaluation by t-test approached a level of significance. (P=0.113). If the same analysis was applied to only those values with a linear regression correlation coefficient of 0.95 or greater, the significant difference improved. (P=0.097). Further restricting the same analysis to only those values with correlation coefficients of 0.95 or greater with initial intracellular sodium loading values of 75 meq/liter cells or greater markedly improved the level of statistical significance. (P=0.027). (Table 9).

TABLE 7

POTASSIUM INFLUX, LINEAR REGRESSION CORRELATION  
COEFFICIENTS FOR  $K^+$  INFLUX AND 0 HR.  $(K^+)_i$  FOR  
14 CONTROL SUBJECTS AND 14 CF HETEROZYGOTES

Incubation conditions and statistical analysis  
are identical to those given for Table 6.

$r_{K^+}$  = linear regression  
correlation coefficient

$(K^+)_i$  0 Hr. = initial intracellular  
potassium concentration  
(meq/liter cells)

$k'_{K^+}$  = potassium influx  
(meq/liter cells hr<sup>-1</sup>)

TABLE 7

POTASSIUM INFLUX, LINEAR REGRESSION CORRELATION  
 COEFFICIENTS FOR K<sup>+</sup> INFLUX AND 0 HR. (K<sub>0</sub><sup>+</sup>) FOR  
 14 CONTROL SUBJECTS AND 14 CF HETEROZYGOTES

r K <sup>+</sup>	<u>Controls</u>		r K <sup>+</sup>	<u>CF Heterozygotes</u>	
	(K <sub>0</sub> <sup>+</sup> ) 0 Hr.	k <sup>+</sup> K <sup>+</sup>		(K <sub>0</sub> <sup>+</sup> ) 0 Hr.	k <sup>+</sup> K <sup>+</sup>
0.97	26.3	3.90	0.99	27.6	3.19
0.99	22.7	3.19	0.96	35.0	2.76
0.99	14.5	2.26	0.99	12.7	4.02
0.99	11.7	3.47	0.99	10.6	3.32
0.97	28.5	2.65	0.99	21.5	3.14
0.99	20.0	2.94	0.96	28.4	2.35
0.99	15.8	2.08	0.99	13.2	2.67
0.99	19.5	2.84	0.99	13.2	2.22
0.93	26.9	2.14	0.99	26.9	3.47
0.99	10.8	3.23	0.99	12.0	2.63
0.99	15.3	2.06	0.98	16.1	2.52
0.98	40.6	1.99	0.99	17.4	2.88
0.99	30.0	2.58	0.99	31.4	2.16
0.89	31.6	1.71	0.99	37.4	3.64
$\bar{x}$		2.65			2.93
S.D.		0.64			0.56
P			0.227		

COMPARISON OF TOTAL INITIAL (0 HR.) INTRACELLULAR SODIUM LOADING  
AND POTASSIUM DEPLETION LEVELS FOR SERIES I AND SERIES II

	Intracellular Sodium (meq/liter cells)		Intracellular Potassium (meq/liter cells)	
	Controls	Heterozygotes	Controls	Heterozygotes
Series I	101.0	94.4	11.4	12.0
range	77.6-111.8	77.1-102.0	9.5-16.2	8.1-18.8
n	20	8	20	8
Series II	77.7	78.2	22.4	21.7
range	57.6-94.7	56.5-98.4	10.8-40.6	10.6-37.4
n	14	14	14	14

For both series loading media contained mM, NaCl,150; MgCl<sub>2</sub>,1; TRIS,5. Sealing media contained mM, NaCl,150; MgCl<sub>2</sub>,1; TRIS,5; Glucose,11. The differences were as follows:

	Loading (PCMBBS)	Sealing (Cysteine)
Series I	0.1mM	2.0mM
Series II	0.01mM	4.0mM

**STATISTICAL ANALYSIS OF SODIUM EFFLUX VALUES BY  
STUDENT'S T-TEST FOR CONTROLS AND CF HETEROZYGOTES  
IN SERIES II**

	All Data		r > 0.95		Restriction below*	
	Control	CF	Control	CF	Control	CF
n	14	14	13	13	7	7
$\bar{x}$	3.44 <sup>+</sup>	3.89	3.44	3.94	3.51	4.40
S.D.	0.55	0.90	0.57	0.91	0.55	0.77
P	0.113		0.097		0.027	

Incubation conditions for these erythrocytes are identical to those given in Table 6. The data in Table 6 was analyzed in total (column 1) after removing the data having correlation coefficients less than 0.95 (column 2) and after removing the data with correlation coefficients less than 0.95 and the data with initial intracellular sodium loading values below 75 meq/liter cells (column 3).

+ Na<sup>+</sup> efflux (meq/liter cells hr<sup>-1</sup>)

\* Restriction for this data

r = > 0.95, initial intracellular sodium loading value equal to 75 meq/liter cells or greater

r = linear regression correlation coefficient

In both Series I and Series II, mean potassium influx for the cystic fibrosis heterozygotes was greater than for the controls. Whereas in Series I, this difference was significant ( $P=0.018$ ), in Series II, the significance disappeared ( $P=0.227$ ). (Figure 4 and Table 7). Application of restrictions similar to those applied to the sodium efflux data did not improve the level of significance of the differences in  $K^+$  influx.

Three cystic fibrosis homozygotes were also evaluated in Series II. Results from these experiments are shown in Table 10. The sodium loading values for these subjects fell within the ranges for both controls and heterozygotes in Series II, but were closer to the lower end of the range. Likewise, the potassium depletion values for these subjects also fell within the ranges for both controls and heterozygotes, but were closer to the higher values in the range.

Table 11A and 11B compares the values for net sodium efflux and potassium influx for the homozygotes with values from the other two populations. Controls and heterozygote values were selected on the basis of having similar initial intracellular sodium loading and potassium depletion values in the erythrocytes corresponding to the flux values. Clearly, the values for both net sodium efflux and potassium influx are quite similar for all three groups when compared at these levels of intracellular sodium loading and potassium depletion.

The population of homozygotes is too small to draw specific conclusions as to the range of sodium loading and potassium depletion values that might characterize a larger homozygote population. However, it should be noted that the sodium loading values for the homozygotes are approximately 14 meq/liter cells less than the other two populations. Likewise, the potassium depletion level for the homozygote cells is approximately 12 meq/liter cells

TABLE 10

**SODIUM EFFLUX, POTASSIUM INFLUX, LINEAR  
REGRESSION CORRELATION COEFFICIENTS AND  
0 HR. (Na<sup>+</sup>) AND (K<sup>+</sup>) FOR  
3 CYSTIC FIBROSIS HOMOZYGOTES**

r Na <sup>+</sup>	Sodium		r K <sup>+</sup>	Potassium	
	0 Hr. (Na <sup>+</sup> )	k' Na <sup>+</sup>		0 Hr. (K <sup>+</sup> )	k' K <sup>+</sup>
-0.99	60.3	-4.31	0.99	37.5	3.52
-0.98	63.8	-3.74	0.98	33.0	2.64
-0.99	66.7	-2.43	0.97	30.4	1.94
$\bar{x}$	63.6	-3.49		33.6	2.70

Incubation conditions for the erythrocytes from the homozygotes were identical to those given for Table 6.

r Na<sup>+</sup> = linear regression  
correlation coefficient

(Na<sup>+</sup>)<sub>0 hr.</sub> = initial  
intracellular  
sodium concentration  
(meq/liter cells)

k' Na<sup>+</sup> = sodium efflux  
(meq/liter cells hr<sup>-1</sup>)

r K<sup>+</sup> = linear regression  
correlation coefficient

(K<sup>+</sup>)<sub>0 hr.</sub> = initial intracellular  
potassium concentration  
(meq/liter cells)

k' K<sup>+</sup> = potassium influx  
(meq/liter cells hr<sup>-1</sup>)

TABLE 11A and 11B

COMPARISON OF Na<sup>+</sup> EFFLUX AND K<sup>+</sup> INFLUX FOR  
CONTROLS, CF HETEROZYGOTES, AND CF HOMOZYGOTES  
RESTRICTED TO SIMILAR INITIAL (Na<sup>+</sup>) AND (K<sup>+</sup>)

Erythrocytes were incubated under conditions  
identical to those given in Table 6.

COMPARISON OF Na<sup>+</sup> EFFLUX AND K<sup>+</sup> INFLUX FOR  
CONTROLS, CF HETEROZYGOTES, AND CF HOMOZYGOTES  
RESTRICTED TO SIMILAR INITIAL (Na<sup>+</sup>) AND (K<sup>+</sup>)

A.

Controls		Heterozygotes		Homozygotes	
(Na <sup>+</sup> ) <sup>*</sup> 0 Hr.	k' Na <sup>+</sup> **	(Na <sup>+</sup> ) 0 Hr.	k' Na <sup>+</sup>	(Na <sup>+</sup> ) 0 Hr.	k' Na <sup>+</sup>
67.6	3.15	69.7	4.68	60.3	4.31
67.6	4.25	60.7	3.52	63.8	3.74
64.2	3.37	66.4	2.56	66.7	2.43
$\bar{x}$ 66.5	3.59	65.6	3.59	63.6	3.49

\* (Na<sup>+</sup>) = intracellular concentration of sodium (meq/liter cells)  
\*\* k' Na<sup>+</sup> = sodium efflux (meq/liter cells hr<sup>-1</sup>)

B.

Controls		Heterozygotes		Homozygotes	
(K <sup>+</sup> ) <sup>*</sup> 0 Hr.	k' K <sup>+</sup> **	(K <sup>+</sup> ) 0 Hr.	k' K <sup>+</sup>	(K <sup>+</sup> ) 0 Hr.	k' K <sup>+</sup>
30.0	2.58	35.0	2.76	37.5	3.52
31.6	1.71	31.4	2.16	33.0	2.64
40.6	1.99	37.4	3.64	30.4	1.94
$\bar{x}$ 34.1	2.09	34.6	2.85	33.6	2.70

\* (K<sup>+</sup>) = intracellular concentration of potassium (meq/liter cells)  
\*\* k' K<sup>+</sup> = potassium influx (meq/liter cells hr<sup>-1</sup>)

higher than for the other two groups. It appears that, considering these observations, the erythrocytes from the cystic fibrosis homozygotes do not load to as high a level nor are they depleted of potassium to as low a level as the other two groups when treated with the same loading and sealing media.

### Series III

In this series, a phosphate buffer system replaced the TRIS buffer system common to Series I and Series II. Adenine and inosine were added to the sealing media. In addition, sealing time incubation was extended from 30 minutes to 45 minutes. Net sodium efflux and potassium influx were measured as previously described. No inhibitors were used. Results from the third series of paired experiments are shown in Table 12A and 12B.

In this series, mean net sodium efflux and potassium influx were higher in the controls than in the heterozygotes, whereas in Series II these values were higher in the CF heterozygotes than in the controls. In both groups the mean net values increased. For the controls, net sodium efflux increased from 3.44 in Series II to 4.65 meq/liter cells  $\text{hr}^{-1}$  and potassium influx increased from 2.65 in Series II to 3.17 meq/liter cells  $\text{hr}^{-1}$ . The increase in sodium efflux for the heterozygotes was from 3.89 in Series II to 4.17 meq/liter cells  $\text{hr}^{-1}$  and potassium influx increased from 2.93 in Series II to 3.10 meq/liter cells  $\text{hr}^{-1}$ .

Sodium efflux increased by 35% in the controls compared to only 7% in the heterozygotes. Likewise, potassium influx increased by 20% in the controls compared to only 6% in the heterozygotes. This marked difference in the percent increase of the net fluxes resulted in an overlap of values for the two groups that abolished the significant difference in sodium efflux observed between the two groups in Series II. Thus, under these experimental condi-

TABLE 12

**SODIUM EFFLUX, POTASSIUM INFLUX,  
LINEAR REGRESSION CORRELATION COEFFICIENTS  
AND 0 HR. ( $\text{Na}^+_i$ ) AND ( $\text{K}^+_i$ ) FOR 6 CONTROL  
SUBJECTS AND 6 CF HETEROZYOTES  
(SERIES III)**

Erythrocytes from 6 control subjects and 6 CF heterozygotes were incubated in a loading medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM Na<sub>2</sub> HPO<sub>4</sub> and 0.01mM PCMBs. They were sealed in a sealing medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM Na<sub>2</sub> HPO<sub>4</sub>, 11mM glucose and 4mM cysteine, 2mM adenine-HCl and 4mM inosine. The sealing phase was extended from thirty minutes to forty-five minutes. Recovery of the gradients occurred in a Na<sup>+</sup>/K<sup>+</sup> Ringers medium containing 145mM NaCl, 5mM KCl, 1mM MgCl<sub>2</sub>, 2mM CaCl<sub>2</sub>, 2.5mM Na<sub>2</sub> HPO<sub>4</sub> and 10mM glucose.

Statistical analysis was by Student's t-test.

$r \text{ Na}^+$  = linear regression  
correlation coefficient

$(\text{Na}^+_i) 0 \text{ hr.}$  = initial  
intracellular  
sodium concentration  
(meq/liter cells)

$k' \text{ Na}^+$  = sodium efflux  
(meq/liter cells hr<sup>-1</sup>)

$r \text{ K}^+$  = linear regression  
correlation coefficient

$(\text{K}^+_i) 0 \text{ hr.}$  = initial intracellular  
potassium concentration  
(meq/liter cells) -

$k' \text{ K}^+$  = potassium influx  
(meq/liter cells hr<sup>-1</sup>)

SODIUM EFFLUX, POTASSIUM INFLUX,  
LINEAR REGRESSION CORRELATION COEFFICIENTS  
AND 0 HR. (Na<sup>+</sup>) AND (K<sup>+</sup>) FOR 6 CONTROL  
SUBJECTS AND 6 CF HETEROZYGOTES  
(SERIES III)

## A. Sodium

	Controls			Heterozygotes		
	r Na <sup>+</sup>	(Na <sup>+</sup> ) 0 Hr.	k' Na <sup>+</sup>	r Na <sup>+</sup>	(Na <sup>+</sup> ) 0 Hr.	k' Na <sup>+</sup>
	-0.99	72.3	-5.87	-0.99	50.0	-3.60
	-0.99	52.0	-4.36	-0.98	52.8	-3.34
	-0.99	82.2	-5.17	-0.99	83.9	-5.10
	-0.97	89.7	-3.95	-0.92	101.1	-3.77
	-0.99	51.8	-3.71	-0.99	53.6	-3.83
	-0.99	86.6	-4.83	-0.99	84.6	-5.39
$\bar{x}$			4.65			4.17
S.D.			0.81			0.85
P				0.66		

## B. Potassium

	Controls			Heterozygotes		
	r K <sup>+</sup>	(K <sup>+</sup> ) 0 Hr.	k' K <sup>+</sup>	r K <sup>+</sup>	(K <sup>+</sup> ) 0 Hr.	k' K <sup>+</sup>
	0.97	26.8	3.88	0.98	49.6	2.90
	0.93	50.1	2.48	0.99	47.0	2.45
	0.98	25.9	4.06	0.99	17.0	4.21
	0.99	14.7	3.33	0.99	11.3	2.30
	0.99	44.6	2.56	0.99	46.8	2.72
	0.99	18.8	2.69	0.99	23.9	4.03
$\bar{x}$			3.17			3.10
S.D.			0.69			-0.82
P				0.88		

tions, there was no significant difference in either net sodium efflux or potassium influx between controls and cystic fibrosis heterozygotes.

#### Investigative Procedures

These experiments were done to determine what changes in methodology might achieve less cell membrane damage, better recovery of competent membrane transport systems and better consistency in results. They also clarified the nature of the reactions occurring during the incubation phases. Marked hemolysis was observed in Series I in the supernatants of the loading phase following centrifugation of the cell suspensions. Variation in hemolysis among individual specimens was also noted. These observations were considered indicative of cell membrane damage and of varied reaction of cell membranes to PCMBS treatment. Consequently, the effect of reducing PCMBS concentration in the loading phase and increasing concentration of cysteine in the sealing phase was evaluated.

As expected, cell sodium increased and cell potassium decreased to a greater extent as the concentration of PCMBS increased. At each individual concentration of PCMBS, sodium loading and potassium depletion increased with increasing time in the loading phase. (Table 13). Hemolysis at 20 hours was greater the higher the concentration of PCMBS and at each concentration of PCMBS hemolysis increased as time in the loading stage increased (Figure 5). The changes were most regular in the lowest concentration of PCMBS. In all cases, it appeared that the rate of loading/depletion decreased with increasing time. This was possibly indicative of the recovery phase described by Sutherland et al. (1967).

Measurements of intracellular sodium and potassium concentration on the same sample after initial processing following the sealing phase and again

TABLE 13

**SODIUM LOADING AND POTASSIUM DEPLETION  
VALUES WITH VARYING LOADING TIME AND  
VARYING PCMBS CONCENTRATION\***

Hour	Cell Na <sup>+</sup> meq/l Cells			Cell K <sup>+</sup> meq/l Cells		
	Conc. PCMBS(mM)			Conc. PCMBS(mM)		
	0.01	0.03	0.05	0.01	0.03	0.05
20	81.3	90.3	93.6	17.0	10.2	7.8
22	85.8	93.5	99.8	13.8	7.8	6.3
24	87.6	92.6	97.4	12.3	6.8	5.5
26	88.8	94.4	99.6	9.8	5.7	4.5

Erythrocytes from a single donor were incubated in a loading medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM TRIS and either 0.01mM, 0.03mM, or 0.05mM PCMBS. They were sealed in sealing medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM TRIS, 11mM glucose and 4mM cysteine. Measurements for intracellular sodium and potassium concentration were made after the cells were washed three times in 150mM choline chloride (4°C) and resuspended in the choline chloride to a hematocrit of 20%-25%.

\*Values are the average of two separate experiments

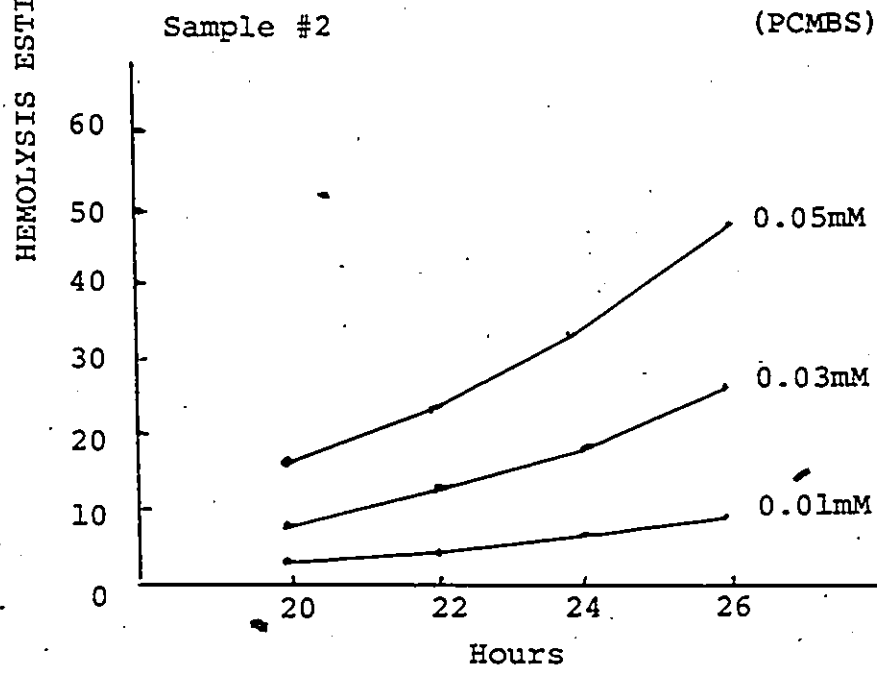
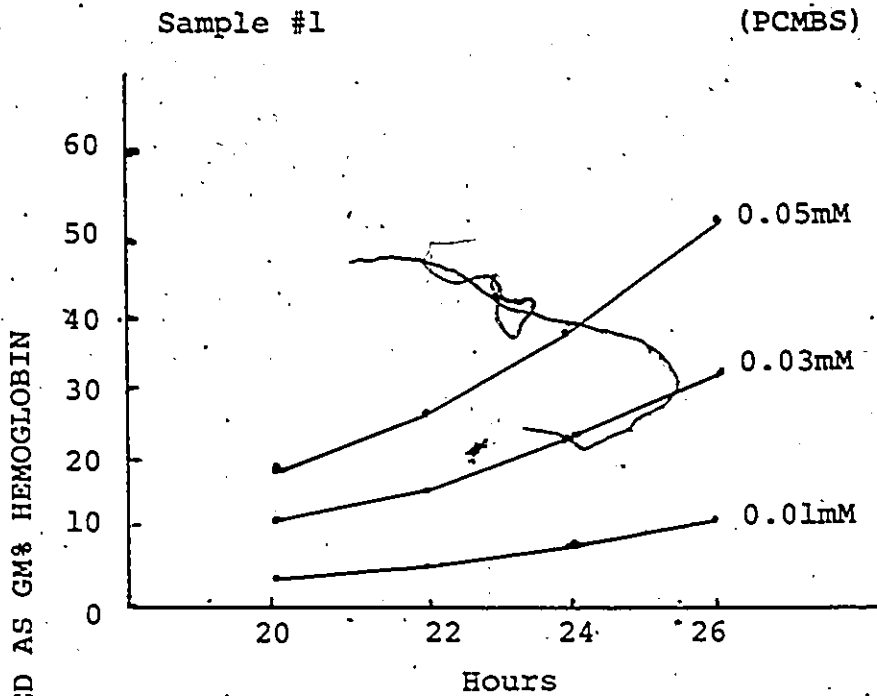
**FIGURE 5**

**HEMOLYSIS IN LOADING SUPERNATANT  
FOR VARIED LOADING TIMES AND  
VARIED CONCENTRATIONS OF PCMBS**

Erythrocytes from two single sources (the same sources as in Table 13) were incubated in loading media as described in Table 13. The supernatants resulting from centrifugation of the loading phase samples were saved and extent of hemolysis was estimated as gm% hemoglobin by reading the supernatants against the loading medium blank in a Coulter Hemoglobinometer (540nm).

FIGURE 5

HEMOLYSIS IN LOADING SUPERNATANT  
FOR VARIED LOADING TIMES AND  
VARIED CONCENTRATIONS OF PCMBS



after a four hour holding period were done to reveal any changes that might occur in sample values when held for varying periods of time prior to measurement. This also reflected the consistency with which the flame photometer could measure the same sample at different times.

Sample value consistency over the holding period prior to measurement is illustrated in Tables 14A, 14B, and 15. Only slight changes occurred in the single samples over varied waiting periods up to a maximum of four hours. Correlation coefficients based on linear regression analysis of the samples for both a control and a CF heterozygote were essentially the same whether based on samples measured at the time of initial processing or on samples measured in succession at the end of the four hour incubation.

In Series III the possibility of extending the sealing phase from thirty minutes to forty-five minutes was considered. Consequently, this phase was monitored to determine what changes in intracellular sodium and potassium values might occur if the cells remained in this medium after reconstruction was virtually complete. Results in Table 16A clearly indicate that for cells from both a control subject and a CF heterozygote, values for intracellular sodium remain virtually constant for the first thirty-five minutes in the sealing phase and then begin to decline, dropping between 8 and 9 meq/liter cells by forty-five minutes. Changes in intracellular potassium concentration were not as marked, however, the values did begin to increase in the interval from thirty-five to forty-five minutes. (Table 16B).

**SAMPLE VALUE CONSISTENCY FOR FOUR HOUR  
HOLDING PERIOD AT VARIED PCMBS CONCENTRATIONS**

A

Concentration of PCMBS(mM)	Loading Time Hour 20 Meq/Liter Cells		Second Reading Time Hour 24 Meq/Liter Cells	
	Na <sup>+</sup>	K <sup>+</sup>	Na <sup>+</sup>	K <sup>+</sup>
.01	80.5	16.9	80.5	16.9
.03	90.0	9.6	90.4	10.8
.05	92.9	7.9	92.1	8.6

B

Concentration of PCMBS(mM)	Loading Time Hour 22 Meq/Liter Cells		Second Reading Time Hour 26 Meq/Liter Cells	
	Na <sup>+</sup>	K <sup>+</sup>	Na <sup>+</sup>	K <sup>+</sup>
.01	84.8	14.3	85.7	14.8
.03	92.2	7.4	91.7	7.8
.05	101.1	6.1	100.0	8.7

Erythrocytes were incubated under conditions identical to those described in Table 13. Intracellular sodium and potassium concentrations were measured on samples taken after 20 hours in the loading phase. These same samples were held at room temperature for four hours and then again measured for intracellular sodium and potassium concentration. Samples taken after 22 hours in the loading phase were treated the same.

TABLE 15

**SAMPLE VALUE CONSISTENCY  
FOR ONE TO FOUR HOUR HOLDING PERIODS  
FOR A CONTROL SUBJECT AND A CF HETEROZYGOTE**

CONTROL

Hours	0	1	2	3	4
0	*87.5				84.8
1		81.7			80.6
2			77.2		75.6
3				74.1	72.7
4					71.2

$r = -0.99$     $-0.98$   
 $k' Na^+ = -4.12$     $-3.51$

CF HETEROZYGOTE

Hours	0	1	2	3	4
0	80.6				80.0
1		76.8			78.7
2			76.3		74.4
3				70.0	70.5
4					70.7

$r = -0.94$     $-0.96$   
 $k' Na^+ = -2.66$     $-2.68$

Incubation conditions were identical to those in Table 6 except that PCMBS concentration was 0.02mM. Values in columns 0-3 and the last value in column 4 are the intracellular sodium concentration, ( $Na_i^+$ ), after sample processing on the hour of collection. The first correlation coefficient,  $r$ , and sodium efflux value,  $k'Na^+$ , result from linear regression analysis of these values. Values in column 4 represent ( $Na_i^+$ ) on these samples after holding at room temperature until collection of sample 4. The second  $r$  and  $k'Na^+$  refer to these values.

\* Intracellular Sodium (meq/l cells)

$r$  = Correlation coefficient for values 0-4 hours

$k' Na^+$  = Sodium efflux as the slope of the line calculated by linear regression analysis

**VALUES FOR INTRACELLULAR SODIUM AND POTASSIUM  
AT TEN MINUTE INTERVALS DURING  
THE FORTY-FIVE MINUTE SEALING STAGE  
IN SERIES III**

A

Time (minutes)	Intracellular Sodium (meq/liter cells)	
	Control	Heterozygote
15	91.3	93.5
25	88.9	88.6
35	89.5	92.8
45	82.0	84.5

B

Time (minutes)	Intracellular Potassium (meq/liter cells)	
	Control	Heterozygote
15	20.9	15.0
25	24.9	14.7
35	22.7	14.8
45	26.2	17.4

Erythrocytes from a control subject and a CF heterozygote were incubated in a loading medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM Na<sub>2</sub>HPO<sub>4</sub> and 0.01mM PCMBS. They were sealed in a sealing medium containing 150mM NaCl, 1mM MgCl<sub>2</sub>, 5mM Na<sub>2</sub>HPO<sub>4</sub>, 11mM glucose and 4mM cysteine. The total sealing time was forty-five minutes. At ten minute intervals, samples of the sealing suspension were taken, washed three times in 150mM cold choline chloride and resuspended in choline chloride to a hematocrit of 20%-25%. Values for intracellular sodium and potassium concentration were then made on these ten-minute samples.


## DISCUSSION

The experimental system used in the initial experiments successfully measured net sodium efflux and potassium influx in human erythrocytes. Although the spread of values for the net fluxes was quite broad, particularly in the case of sodium efflux, the net flux rates over the four hour incubation period were consistently linear with time and net changes in intracellular sodium and potassium were of sufficient magnitude to be measured accurately using flame photometry.

Results using the inhibitors ouabain and furosemide indicated that their use in this test system to isolate the ouabain-insensitive, furosemide-sensitive  $\text{Na}^+, \text{K}^+$ -co-transport component was not feasible. When operationally defining this sodium transport component, previous work indicates that the inhibitors ouabain and furosemide must be used in combination. (Sachs, 1971; Dunn, 1973; Wiley and Cooper, 1974). Since no consistent amount of ouabain-insensitive sodium efflux or potassium influx could be isolated, the use of the combined inhibitors was not possible. Although furosemide used alone consistently inhibited a portion of each flux, the values were quite variable possibly indicating that in this system, the target of furosemide inhibitory action was not clearly enough defined to make its use informative. The magnitude of the contribution of the ouabain-insensitive, furosemide sensitive transport component of sodium and potassium to total net movements of these ions under normal physiological conditions, such as in this experimental system, was apparently not large enough to be measured with a procedure of this sensitivity. The lack of success in isolating an appreciable amount of

ouabain-insensitive sodium efflux and potassium influx was surprising. Based on previous work by Garay and Garrahán (1973), it would be expected that total sodium efflux would increase as intracellular sodium increased. Additionally, these authors present data showing that in PCMBS treated human erythrocytes suspended in Na/K media, as intracellular sodium increases beyond 75m moles/liter cell water, the proportion of total sodium efflux that is ouabain-insensitive exceeds the ouabain-sensitive component. Below 75m moles/liter of cell water, the opposite situation applies. The ouabain-sensitive component is saturated at this level and the ouabain-insensitive component increases linearly with increasing intracellular sodium in parallel with the total sodium efflux. In Series I, the majority of intracellular sodium values measured after the sealing phase and before the fluxing phase were greater than 90 meq/liter cells. Even so, the highest value for ouabain-insensitive sodium efflux that was measured was 0.87 meq/liter cells hr<sup>-1</sup> in a control, and in three controls and three heterozygotes none could be measured.

In Series I, marked hemolysis was observed in the supernatant of the loading phase following the twenty hour incubation with 0.1mM PCMBS and was interpreted as an indication of cell damage. Combined with the low concentration of cysteine, 2mM, in the sealing phase, this quite possibly could have resulted in leaky cell membranes in the fluxing phase. Consequently, increased leak of ions through the membranes would have obscured the net movements of the ions resulting in lower total sodium efflux even with high concentrations of intracellular sodium initially. Further, the broad variation in net flux measurements might well reflect a broad variation in cell membrane damage and recovery. The large increase in permeability could also have resulted in a considerable amount of PCMBS remaining intracellular after the sealing phase and incompletely sealed cells might have allowed some influx



of calcium from the Na/K Ringers media. Both of these factors could affect variable inhibition of the ouabain-sensitive pump, and therefore, be additional causes of inconsistent results in Series I. (Dunham and Hoffman, 1978; Hoffman, 1972, 1979). Additionally, the values are all below 1 meq Na<sup>+</sup>/liter cells hr<sup>-1</sup>. At this level, the ability of the flame photometer to resolve differences in (meq Na<sup>+</sup>/liter is exceeded, as the manufacturer's manual specifies a repeatability of ± 1.1 meq Na<sup>+</sup>/liter for any one sample. Inconsistent results could, therefore, also be partially due to the limit of sensitivity of the method of measurement. Garay and Garrahan (1973) used flame photometry to measure total intracellular sodium and potassium concentrations, but measured unidirectional fluxes with <sup>22</sup>NaCl, a method better able to resolve ion movements of low magnitude.

The higher potassium influx and the resulting lower net Na<sup>+</sup>/K<sup>+</sup> ratio in the CF heterozygotes seen in Series I follow the pattern seen by Garay in the essential hypertensive patients. (Garay and Meyer, 1979). Since further work by Garay more specifically isolated the abnormality in the ouabain-insensitive Na<sup>+</sup>,K<sup>+</sup>-co-transport system, this gave some support to the possibility that the abnormality in sodium transport in cystic fibrosis might be related to this transport component. Previous work in CF has identified reduced ouabain-insensitive sodium transport in CF patients and heterozygotes. (Balfe et al., 1968; Lapey and Gardner, 1970; Cole and Dirks, 1972). These studies have not been successful in significantly separating CF heterozygotes from normal controls. If the abnormal component could be more narrowly defined, possibly differences could be measured that have previously been overshadowed by other larger sodium transport components in the experimental systems used. In light of the results presented in Series I, further investigation into net flux values in CF heterozygotes appeared justified. However, the physical compe-

tence of the reconstructed membranes and transport systems was in question as evidenced by the low net fluxes and lack of correlation between intracellular sodium concentration and net sodium efflux.

Reasoning that reconstruction of the cell membrane would improve with less harsh treatment, the feasibility of reducing PCMBs concentration without significantly compromising loading levels, was investigated. These investigations confirmed in this test system some of the major characteristics of the PCMBs reaction with human erythrocytes first observed by Sutherland in 1967. The lower PCMBs concentrations produced less hemolysis over the time of the loading stage and appeared not to compromise the extent of alteration of the intracellular sodium and potassium ion concentrations. The concentration of PCMBs could be reduced to 0.01mM without lowering loading levels below 75 meq/liter cells. (Table 13). The original cation loading procedure used 2.0mM cysteine in the sealing phase. (Garrahan and Rega, 1967). Garay, however, used 4.0mM cysteine and in a later study in which he introduced the use of ouabain and furosemide, he reduced the concentration of PCMBs to 0.02mM. (Garay and Meyer, 1979; Garay et al. 1980). Consequently, modifications in Series II, lowered PCMBs concentration and increased cysteine concentration, were designed to cause less cell membrane damage and better reconstruction of membrane transport systems without compromising loading values.

Clearly, better results were observed on a net flux basis in Series II. There was marked reduction in hemolysis in the supernatant of the loading phase reflecting less cell damage. Net sodium efflux and potassium influx increased in both controls and CF heterozygotes. These factors would appear to reflect better membrane repair and more complete recovery of ion

transport systems in that they indicate a better balance between the pump and leak components of sodium transport.

Two observations in Series II add further support to the identification of the CF sodium transport defect in the ouabain-insensitive transport component. With the apparent better recovery of competent erythrocyte membranes, the stimulus to increased sodium efflux was greater in the CF heterozygotes than in the controls. The ouabain-insensitive fluxes have so far been shown non-energy dependent as far as ATP hydrolysis is concerned. (Hoffman and Kregnow, 1966; Dunn, 1973). Modifications in Series II involved only better reconstruction without changes in energy supply. Hence, increased sodium efflux values most likely reflected increased transport by the non-active components. Although better reconstruction would have aided both active and nonactive transport systems in general, it is possible that it would have a greater proportional effect on the nonactive components. It is interesting to note the lack of ouabain-insensitive potassium influx observed in Series I. Previous work in non-PCMBS treated cells demonstrates that ouabain-insensitive potassium influx contributes approximately 20% to the total potassium influx. (Glynn, 1957). Evidently, the PCMBS treatment of the cells affects the cell membrane in such a way that this component of potassium influx is seriously compromised. Referring to reconstructed erythrocyte ghosts, Ferriera and Lew (1977) suggest that the  $\text{Na}^+$ ,  $\text{K}^+$ -pump appears to recover well, but competent reconstruction of all membrane properties, especially the passive fluxes, cannot be assumed. The same line of reasoning might be applicable to PCMBS treated cells. Thus, less damage and better reconstruction might logically be expected to favor improved nonactive fluxes over active fluxes even though both would benefit. If the assumption is valid, the predominance of the increased net sodium efflux in the CF heterozygotes

as opposed to controls would reflect a greater response in the nonactive component in the heterozygotes. Secondly, since the difference between the two groups was most marked when intracellular sodium concentration was 75 meq/liter of cells or greater, it appears that the difference resided in the ouabain-insensitive component that predominates at that point. (Garay and Garrahan, 1973).

In Series III, the addition of adenine and inosine to the sealing media was designed to increase the ATP content of the cells providing further energy repletion for membrane reconstruction as well as for energy dependent transport systems. The increase in sealing time allowed a longer period for more complete reconstruction. The sodium phosphate buffer system was introduced to provide the inorganic phosphate required for nucleotide synthesis. (Whittam and Wiley, 1967, 1968). It has been reported that some loss of intracellular sodium load occurs as sealing time is prolonged and transport systems begin to recover. (Kregnow, 1974). Monitoring of the intracellular sodium concentration at ten minute intervals during a sealing phase demonstrated that intracellular sodium did fall by approximately 10 meq Na<sup>+</sup>/liter cells in both a control and a CF heterozygote between ten minutes and forty-five minutes in the sealing phase, most of the fall occurring after thirty-five minutes. This situation would not be detrimental as long as initial loading of intracellular sodium was not below approximately 90 meq/liter cells. At this level of intracellular sodium load, a concentration of 75 meq Na<sup>+</sup>/liter cells could be expected even with a forty-five minute sealing stage. However, the sodium load varied widely in Series III and in fifty percent of the paired experiments, the final concentration of intracellular sodium at the end of the sealing stage fell below 75 meq/liter cells. (Table 12). As previously explained, below this level of intracellular sodium, the ouabain-sensitive

component of sodium efflux predominates. (Garay and Garrahan, 1973). Combined with increased ATP, the stimulation of the active fluxes was favored. Consequently, the active transport component overshadowed the ouabain-insensitive component and the difference between controls and CF heterozygotes disappeared. This adds indirectly to the evidence implicating defective ouabain-insensitive sodium transport in cystic fibrosis.

The difficulty encountered in measuring the ouabain-insensitive, furosemide-sensitive  $\text{Na}^+, \text{K}^+$ -co-transport system under these experimental conditions is not totally unexpected. To begin with, this component contributes only about 0.5m moles  $\text{Na}^+$ /liter cells/hour out of a total sodium efflux of approximately 3-4m moles  $\text{Na}^+$ /liter cells/hour. (Dunn, 1970). Additionally, this furosemide sensitive component has been shown to accomplish equal bidirectional movements of sodium, under physiological conditions demonstrating movements of the ions against their gradients only when the steady state is perturbed. (Wiley and Cooper, 1974). Gunn suggests that furosemide inhibition of this co-transport component can be seen only in specially constructed media that stimulates it by disturbing the steady state. (Gunn, 1980). The use of the cation loading procedure does disturb the steady state by reversal of the sodium and potassium gradients. However, the extent of this disturbance is apparently insufficient to stimulate the co-transport of the ions to a magnitude that demonstrates hourly changes in intracellular sodium large enough to be resolved accurately by monitoring net transport using flame photometry. Only if, as in Series II, the reversal of the gradients is combined with experimental conditions that favor inactive fluxes over active fluxes is the ouabain insensitive component influential enough to be "visible" in net flux measurements in a physiological  $\text{Na}^+/\text{K}^+$  recovery media.

Finally, the target of furosemide inhibition in the human erythrocyte is as yet controversial. Most specifically, it inhibits chloride-chloride self-exchange. (Brazy and Gunn, 1976). As a diuretic, it is most effective on active chloride transport in renal tissue and it has been suggested that the low contribution of chloride movements to cation movements in non-renal tissue makes the inhibitory action of furosemide difficult to demonstrate in these tissues. (Lassiter, 1975).

Evaluation of the procedure as modified in Series II, as a clinical diagnostic test is discouraging. From a positive standpoint, sample requirement is small, risk to the patient is minimal, and technical manipulations are not difficult. Further, required equipment is not complicated and is usually available in a clinical laboratory. However, the length of time required for the test must be considered especially in light of the fact that to yield a significant distinction between controls and CF heterozygotes, the linearity of the changing hourly values of intracellular sodium concentration must be 0.95 or greater by linear regression analysis and sodium loading must be 75 meq/-liter cells or greater. Although linearity is reasonably repeatable, on the basis of sodium loading values, 43% of the CF heterozygotes and 36% of the control tests would require repeated tests. The difficulty encountered in controlling sodium loading values was quite puzzling. Clearly, this variable must be more narrowly controlled to reduce the rate of equivocal tests. The test requires the undivided attention of one laboratory technologist for two days which is not compatible with allocation of laboratory personnel in most hospitals. Finally, as developed here, the procedure is not yet amenable to the multiple sample analysis required of the clinical diagnostic laboratory. For these reasons, it would also clearly be impractical as a genetic screening procedure except for screening populations at high risk for carrying the gene such as

siblings of identified CF homozygotes. Nevertheless, further refinements in the procedure as well as tighter controls on the problem variables discussed here might improve its prospects in the identification of the CF heterozygote. Clearly once refined, its application to a much broader population in clinical trials would be necessary to establish the true extent of overlapping values between controls and CF heterozygotes. The gene frequency of 5% must be considered when assessing control values and the distribution of altered  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport in other disease states, especially those similar symptomatically to CF, must be evaluated.

## CONCLUSION

The procedure for the measurement of net sodium efflux and net potassium influx in human erythrocytes was successfully developed in this clinical laboratory setting. However, difficulties encountered with broad ranges both for net flux values and total intracellular sodium loading make its application on a routine basis inadvisable at the present time. The PCMBS treatment of the cells is clearly damaging as evidenced by the hemolysis observed in the supernatant of the loading phase. The inconsistent net flux results in Series I and their subsequent improvement in Series II along with the marked reduction in hemolysis are further evidence of the damaging effect of PCMBS.

Clearly, the final net flux results depend on a combination of a set of experimental conditions in the three stages of the procedure. First, a balance must be maintained between the extent of damage to the cell membranes and adequate conditions for reconstruction of competent membrane processes in the sealing stage. The extent of damage depends not only on the concentration of PCMBS but also on individual membrane responses to PCMBS treatment and repair as evidenced by the broad ranges of net flux values. Secondly, conditions of repletion in the sealing phase can also affect the proportional contribution of different transport systems in the final recovery stage. Thirdly, the construction of the recovery media itself also dictates the proportional activity of transport systems based on kinetic considerations. All of these conditions must be carefully controlled if valid interpretation of the final net flux values is to be made. The use of the inhibitors, ouabain and furosemide, to operationally define the  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport system under the

experimental conditions used here is not feasible. It appears that although reversal of the gradients disturbs the steady state, in physiological media the contribution of the co-transport system to the recovery of the gradients is still overshadowed by the  $\text{Na}^+$ ,  $\text{K}^+$ -pump. Consequently, the movements of the ions by the furosemide-sensitive co-transport system are too small to be measured reliably by flame photometry.

Application of the net flux procedure to cystic fibrosis heterozygotes also successfully demonstrated significant differences between controls and heterozygotes. The restrictions on the data required to demonstrate the difference, however, make tighter control on intracellular sodium loading values mandatory. Additionally, the construction of the experimental conditions in the three stages had direct effect on the significant difference that was seen. In Series I, the significant difference between the two groups was in a higher potassium influx for the CF heterozygotes with no difference in sodium efflux. The situation was reversed in Series II with a higher sodium efflux for the CF heterozygotes and no difference in potassium influx. No difference was seen in either net flux in Series III.

The changes in the significant differences do offer circumstantial evidence implicating abnormal nonactive sodium efflux in cystic fibrosis. Further investigation of both net sodium efflux and ouabain-insensitive, furosemide-sensitive sodium efflux using modifications of this system are clearly justified. Application of the procedure developed by Dagher and Garay, (1980) for the measurement of the  $\text{Na}^+$ ,  $\text{K}^+$ -co-transport component in human erythrocytes may be the answer to the need for more definitive isolation of the sodium transport abnormality in cystic fibrosis. The modifications made in the experimental solutions used deviate markedly from physiological conditions, however, they achieve perturbation of the steady

state from two standpoints. Not only are the sodium and potassium gradients reversed with PCMBS treatment, but also, the recovery media is constructed free of sodium and potassium further deviating from physiological conditions. Results in the present study in Series II indicate that this additional deviation from the steady state may be necessary to stimulate the nonactive fluxes to levels of ion transport that can be reflected in net flux movements sufficient to be measured by flame photometry.

Application of this procedure to routine clinical laboratory testing is not indicated at the present time. However, several problem areas have been identified in this study and differences between controls and CF heterozygotes have been demonstrated. Once the problems are resolved, the procedure may well be applicable if subsequent large scale clinical trials demonstrate its efficacy in reliably identifying cystic fibrosis heterozygotes. Bearing in mind the broad ramifications and persistence of this disease as well as past defeats in definitively identifying the sodium transport defect, it is clear that efforts in this area of CF research must continue. Results in this study appear to offer a clear direction for further research in this area.

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