CELLULAR AND BIOCHEMICAL ASPECTS OF CYSTIC FIBROSIS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
JOHN MUNRO NICKERSON
1976

ABSTRACT

CELLULAR AND BIOCHEMICAL ASPECTS OF CYSTIC FIBROSIS

By

John Munro Nickerson

Three lines of investigation were used to obtain baseline data for further studies of the disease cystic fibrosis.

Carboxypeptidase B-like activity from Cohn Fraction IV-1 was purified using TEAE cellulose and Sephadex G-200. An 86.5 fold purification with 3.99% yield was obtained. Possible serum isozymes of carboxypeptidase B were found.

Using a modified method for phosphate determination, ATPase activities from human erythrocytes and mouse brain microsomes were measured with and without human serum proteins present. The serum proteins activated the ATPase activities, and possibly acted heterotropically.

Rabbit trachea epithelium was treated with cystic fibrosis or control serum and was examined after various incubation times by phase contrast microscopy. The differences in response of the tissue to the two sera were significant after 15 to 20 minutes incubation.

CELLULAR AND BIOCHEMICAL ASPECTS OF CYSTIC FIBROSIS

By

John Munro Nickerson

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Anatomy

1976

DEDICATION

To my parents, Mr. and Mrs. E. W. Nickerson, and my fiancée, Kathleen O'Neill.

ACKNOWLEDGMENTS

I wish to express my thanks to my committee, N. S. Henderson, C. W. Smith, C. C. Sweeley, and W. W. Wells. I would also like to thank W. F. Howatt, G. Polgar, and C. Wortley for obtaining serum samples; the National Cystic Fibrosis Foundation, the Michigan National Bank Trust Fund, and the Department of Anatomy for research funds; and K. M. O'Neill and D. Schmuck for technical assistance.

I would especially like to thank Dr. Henderson for her guidance and suggestions during this research program.

TABLE OF CONTENTS

	Page
LIST OF TABLES	vi
LIST OF FIGURES	vii
INTRODUCTION	1
LITERATURE REVIEW	3
METHODS	24
Preparation of Erythrocyte Ghost Membranes	24
Preparation of Brain, Skin, and Lung ATPases	25
ATPase Assay	26
Phosphate Determination	27
Protein Determination	28
Carboxypeptidase B-Like Activity	28
Assay Method	28
Fractionation of Cohn Fraction IV-1	29
Rabbit Trachea Ciliary Assay	29
Preparation of Tissue	29
Assay	31
RESULTS	32
CBLA Assay Validation	32
CBLA Purification From Cohn Fraction IV-1	32
Phosphate Determination	39
Results of ATPase Assays	52
Serum Fractions	59
Rabbit Trachea Ciliary Response to Sera	70
Incorporation of (^{3}H)-glucosamine Into Rabbit Trachea in the Presence of Sera (Control, C/F and FCS)	79
DISCUSSION	84
CBLA: Introduction	84
CBLA Assay and Purification	85

Page

Phosphate Determination 87
ATPase Assay Validation 88
The Effect of Serum Proteins on ATPase Activities 89
Rabbit Trachea Ciliary Bioassay 90
Future Experiments Using the Ciliary Bioassay 92
Incorporation Experiments 92

BIBLIOGRAPHY 96

LIST OF TABLES

Table		Page
1.	Clinical Symptoms of C/F	4
2.	CBLA From Cohn Fraction IV-1 Enzyme Purification Summary Sheet	44

LIST OF FIGURES

Figure		Page
1.	CBLA Assay Validation: Linearity of Hippuric Acid formation with time	34
2.	CBLA Assay Validation: Linearity of enzyme velocity (μMol hippuric acid formed per hour) with enzyme concentration	36
3.	Elution profile of CBLA and protein concentration from the 5 x 80 cm column of TEAE cellulose	38
4.	Elution profile of CBLA and protein concentration from the 3.5 x 170 cm Sephadex G-200 column	41
5.	Elution profile of CBLA and protein concentration from the 2.3 x 100 cm column of Sephadex G-200	43
6.	Estimate of phosphate concentration by determination of reduced phosphomolybdate	47
7.	Absorbance loss with time	49
8.	Rate of optical density loss vs. phosphate concentration	51
9.	Optical density of reduced phosphomolybdate with ethanolamine at wavelengths from 500 to 750 nm	54
10.	Optical density of molybdate, ascorbic acid and monoethanolamine compared to distilled water	56
11.	ATPase Assay Validation: Linearity of product (P_i) release with time	58
12.	ATPase Assay Validation: Linearity of enzyme velocity with enzyme concentration	61

Figure Page

13. Lineweaver-Burk plot: total ATPase of RBC ghosts with varying concentration of 30-50% cut of pooled human serum 63

14. Lineweaver-Burk plot: total ATPase from RBC ghost membranes with varying concentrations of the 30-50% cut 65

15. Lineweaver-Burk plot: total ATPase from RBC ghost membranes with varying concentrations of the 30-50% cut 67

16. Lineweaver-Burk plot: Na^+/K^+ ATPase from RBC ghost membranes 69

17. Lineweaver-Burk plot: ouabain-insensitive ATPase from RBC ghost membranes with varying concentrations of 30-50% cut 72

18. Lineweaver-Burk plot: ouabain-insensitive ATPase from RBC ghost membranes with varying concentrations of 30-50% cut 74

19. Lineweaver-Burk plot: total ATPase from mouse brain microsomes with varying concentrations of 30-50% cut 76

20. Expression of secretory and dyskinetic responses of rabbit trachea epithelium to sera with time 78

21. Incorporation with time of radioactive glucosamine into TCA precipitable material 81

22. Incorporation with time of radioactive glucosamine into TCA precipitable material 83

INTRODUCTION

The subject of this thesis is cystic fibrosis, a lethal genetic disease of high frequency, for which the basic defect is not known. The results presented within serve as requisite background for further investigations which will lead to an understanding of the pathology of cystic fibrosis and the corresponding normal physiologic situation.

The topics of investigation presented here are: carboxypeptidase B-like activity in Cohn Fraction IV-1, the effect of serum proteins on ATPases, and the effect of serum on rabbit trachea epithelium *in vitro*. The results reported here on the first two topics are concentrated on the investigation of the normal physiological condition, and the results on the third topic were directed toward the comparison of the effects of cystic fibrosis and control sera.

The reasons for the investigation of carboxypeptidase B-like serum activity was that a deficiency in the activity was suggested to be the basic defect in cystic fibrosis, and the question of possible existence of isozymes was raised.

The basis for the investigation of the effect of serum proteins on ATPases was that ATPase inhibitor materials of high molecular weight were reported in cystic fibrosis sera, saliva, and conditioned media; however, the mechanism of action of the corresponding control materials (which appeared to activate ATPases) was not analyzed.

The rationale for investigating the effect of sera on rabbit trachea epithelium was that the mechanism of the response of the ciliated epithelium to sera has not been elicited.

For each topic recommendations for further experimentation are given. Some of those experiments are already underway, while others will be started in the immediate future.

LITERATURE REVIEW

C/F (cystic fibrosis) is a simple autosomal recessive disease affecting 1 in 2,000 Caucasians, with lower frequencies in other races. The final result of homozygosity of the gene for C/F is death at an early age. Based on data obtained in 1972 Warwick et al. (1975) showed that the mean age for survival of 50% of C/F patients treated at C/F centers was 18.1 years. The most common cause of death in C/F is cor pulmonale, resulting from progressively increasing pulmonary hypertension, whose antecedent is chronic lung disease. The cause of disease in the lung is attributed to the buildup of thick, viscous mucus on which microorganisms can rapidly grow. This buildup of mucus is also found in the GI (gastrointestinal) tract. A particularly significant GI problem is that the pancreatic duct in many patients becomes clogged, and this blockage results in the degeneration of the pancreas with concommitant deficiency of pancreatic enzymes in duodenal fluid (di Sant'Agnese and Talamo, 1967). Clinical symptoms are listed in Table 1.

Despite the many and protean manifestations of C/F, one abnormality of C/F is present in all cases. di Sant'Agnese et al. (1953) showed that the sweat of patients with C/F was invariably high in the concentration of sodium and chloride. Mangos et al. (1967)

Table 1.--Clinical Symptoms of C/F.*

Failure to thrive
Shortness of breath
Barrel chest
Labored breathing
Noisy respiration
Muscular weakness
Cyanosis
Digital clubbing
Enlarged liver - Cirrhosis
Meconium ileus
Distended abdomen
Poor weight gain
Lack of subcutaneous fat
Pancreatitis
Diabetes
Salt depletion
Absence of vas deferens
Cervical polyps
Increased viscosity of sputum

*No one symptom is unique to C/F.

attributed these high concentrations to the lack of reabsorption of electrolytes in the straight portion of the sweat gland duct.

The available treatment of C/F is purely palliative. A rationale for more effective treatment awaits the discovery of the basic defect in C/F.

Researchers have been actively pursuing the basic defect in C/F for many years. Investigations comparing mucins from C/F and control subjects have been carried out. Dishe et al. (1959) were not able to find qualitative changes in the sugar contents of the mucins of C/F subjects, except that the fucose content was slightly elevated. However, Gugler et al. (1967) showed that the properties of C/F and control mucins were different. Using salivary mucins, the electrophoretic patterns on polyacrylamide gels were different. However, when EDTA (ethylene-diamine tetraacetic acid) was added to C/F saliva the pattern changed to that of the normal saliva, and when calcium was added to normal saliva the same pattern as with C/F saliva was obtained.

Other researchers have investigated differences in serum factors by biophysical methods. Wilson et al. (1975) distinguished C/F homozygotes, normals, and C/F heterozygotes using isoelectric focusing on thin layer polyacrylamide gels in a pH 5 to 10 gradient. Volumes of individual sera were applied to the gels with a standardized amount of IgG (300 μg). A band focusing with a pI = 8.46 was found in C/F homozygotes and C/F heterozygotes that was absent in control sera. Another band with an isoelectric point of 5.48, found

in control sera, was absent in the C/F homozygotes and C/F heterozygotes.

Researchers have used rabbit trachea as a model tissue for simulating the effect of C/F serum in vivo on human trachea. Spock et al. (1967) compared the effect of C/F and normal sera and plasma on rabbit tracheal epithelium. C/F sera were found to cause a dyskinetic beat in the rabbit tracheal ciliary movement. Seventy-five out of 75 C/F patients' sera gave the dyskinetic effect, while sera from none of the 75 patient controls with a wide variety of other diseases gave the dyskinetic effect. The test procedure was as follows. Rabbit ciliated tracheal epithelium was dissected from cartilage rings and was cut into small fragments. The tissue was maintained in 20% (V/V) rabbit serum and 80% (V/V) Medium 199 for 4 to 6 days. The assay was performed at room temperature by placing a drop of test serum on a piece of actively beating ciliated epithelium and observing the tissue under phase contrast microscopy at 550X. In attempts to identify the serum factor having the dyskinetic effect, serum from a C/F patient was fractionated on Sephadex G-200, and dyskinetic activity was found in a fraction of at least 200,000 daltons and in a fraction which eluted between 60,000-156,000 daltons.

Other researchers have used oyster gill tissue to investigate toxic factors in serum of C/F subjects. Bowman et al. (1969) reported that the time required for the oyster gill cilia dyskinetic response caused by serum of C/F patients and heterozygotes was significantly less than that caused by serum from control subjects. In 47 of 47 C/F subjects, in 19 of 19 heterozygotes, and in 2 of 64 control

subjects, the time required for ciliary dyskinesis was less than 35 minutes. The oyster gill cilia test was performed by examination of a 3 x 3 mm piece of gill tissue in a hanging drop preparation under phase contrast microscopy. A CD+ (ciliary dyskinetic positive) response was characterized by the accumulation of debris on the cilia and gill mounds and arhythmic beating of cilia. These investigators also noted a considerable variation in response between differing gill preparations, a loss of CD+ response after samples were repeatedly frozen and thawed, and a lack of repeatability in the time required for CD+ response using hemolyzed serum samples.

Bowman et al. (1970) used the oyster gill ciliary inhibition assay to show that a cationically migrating fraction of serum from C/F subjects on starch gels at pH 8.6 caused a CD+ response. No other fraction from the starch gel caused the dyskinetic response. They also demonstrated that on gel filtration using Sephadex G-200 a fraction of sera from 2 C/F patients and 1 heterozygote corresponding to roughly 125,000 to 200,000 daltons caused a CD+ response. Proteins found in this fraction included IgG (immunoglobulin G), IgA (immunoglobulin A), ceruloplasmin and haptoglobin. After DEAE (diethylaminoethyl) cellulose chromatography of serum from C/F, normal, and C/F heterozygotes, fractions containing mainly IgG as shown by immunoelectrophoresis were isolated and compared for oyster gill ciliary dyskinesis. The fractions from C/F and heterozygote serum caused CD+ response, while control sera fractions caused no dyskinesis.

Wood and di Sant'Agnese (1973) reported on the reliability of the bioassays for the C/F factor. They sent coded samples of C/F patient sera, heterozygote sera, and control sera to three research laboratories where the rabbit trachea or oyster gill cilia test was routinely used. The sera were prepared and shipped according to the individual laboratory's requests. Sera was stored at -30°C or on dry ice prior to and during shipment. No laboratory reported results that showed significant differences between C/F and control sera when Wood and di Sant'Agnese broke the code and analyzed the data.

Doggett and Harrison (1973) reported that 43 to 500 units of heparin per ml of secretion can prevent saliva of C/F and heterozygote subjects from causing ciliary dyskinesis on oyster gills. The addition of 1,000 units of heparin per ml of whole blood prevents ciliary dyskinesis, but after incubation overnight, the plasma regains its ciliary dyskinetic activity.

Researchers have attempted to demonstrate that explants of rabbit tracheal epithelium to which C/F serum is applied serve as an accurate model of the properties of C/F subject tracheal epithelium in vivo. Yeates et al. (1975) measured the rates of transport of albumin microspheres (0.5 μ mean diameter) coated with radioactive (^{99}Tc)-pertechnetate through the major air pathways. The progress of the radioactive spheres was monitored by a gamma camera or a scanning scintillation probe. The transport rates were tested for correlation against lung function tests, ciliary dyskinetic activity of the same patients' sera, Schwachman score, and clinical history. High ciliary dyskinetic activity and high maximum mid-expiratory flow

rate correlated with high values of mucociliary tracheal transport rates. In the performance of the ciliary dyskinesis assay, they noted that material was discharged prior to the interruption of ciliary rhythm. They suggest that the discharge of material is related to the abnormal mucus secretion in C/F subjects in vivo.

Conover et al. (1973a) modified the rabbit ciliary technique of Spock et al. (1967) to produce a technique giving higher reliability in the detection of CD+ response. The modifications were: first, maintenance of a 37°C temperature while the assay was performed; second, prior screening of tissue for use in the assay; and third, fast set-up and handling of the tissue to minimize tissue trauma. Conover et al. reported 12 out of 12 ciliary dyskinesis positive sera from C/F patients and 2 out of 29 ciliary dyskinesis positive sera from the control group. Conover et al. also suggested that the ciliary dyskinesis factor is a normally present molecule which is over-produced in C/F, not properly inactivated, or inhibited in C/F.

Conover et al. (1973b) cultured leukocytes from 3 C/F patients, 2 heterozygotes, and 4 controls. They found that the conditioned medium from leukocytes of the 3 C/F patients and the 2 heterozygotes caused ciliary dyskinesis, but that none of the controls' conditioned media caused ciliary dyskinesis with rabbit trachea.

The conditioned media from long term lymphoid lines from 3 C/F patients and from 4 heterozygotes also produced ciliary dyskinesis; but in 2 lymphoid lines from controls, there was also a

ciliary dyskinetic response from the conditioned medium. Rabbit anti-human IgG added to ciliary dyskinetic positive medium (incubated overnight at 4°C and centrifuged to remove the precipitate) caused the removal of the ciliary dyskinetic positive response from the supernatant solution.

Beratis et al. (1973) cultured skin fibroblasts from C/F patients, heterozygotes, and controls. When conditioned media were tested for ciliary dyskinesis by the rabbit tracheal ciliary test of Conover et al. (1973a), 1 out of 16 control subject media were CD+, 13 out of 13 C/F patient media were CD+, and 6 out of 6 heterozygote media were CD+. Beratis et al. also cultured amniotic fluid cells and found one CD+ medium in 10 control amniotic cell lines and in 4 out of 4 amniotic cell lines in which both parents' sera were CD+. They reported that the addition of IgG was necessary to obtain CD+ results in all fibroblast lines, but that the addition of IgG had no effect on the ciliary dyskinetic negative fibroblast lines.

Rabbit anti-Human IgG treated media gave all ciliary dyskinetic negative results. By fractionation of ciliary dyskinetic positive media using Amicon ultrafiltration, a fraction of approximate molecular weight of 1,000 to 10,000 could be obtained to which IgG was added caused a CD+ response. A similar fraction of ciliary dyskinetic negative media to which IgG was added did not give a ciliary dyskinetic positive response.

Cheung and Jahn (1976a) reported that there is no change in the shape of a rabbit tracheal cilium throughout its forward and return bowing motion. They observed cilia via high speed

microcinematography and documented that the cilia penetrate into the mucus layer for 5-8° of the arch through which the cilium moves (a total of 35-40°). They also demonstrated that the cilium beat is more vigorous in the cephalic direction than in the return stroke.

Using the same methods Cheung and Jahn (1976b) reported that by studying high speed microcinematography, C/F serum has no effect on the rhythm or the beat pattern of rabbit trachea ciliated epithelium. They reported that the C/F serum causes the expulsion and release of cells and debris from the epithelium and that so-called dyskinesia is a secondary effect of the C/F serum caused by drag forces applied to the functioning cilia.

Satir (1975) found that the addition of calcium with ionophore A23187 arrested the lateral cilia of mussel gills. Neither calcium alone nor A23187 alone caused ciliary arrest. Satir suggested the hypothesis that a local rise in internal calcium concentration causes ciliary arrest.

Bowman et al. (1973) fractionated conditioned medium of fibroblast cultures from C/F patients and heterozygotes on DEAE cellulose and Sephadex G-200. Three fractions were obtained that exhibited a ciliary dyskinetic positive response. No IgG could be detected in any of the 3 fractions. These 3 fractions corresponded to molecular weights of approximately 13,000, 20,000 and 150,000 daltons.

The molecular weight of the ciliary inhibitor from C/F fibroblast conditioned medium was estimated by Barnett et al. (1973a) using sucrose density gradient centrifugation, membrane filtration

and gel filtration. About 500 ml of medium was passed through Sephadex G-15, and the protein fraction eluting at the void volume was applied to a DEAE cellulose column. The fractions eluted from that column were concentrated by membrane ultrafiltration on a filter which retained material of molecular weight greater than approximately 1,000 daltons. The concentrated fractions were tested for ciliary dyskinetic activity. An aliquot of the fraction causing ciliary dyskinesis was put on a 5-20% (W/V) sucrose gradient and was centrifuged. After dialysis, using 3,500 dalton retaining dialysis tubing, the fractions of the sucrose gradient were assayed for ciliary dyskinesis. The fraction exhibiting a CD+ response correlated to a substance of molecular weight of about 9100 daltons. On a calibrated Sephadex G-50F column, the ciliary dyskinetic activity eluted at a volume corresponding to a molecular weight of 4,500 to 10,000 daltons. By assaying the eluants and retentates of ultrafilters having molecular weight retention of greater than 100,000, 50,000, 10,000 and 1,000 daltons, the ciliary inhibitor was estimated to have a molecular weight between 10,000 and 1,000 daltons.

Barnett et al. (1973b) reported the fractionation of conditioned medium from C/F patient-derived fibroblasts on DEAE cellulose. The fraction containing CD+ material was treated with pepsin or papain. Both treatments removed ciliary dyskinetic activity. Other aliquots of CD+ material were treated for 15 minutes at temperatures of 25°C, 50°C, 80°C, and 100°C. Only the material treated at 25°C for 15 minutes retained the ciliary dyskinetic positive response.

Schmoyer et al. (1972) fractionated C/F heterozygote serum on DEAE cellulose equilibrated with 0.005 M Tris at pH 7.5 and found that the material responsible for ciliary dyskinetic activity did not bind to DEAE cellulose. The fraction of serum not binding to the DEAE cellulose was applied to a CM (carboxymethyl) cellulose column, and material was eluted in a stepwise fashion. The fraction causing ciliary dyskinesis was eluted only in the first stepwise eluant which contained 0.04 M Tris-phosphate at pH 7.5. This fraction contained less than 2% of the original amount of protein. The first fraction of the CM cellulose eluate was subjected to isoelectric focusing on pH gradients of 7 to 9 and 3 to 10. On the 3 to 10 gradient the fraction exhibiting ciliary dyskinetic activity was between pH 8 and 9. When fractionated on the 7 to 9 gradient, the fraction exhibiting ciliary dyskinetic activity was resolved into three fractions. These three fractions yielded one single band which migrated the same distance on polyacrylamide gels.

When the isoelectric-focused fraction giving ciliary dyskinesis was tested for antigenicity against rabbit monospecific anti-IgG, -IgM and -IgA, only anti-IgG cross-reacted. Schmoyer et al. concluded that the ciliary dyskinesis factor belonged to the IgG class or was bound to IgG.

Herzberg et al. (1973) showed that the oyster gill ciliary inhibitor did not behave as an antibody. A preparation of C/F IgG (the IgG fraction of C/F patient serum) was shown to cause a CD+ response. On double immunodiffusion with sonicated oyster gill ciliary epithelium, anti-ciliary epithelium, saline control, and the

C/F IgG fraction, there were two precipitation lines shown--two between anti-ciliary epithelium and ciliary epithelium. There was no precipitin line between C/F IgG and the ciliary epithelium. There was no alteration of the precipitin lines by the C/F IgG.

Barnett et al. (1973c) reported that the ciliary inhibitor has an isoelectric focusing point (pI) of 9.1 to 9.3 and was associated with IgG1. The inhibitor was obtained from conditioned media and purified on DEAE cellulose by the method of Barnett et al. (1973a).

Danes et al. (1973) presented evidence that the cystic fibrosis factor (CFF) is bound to immunoglobulin. Using the oyster gill cilia assay and used medium from C/F and normal fibroblasts, they noted that the factor had the following properties: had a molecular weight less than 5,000 daltons, contained no uronic acid, was pH and heat labile, was negatively charged, and was bound to either IgG1 or IgG2. They showed that the interaction of the CFF with the immunoglobulins was not an antigen-antibody reaction. Their method was to incubate CD+ medium with immunoglobulin fragments containing antibody binding sites. Then the immunoglobulin fragments were precipitated. The CFF activity remained in the supernatant. When the constant regions of immunoglobulins were incubated with CD+ medium and the immunoglobulin fragments precipitated, the CD+ activity was removed from the supernatant.

Conover et al. (1974) presented evidence that suggested that the factor causing ciliary dyskinesia is a complex of the complement factor C3a and IgG. Beratis et al. (1973) had previously shown that IgG was required to elicit ciliary dyskinetic activity and that

there was a factor of molecular weight between 1,000 and 10,000 daltons present in fibroblast media of C/F patients that was also necessary for dyskinetic activity. Conover et al. (1974) showed that the second factor was also present in the 1,000 to 10,000 molecular weight fraction of serum. They also showed that when control serum, previously shown not to be CD+ was incubated with EACA (ε-aminocaproic acid), the serum caused a CD+ response. The effect of EACA alone on rabbit trachea cilia was not noted in this paper. (EACA is known to inhibit anaphylatoxin inhibitor.) They also noted that when CD+ sera, either from EACA-stimulated or from C/F patients, were incubated with carbosypeptidase B (di-isofluorophosphate treated, highly purified) that the responses on rabbit trachea cilia changed to ciliary dyskinetic negative.

Conover et al. (1974) tested purified C3a with IgG on rabbit trachea and reported a ciliary dyskinetic positive response. When an anti-C3a was added to C3a plus IgG, a slightly ciliary dyskinetic response was noted, and when the 1,000 to 10,000 molecular weight fraction and IgG were treated with anti-C3a, the same response was noted.

Conod et al. (1975) reported that sera from C/F patients caused more degranulation of lysosomal enzymes from sensitized PMN's (polymorphonuclear leukocytes) than the sera of control subjects. They also reported that heterozygote sera caused the degranulation of an intermediate amount of lysosomal enzymes. They tested sera from 5 control subjects, 7 C/F patients, and 8 heterozygotes. PMN's from healthy donors were sensitized with Cytochalasin B, a fungal

metabolite which prevents phagocytosis and allows lysosomes to open at the cell membrane when a phagocytic-inducing material is detected at the cell surface. The amount of lysosomal release was estimated by the measurement of activity of B-glucuronidase and myeloperoxidase released into the media surrounding the PMN's. Serum levels of both enzymes were measured and subtracted from the appropriate sample of PMN's treated with serum. Conod et al. suggested that the molecules which caused the greater amount of degranulation of PMN's by C/F sera might also cause excessive degranulation of exocrine gland cells, resulting in blockage of exocrine gland ducts. They also suggested that the PMN degranulators are the same as the ciliary inhibitors.

Conover et al. (1973c) presented data that implicated anaphylatoxin inactivator as the site of the primary gene defect in C/F. They showed that in 3 C/F, 3 heterozygote and 3 control sera samples, there were no significant differences in the levels of complement components C1 through C9, whole complement, or C3 inactivator (by hemolytic complement assays). In tests of the amounts of C1q, C4, C3 and C3-proactivator by radial immunodiffusion, C3 was found to be elevated. These investigators did not indicate the level at which the differences were significant. They stated that commercial rabbit anti-human C3 will also cross-react with C3a or C3a bound to IgG, and suggested that high levels of C3a, not C3, were detected by radial immunodiffusion.

Lieberman (1975) studied the hypothesis put forward by Conover et al. (1974) that CBLA should be deficient in C/F patients and give rise to high C3a levels in C/F serum. (C3a was hypothesized

to be the cystic fibrosis factor.) Lieberman tested the levels of serum CBLA (carboxypeptidase B-like activity) in C/F patients, control patients with chronic lung disease, and healthy control subjects. He found no significant difference between C/F patients and healthy controls. But he found that patients with other chronic lung diseases had significantly higher levels of CBLA than healthy controls.

In addition, Lieberman (1974) found that C3 levels in C/F patients were not significantly different from controls. He tested 14 C/F patients and 15 controls by radial immunodiffusion.

CBLA in serum was characterized by Erdös et al. (1964, 1967), and its function as an inactivator of C3a was described by Bokisch and Müller-Eberhard (1970).

Erdös et al. (1964) described a carboxypeptidase that cleaves C-terminal basic amino acids from polypeptides and hydrolyzes hippuryl-L-lysine, hippuryl-L-arginine, Hippuryl-L-ornithine, and N-acetyl-phenylalanyl-L-arginine. They found this carboxypeptidase B-like activity in sera of many animals, in human urine and thrombocytes, but did not find this activity in erythrocytes. The enzyme was activated by $CoCl_2$ and, to a lesser extent, by $NiSO_4$. It was inhibited by EACA, benzoyl-L-arginine, and chelating agents. The optimum pH for CBLA varied according to the choice of buffer. But with most buffers tested, the optimum was between pH 7.0 and 8.5. Hippuryl-L-phenylalanine as a substrate gave no measurable activity with human serum, indicating to Erdös et al. that there was no carboxypeptidase A-like activity in serum.

Erdös et al. (1967) partially purified the CBLA of human plasma by: first, precipitation between 30% and 55% saturated ammonium sulfate; second, dialysis and fractionation on DEAE-Sephadex A-50 with stepwise elution; and, finally, a second fractionation on a DEAE-Sephadex A-50 column using smaller increments of increasing salt concentrations in stepwise elution. The purification achieved was about 250-fold.

Many experiments have used fibroblasts in cell culture derived from skin biopsies of C/F and control subjects. Differences between C/F fibroblast cells and control fibroblast cells have been demonstrated to complement the differences indicated above with the conditioned medium from the fibroblast cultures.

Barnett et al. (1973d) showed that conditioned media from fibroblasts of C/F patients caused ciliary dyskinetic positive responses. They also showed that the concentration of ciliary inhibitor increased linearly with increasing cell number and that the inhibitor continues to be produced in stationary phase C/F fibroblasts.

Barranco et al. (1976) reported that C/F fibroblasts incorporated only one half the tritiated thymidine that normal fibroblasts did. Heterozygote fibroblasts incorporated an amount between the 2 above quantities. Barranco et al. matched fibroblasts according to culture age, growth fraction, and doubling time. They tested the possibility that TK (thymidine kinase) of C/F and heterozygote fibroblasts were lower than normal fibroblasts, suggesting that altered TK activity could account for the lower amount of thymidine

incorporated into DNA. Barranco et al. found the activity of TK to be the same in all fibroblast lines--C/F, heterozygote and control. Another finding was that C/F fibroblasts could not be synchronized by the excess thymidine block technique, while control fibroblast lines were quickly synchronized. These 2 results indicated to Barranco et al. that thymidine transport was altered. They further suggested that this hypothesis was consistent with other data indicating that membrane transport is abnormal in C/F.

In a number of studies the transport of electrolytes and the electrolyte levels in C/F and control subjects' fluids and cells have been investigated.

Blomfield et al. (1976) collected parotid saliva from C/F patients and child controls and compared the levels of calcium, potassium, sodium, phosphate, salivation flow rate, and amylase. Of the above materials, only calcium concentrations differed significantly, with Ca^{2+} concentrations from C/F patients being the higher. Salivation in these studies was stimulated by 5% (W/V) ascorbic acid solution placed on the tongue every 15 seconds. Previous studies using lozenges or other materials to stimulate salivary secretion had given inconclusive results.

Duffy et al. (1973) reported that C/F serum, plasma and IgG fractions have no increased capacity for binding Ca^{2+}. Ten control serum samples and 9 C/F serum samples were used. It was shown that plasma from 8 patients with IgG myeloma had an elevated capacity to bind Ca^{2+}.

Araki et al. (1975) demonstrated that C/F, heterozygotes and control sera differentially affect the resistance and the short circuit current of rat jejunum. SCC (short circuit current) is defined as the potential difference between the luminal side of the rat jejunum and the serosal side divided by the membrane resistance. It was shown that C/F serum caused the SCC to drop by almost a factor of 2, while heterozygotes' sera caused a drop by about a factor of 1.5. When C/F and control sera were heat treated (56°C for 30 minutes), there were no significant differences between SCC's of the two sera. There were no differences between heat-treated control sera and control sera not heat-treated. These effects were only noted when glucose was included in the bathing solution, and the effects on the SCC were greater when glucose was added only to the luminal side. Araki et al. suggested that the effect on SCC was due to glucose dependent Na^+ transport. However, they stated that the stimulation of Na^+ transport by glucose did not alter SCC. Preliminary experiments suggested that the effect of C/F serum added to the serosal side was less effective than the luminal.

Rennert (1976) presented data which showed that RNA from C/F patient and heterozygote fibroblasts or leukocytes is undermethylated. He isolated RNA by phenol extraction from cells that had been grown in the presence of tritiated uridine and (^{14}C-methyl)-methionine. Studies on the transport of methionine indicated that there was no difference between C/F and control fibroblasts. Rennert suggested that there is a defect in the metabolism of methionine, not involving the RNA methylases. He indicated that undermethylation was due to

overproduction of polyamines. Additional studies showed that there were statistically significant increases in spermidine and an unidentified polyamine product in C/F patient serum as compared to control serum. Rennert (1976) in conjunction with Mangos, has shown that 0-methyl glucose transport in the rat jejunum is inhibited by spermidine added to control serum, by C/F patients' serum, and heterozygote serum when compared to control serum. Another experiment showed that C/F serum treated with a crude preparation of polyamine oxidases gave the same effect on the transport velocity of 0-methyl glucose as control serum.

Cole and Dirks (1972) reported that there were no significant differences between the ouabain-sensitive component of ATPase in erythrocyte ghost membranes from C/F and control subjects. They found a significant decrease in the activity of ouabain-insensitive ATPase from C/F erythrocytes when compared to control erythrocytes. In a second series of experiments, Cole and Dirks incubated normal erythrocyte ghost membranes with serum from control and C/F subjects. After a 16 hour incubation at 37°C, the ouabin-sensitive ATPase activity of the ghosts was determined. A significant decrease in activity of the C/F serum-treated preparation compared to the control serum-treated ghosts was noted.

Cole and Sella (1975) presented data that saliva from patients with C/F causes a decrease in ouabain-sensitive ATPase of erythrocytes when compared to the effects of saliva from control subjects. They incubated saliva adjusted to isotonicity with 1.0 M NaCl plus human erythrocytes for 18 hours at 37°C. Then they prepared ghost membranes

from the incubated erythrocytes and assayed the membranes for ATPase activities. Ghost preparations incubated with C/F patient saliva had significantly lower specific activities of Na^+, K^+, Mg^{2+} ATPase, ouabain-sensitive ATPase and Ca^{2+} activated ATPase.

In a second experiment, samples of C/F and control saliva were filtered in a Millipore filtration cell with a membrane filter that retained molecules of molecular weight greater than 1,000 daltons. To the retained material, water was added and the procedure was repeated until the phosphate concentration in the retentate of the saliva was less than 0.2 mM. The retentate was added to prepared erythrocyte membrane ghosts, and the ATPase activities were assessed. No significant difference was found between total ATPase activities of C/F and control retentate treated membranes. However, the ouabain-sensitive ATPase activities were significantly different with the C/F treated samples lower by almost 16% ($P < 0.05$). In 9 of 10 samples the ouabain-sensitive component was less. Ca^{2+} ATPase was not assessed in the second set of experiments.

Schmoyer and Baglia (1974) reported that a factor was present in the conditioned medium from stationary phase fibroblast cultures from C/F subjects that resulted in decreased activities of human erythrocyte ghost Na^+/K^+ ATPase, Mg^{2+} ATPase, and Ca^{2+} ATPase when compared to used medium of a normal fibroblast line. With both C/F and control conditioned media, the activities were higher than the activities of the ATPase when 0.02 M TES at pH 7.5 was added to the erythrocyte ghost membranes in place of the conditioned media. However, the activities of the ghost membranes were higher when unused

medium was added to the ghost membranes in place of the used media. From these data it cannot be ascertained whether there is an activator and inhibitor, a differential inactivation of the activator, or some other form of action on the ghost ATPases. However, it is clear that the C/F media effect on the ghost membranes was different from the control media.

METHODS

Preparation of Erythrocyte Ghost Membranes

Membrane ghosts were prepared by the osmotic hemolysis method of Hanahan and Eklolm (1974). Outdated whole blood, usually A positive or 0 positive, was obtained from the Lansing Regional American Red Cross. Plasma and residual buffy coat were separated from red blood cells by the following steps. About 50 ml whole blood were mixed with 200 ml of 0.15 M NaCl, and the solution was centrifuged at 4°C for 10 minutes at 2000 X g. The supernatant and buffy coat were removed by suction aspiration. The above steps were performed 3 times. The washed red blood cells were lysed by resuspension in 11.1 mM Tris at pH 7.5. Two ml of the packed red blood cells were transferred to 50 ml centrifuge tubes and 10 ml of the 11.1 mM Tris buffer were jetted into the centrifuge tube and the mixture was briefly shaken on a Vortex mixer at setting 4. Then an additional 20 ml of 11.1 mM Tris buffer were added. The tubes were centrifuged at 40,000 X g for 20 minutes. This centrifugation was sufficient to bring down the red cell membranes. The supernatant solution was removed by suction aspiration and the membranes were resuspended in 11.1 mM Tris buffer as above. The ghost membranes were washed by this method until they were pearly white, taking three to four centrifugations typically.

The ghost membranes were stored at 4°C, if they were to be used within 2 days; otherwise they were stored at -70°C.

Preparation of Brain, Skin, and Lung ATPases

The microsomal fractions of brain, skin and lung tissue from mice of the CD-1 strain were isolated by the method of Akera and Brody (1968, 1969). Tissue was homogenized with a Teflon pestle glass homogenizer in 5 ml HS (homogenizing solution: 0.25 M sucrose, 5.0 mM histidine, 5.0 mM disodium ethylenediamine tetraacetic acid, and 0.01 mM DTT (dithiothreiotol) at pH 7.0) plus 0.15% (W/V) sodium deoxycholate. Fifteen ml of HS were added to the homogenate, and the mixture was centrifuged at 11,000 X g for 15 minutes at 4°C. The supernatant was decanted and saved. The pellet was resuspended in 10 ml HS plus 0.1% (W/V) sodium deoxycholate and homogenized for about half as long as the first homogenization. The mixture was centrifuged at 11,000 X g for 15 minutes at 4°C. The resulting supernatant was combined with the first supernatant, and this solution was centrifuged at 100,000 X g_{av} for 30 minutes at 4°C in a Type 40 rotor. The supernatant was discarded and the pellet was resuspended in RS (resuspending solution: 0.25 M sucrose, 5.0 mM histidine, and 1 mM disodium ethylenediamine tetraacetic acid at pH 7.0) plus 0.01 mM DTT. Care was taken to thoroughly disperse pellets to prevent aggregated microsomes from being spun down with faster sedimenting material in the following centrifugations. This was done by homogenizing the pelleted material briefly in a Teflon pestle glass homogenizer. The next centrifugation was performed to separate the microsomal fraction from the mitochondrial fraction. The solution was

centrifuged at 20,000 X g_{av} for 20 minutes. The supernatant containing the microsomal fraction was centrifuged at 100,000 X g_{av} for 30 minutes. The pelleted microsomes were resuspended in NaI solution (2.0 M NaI, 2.5 mM Na_2EDTA, 3.0 mM $MgCl_2 \cdot 6H_2O$, and 5.0 mM histidine) plus Na_2ATP to 2.0 mM and stirred gently for 30 minutes at 4°C. The resuspended material was effectively solubilized at 2.0 M NaI, and the solution was adjusted to 0.8 M NaI with 1.0 mM Na_2EDTA. The mixture was centrifuged at 100,000 X g_{av} for 30 minutes and the resulting pellet, containing material insoluble at 0.8 M NaI was found to contain the ATPase containing microsomes. The pellet was resuspended in Tris-EDTA (10 mM Tris and 1.0 mM Na_2EDTA at pH 7.5) to wash out the previous solution and centrifuged at 100,000 X g_{av} for 30 minutes. The pellet was resuspended in RS, aliquoted, and stored at -70°C.

ATPase Assay

ATPase activity was estimated by determining the amount of P_i (inorganic phosphate) present at the end of an incubation period. A blank containing all reagents but omitting the enzyme or containing inactivated enzyme was also incubated and any P_i in it was subtracted from the first tube to determine the amount of P_i released. All assay tubes were incubated in a 37°C water bath. All reactants were added to each tube in an ice bath, and the reaction was initiated with the addition of enzyme. The tube was shaken on a Vortex mixer (setting #4), and the tube put in a 37°C water bath. The total volume in each tube was 1.0 ml. Each tube typically contained 100 µl (550 µg protein) RBC ghost membranes, skin microsome (33 µg protein),

or lung microsomes (39 µg protein), or 50 µl brain microsomes (33 µg protein), 200 µl of 5X concentrated ATP solutions, 0 to 200 µl activator or inhibitor solution and 11.1 mM Tris at pH 7.5 to 1.0 ml final volume. Each tube contained final concentrations of 100 mM NaCl, 20 mM KCl, and 20 mM Tris at pH 7.5. ATP final concentrations were 1.5, 1.0, 0.75, 0.60, 0.50, 0.40, or 0.33 mM disodium adenosine triphosphate trihydrate. The tubes were commonly incubated for 20 to 40 minutes and the reaction was terminated by the addition of 0.4 ml of ice-cold 10% (W/V) TCA (trichloracetic acid). The tubes were immediately put in an ice bath and then centrifuged at 1500 X g_{av} for 30 minutes at 4°C. One ml of the supernatant was transferred to a clean test tube and the concentration of P_i was determined as below. Phosphate standards of 0.30, 0.25, 0.20, 0.15, 0.10, 0.05 and 0.00 mM P_i were run with each set of assays.

Phosphate Determination

The P_i concentration was estimated spectrophotometrically via modification of the method of Ames (1966) and the method of Drewes (1972). To 1 ml of TCA-deproteinized solution, 0.4 ml of acid molybdate (4.4% (W/V) ammonium molybdate in 1.0 N H_2SO_4) was added, then 0.4 ml of 8% (W/V) ascorbic acid was added. Within 30 seconds of the addition of ascorbic acid, 1.0 ml of monoethanolamine in a syringe was jetted into the tube while it was being shaken on a Vortex mixer (setting #4). The absorbance of the sample was read at 580 nm within 2 to 3 minutes following the addition of monoethanolamine.

Protein Determination

Protein concentration was determined by the method of Lowery et al. (1951). BSA (bovine serum albumin) standards of 0, 40, 80, 120, 160, 200 µg BSA/ml were tested with each set of unknowns.

Carboxypeptidase B-Like Activity

Assay Method

CBLA (carboxypeptidase B-like activity) was assayed by the method of Lieberman (1975) using HLL (hippuryl-L-lysine) as substrate. Buffer N (500 mM potassium phosphate and 750 mM NaCl at pH 8.0) was made 12.5 mM HLL. Fifteen one-hundredths ml of enzyme preparation (either serum, Cohn IV-1, or an aliquot of fractionated serum or fractionated Cohn IV-1) was added to 0.1 ml of Buffer N plus HLL. The mixture was briefly shaken on a Vortex mixer and incubated at 37°C in a water bath or in a 37°C room for 60 minutes. To the incubation mixture 0.25 ml of 1 N HCl was added to stop the reaction. Hippuric acid was extracted from HLL by adding 1.5 ml of ethyl acetate and shaking the tube containing the reaction mixture and ethyl acetate on a Vortex mixer (setting #4) for 15 seconds. The tube was centrifuged for 2 minutes at 1,000 X g to separate the two phases. One ml of the upper phase containing the ethyl acetate solution was transferred to a clean test tube using a semiautomatic pipettor. The ethyl acetate was evaporated at 120°C for 30 minutes. The dried contents were resuspended in 1 ml of water and the optical density at 228 nm was read using a Beckman 24 spectrophotometer.

Fractionation of Cohn Fraction IV-1

Forty grams of Cohn Fraction IV-1 paste were suspended in 2,000 ml potassium phosphate buffer (50 mM at pH 8.0), and the solution was filtered twice through approximately 2,000 ml of packed TEAE (triethylaminoethyl) cellulose in a 24 cm Buchner funnel. TEAE cellulose was precycled by suspending the powder to 7% (W/V) in 0.5 N HCl, filtering, and resuspending to 7% (W/V) in 0.5 N NaOH, and washing on a Buchner funnel with equilibration buffer (50 mM potassium phosphate at pH 8.0). Material was eluted from the TEAE cellulose with 2,000 ml each of 0.1 M, 0.3 M and 1.0 M NaCl in equilibration buffer. The eluant from the 0.3 M NaCl wash and the latter half of the 0.1 M NaCl wash was applied to a 5 x 80 cm column of TEAE cellulose and eluted with a linear 0 to 0.3 M NaCl gradient of 2,000 ml volume. Pooled fractions were concentrated using XM 100A Amicon ultrafilters. Ultrafiltration was carried out at 4°C and the concentrate was applied to a 3.5 X 170 cm column of Sephadex G-200. The eluant was equilibration buffer. The active fractions were pooled and concentrated by ultrafiltration as before. The concentrate was applied to a second Sephadex G-200 column with dimensions of 2.3 X 100 cm and was eluted with equilibration buffer. The active fractions were lyophilized.

Rabbit Trachea Ciliary Assay

Preparation of Tissue

The ciliated epithelium used in the rabbit trachea ciliary assay was obtained from a New Zealand white rabbit weighing more than

7 pounds. The rabbit was killed by cervical dislocation and the trachea was exposed as rapidly as possible. The trachea was cut first at the carina and then at the larynx, and put in sterile Hank's Balanced Salt Solution. The excised trachea was typically 4 to 5 cm in length. All further steps were performed in a laminar flow hood using sterile technique. All media were warmed to 37°C before use. Surrounding serosa, fat, and debris were dissected away from the trachea. The trachea was rinsed with sterile Medium 199 by transferring the tissue into and out of two 15 X 100 mm petri dishes containing the solution. The trachea was cut into much shorter cylinders containing two cartilage rings by using a #22 surgical blade. These rings were cut at the cartilage defect (posterior wall) and 5 or 6 pieces were put into 10 X 35 mm culture dishes containing Medium 199 plus 10% (W/V) FCS (fetal calf serum). The pieces of tissue were temporarily put in a 37°C, 5% (V/V) CO_2 incubator. The ciliated epithelium was removed from the cartilage rings by: first, pinning the tissue to a sterilized wax block using two 25 gauge needles; second, nicking the epithelium at one end of the tissue; third, pulling up on the ciliated epithelium with very fine dissecting forceps, while gently nicking between the epithelium and the submucosa with a #10 surgical blade. After the epithelium was dissected off, it was cut into 15 to 20 pieces of roughly 1 X 1 mm size. These explants were transferred to 10 X 35 mm culture dishes containing 1.5 ml of Medium 199 with 10% (V/V) FCS. The medium was changed 4 or more times in 48 hours. Explants were kept at 37°C and 5% (V/V) CO_2 in a water jacketed incubator.

Assay

An explant was transferred to an ethanol-cleaned coverslip, and excess culture medium was removed with a pasteur pipet. Two drops of test serum were put on the explant, excess serum was removed, and the coverslip was mounted on a hanging drop slide. The hanging drop preparation was put on a microscope stage, maintained at approximately 37°C by an air curtain incubator. The explant was observed with a conventional phase contrast microscope at 200 X. Microcinematography was performed using the above method with a 16 mm film camera. When films were not being made, the explant was put on an ethanol-cleaned microscope slide and two drops of serum were added. The explant was observed under phase contrast with an inverted microscope. If the air curtain incubator was not used, the samples were kept in a 5% (V/V) CO_2 incubator and observed for 10-15 seconds at room temperature at 5 minute intervals for up to 30 minutes. Characteristics noted were: the appearance of secreted or expulsed debris at the ciliary border, the change from rhythmic to dyskinetic beating of cilia, and the stoppage of beating of cilia.

RESULTS

CBLA Assay Validation

The method used by Lieberman (1975) to assay for CBLA was tested to check assay validity. In Figure 1, product formation (hippuric acid) was shown to be linear with time, up to 2 hours incubation at 37°C between 0.025 mg/ml and 0.1 mg/ml of enzyme preparation. In Figure 2, it was shown that the rate of reaction is linearly proportional to the enzyme concentration.

CBLA Purification From Cohn Fraction IV-1

The procedure used to purify CBLA from Cohn Fraction IV-1 was given in the Methods section. Approximately 240,000 units (1 unit equals 1 μMol hippuric acid released per hour) of CBLA was applied to a 24 X 5 cm column of TEAE cellulose in a Buchner funnel as a batch separation. The last 1,000 ml of the 0.1 M NaCl eluant and all 2,000 ml of the 0.3 M NaCl eluant were pooled. This fraction contained 85.2% of the original activity and gave a fold purification of 1.95. This material was applied to a 5 X 80 cm column of TEAE cellulose. The elution profile is shown in Figure 3. One peak of CBLA is seen between fractions 97 and 121. A second peak of CBLA is seen between fractions 133 and 148. A peak of high protein concentration is seen centered at fraction 109. The protein concentration tailed off from a

Fig. 1.--CBLA Assay Validation: Linearity of Hippuric Acid formation with time. Hippuryl-L-lysine was incubated with a preparation of 147 units activity per mg protein. (●), 100 µg/ml; (■), 75 µg/ml; (▲), 50 µg/ml; and (⬢), 25 µg/ml enzyme final concentration.

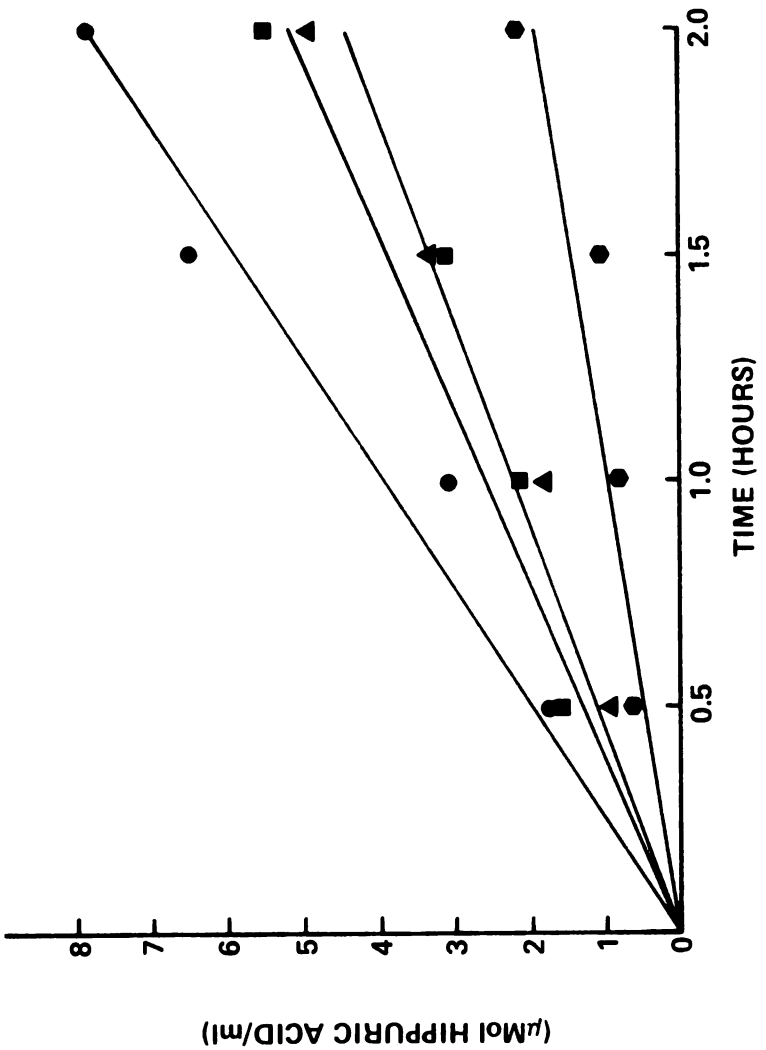

(μMol HIPPURIC ACID/ml)
0
1
2
3
4
5
6
7
8
0.5
1.0
1.5
2.0
TIME (HOURS)

Fig. 2.--CBLA Assay Validation: Linearity of enzyme velocity (μMol hippuric acid formed per hour) with enzyme concentration. The slopes (velocities) of the lines in Figure 1 were plotted against enzyme concentration.

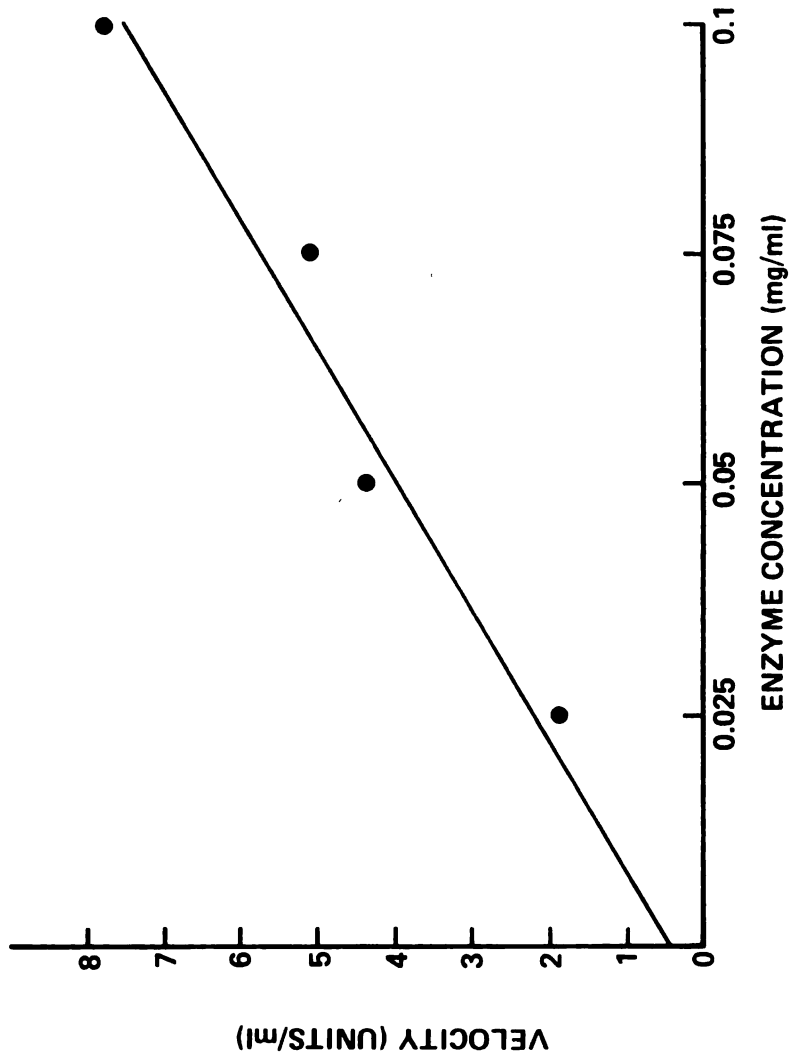
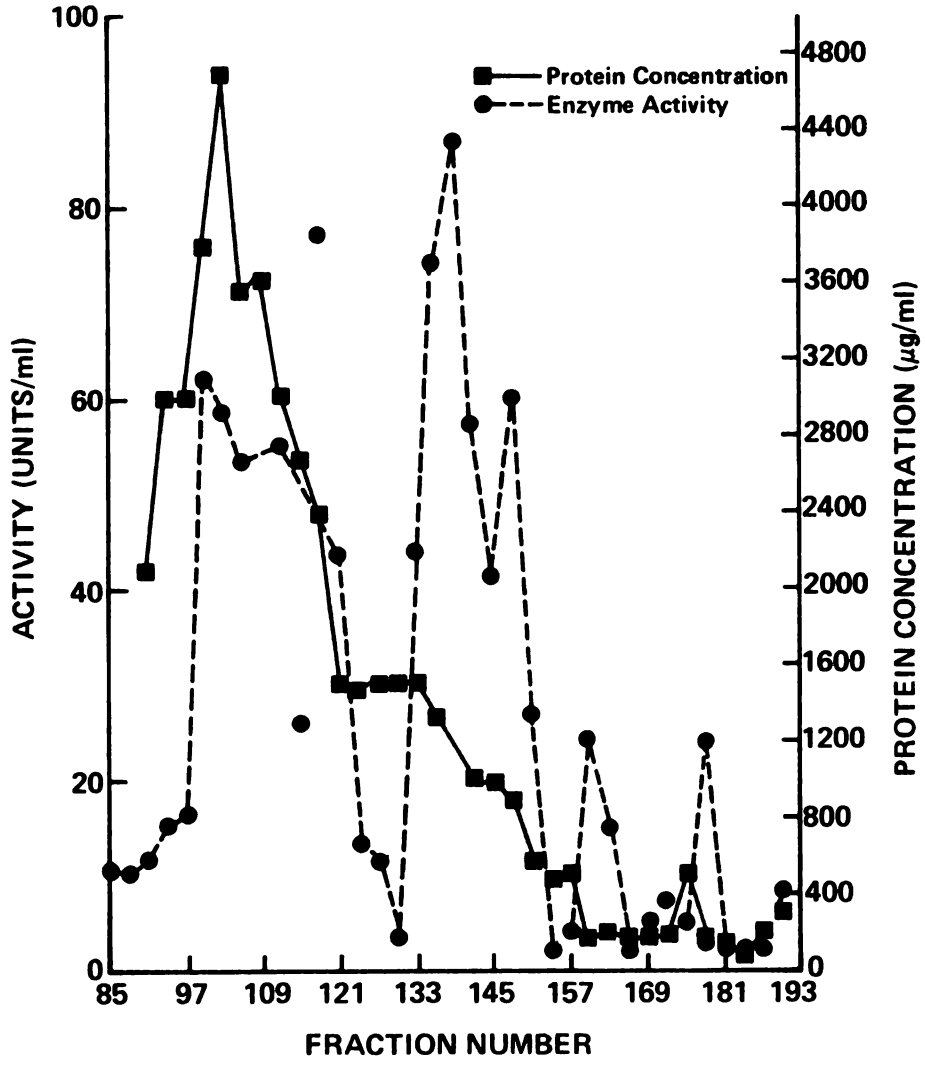

VELOCITY (UNITS/ml)
0
1
2
3
4
5
6
7
8
0.025
0.05
0.075
0.1
ENZYME CONCENTRATION (mg/ml)

Fig. 3.--Elution profile of CBLA and protein concentration from the 5 x 80 cm column of TEAE cellulose. A batch of 3000 ml of dialyzed material from the Buchner funnel column was applied to the column and developed with a linear 0.0 to 0.3 M NaCl gradient. Each fraction contained 15.2 ml eluant. (■), protein concentration; (●), enzyme activity.


Protein Concentration
Enzyme Activity
ACTIVITY (UNITS/ml)
PROTEIN CONCENTRATION (μg/ml)
FRACTION NUMBER
100
80
60
40
20
0
4800
4400
4000
3600
3200
2800
2400
2000
1600
1200
800
400
0
85
97
109
121
133
145
157
169
181
193

maximum of 4.6 mg/ml at fraction 109 to its lowest concentration of 100 µg/ml from fraction 177 to the end of elution.

The fractions containing the second peak of CBLA activity (fractions 132 to 153) were pooled, concentrated by membrane ultrafiltration to 19 ml, and applied to a 3.5 X 170 cm Sephadex G-200 column. The elution profile of activity and protein concentration is shown in Figure 4. One major peak of CBLA is seen between fractions 88 and 106. One major peak of protein has a maximum of 1,200 µg/ml at fraction 72 and tails off to roughly 300 µg/ml at the end of elution (fraction 149).

Fractions 92 through 101 were pooled, concentrated from 47 ml to 6 ml by membrane ultrafiltration and applied to a 2.3 X 100 cm column of Sephadex G-200. The material was eluted with equilibration buffer. The elution profile of that column is given in Figure 5. Protein eluted in one main peak between fractions 37 and 63. CBLA eluted in one peak between fractions 41 and 51. Fractions 43 to 50 were pooled and lyophilized. Table 2 summarizes the results of the CBLA purification from Cohn Fraction VI-1.

The activities of aliquots of the pooled fractions shown on Table 2 were measured in the presence of 10^{-4} M $CoCl_2$, which activated the enzyme. However, the activities of individual fractions shown on the elution profiles were not measured with $CoCl_2$ to help minimize enzyme self-destruction.

Phosphate Determination

The procedure described in the Methods section was used for determining phosphate concentrations. Phosphate was allowed to

Fig. 4.--Elution profile of CBLA and protein concentration from the 3.5 x 170 cm Sephadex G-200 column. Pooled, concentrated fractions of CBLA from the 5 x 80 cm column totaling 19 ml were applied and eluted with equilibration buffer. Fractions of 4.3 ml were collected. (●), protein concentration; (■), enzyme activity.

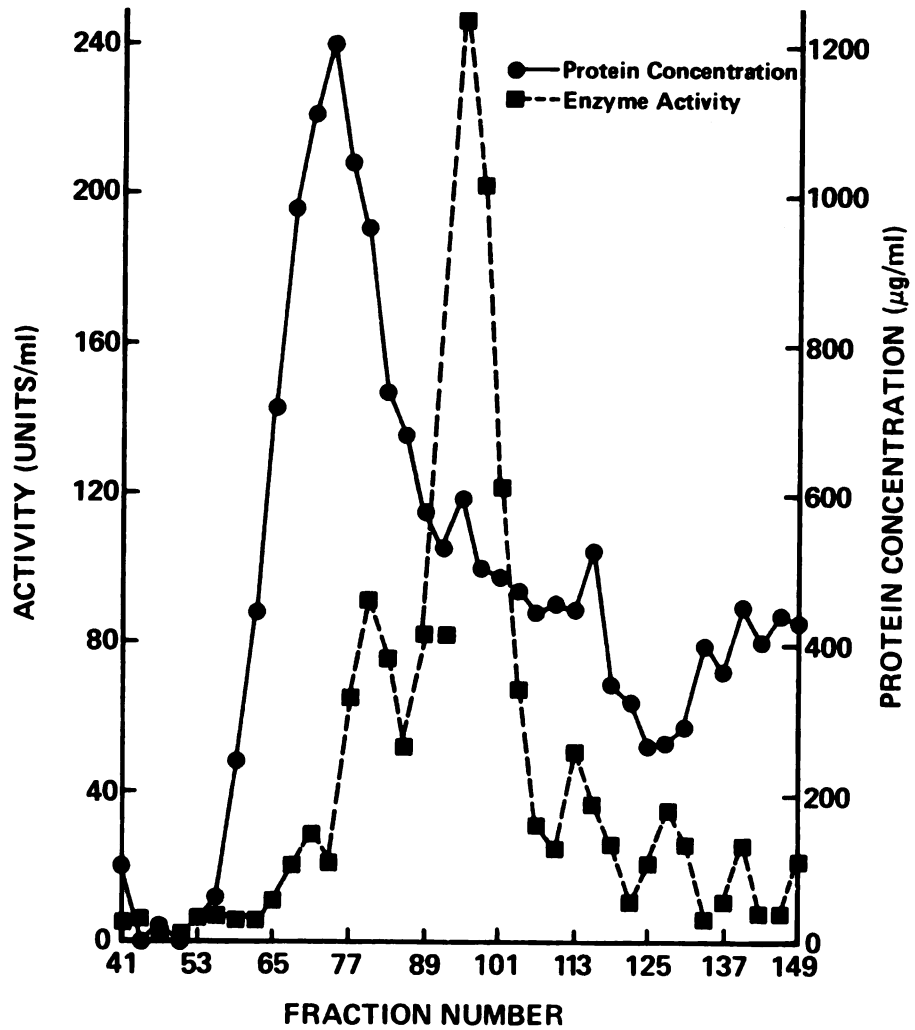

Protein Concentration
Enzyme Activity
ACTIVITY (UNITS/ml)
PROTEIN CONCENTRATION (μg/ml)
FRACTION NUMBER
0
40
80
120
160
200
240
200
400
600
800
1000
1200
41
53
65
77
89
101
113
125
137
149

Fig. 5.--Elution profile of CBLA and protein concentration from the 2.3 x 100 cm column of Sephadex G-200. Pooled, concentrated active fractions from the 3.5 x 170 cm column totaling 6 ml were applied to the column and eluted with equilibration buffer. Fractions of 2.6 ml were collected. Solid lines and circles, protein concentration; dotted lines and squares, enzyme activity.

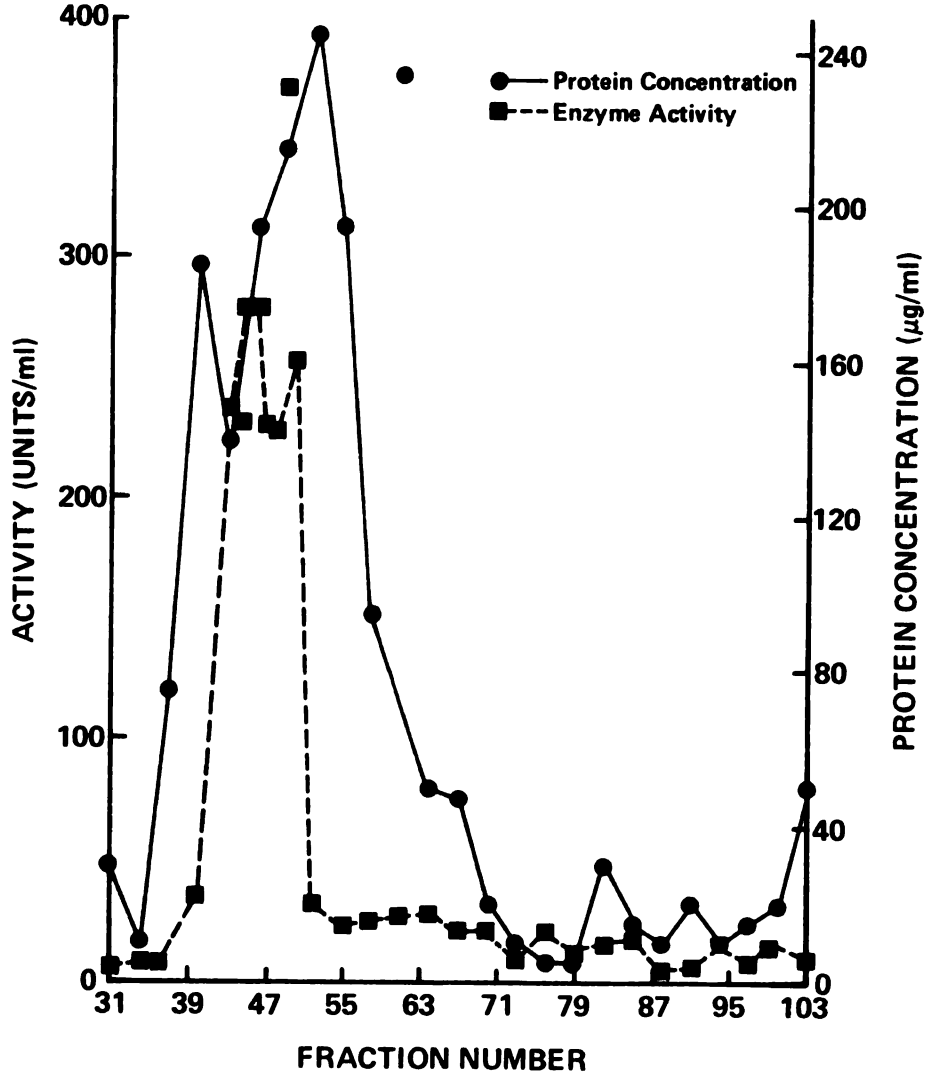

Protein Concentration
Enzyme Activity
ACTIVITY (UNITS/ml)
PROTEIN CONCENTRATION (μg/ml)
FRACTION NUMBER
400
300
200
100
0
240
200
160
120
80
40
0
31
39
47
55
63
71
79
87
95
103

Table 2.--CBLA From Cohn Fraction IV-1 Enzyme Purification Summary Sheet.

Step	Volume	Activity Total Units	Percent Recovery	Protein Total mg	Specific Activity	Fold Purification
(1) 40 gm Cohn Fraction IV-1 in KPO_4 Buffer	2,000 ml	241,000	100%	12,200	19.7	1
(2) Pooled Eluants from TEAE cellulose in 24 cm Buchner Funnel	3,000 ml	206,000	85.2%	5,360	38.4	1.95
(3) Pooled Fractions from 5 x 80 cm TEAE column	335 ml	52,100	21.6%	355	147	7.45
(4) Pooled Fractions from 35 x 170 cm G-200 column	47 ml	10,700	4.46%	24.7	429	21.7
(5) Pooled Fractions 2.3 x 100 cm G-200 column	35 ml	9,650	3.99%	5.66	1,710	86.5

complex with an excess of molybdate, and the phosphomolybdate was reduced with ascorbic acid. After treatment with monoethanolamine, optical density was measured at 580 nm. The final pH of the mixture was 10.4. This determination was performed with phosphate standards of 0.00, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mM P_i each time an ATPase assay was performed. The results of testing five sets of phosphate standards on five different days have been compiled and are shown in Figure 6. The molar absorbance of reduced phosphomolybdate at 580 nm was found to be 2,150 A/M/cm (absorbance per Mole per liter per cm) by determination of the slope by least-squares fit. It was observed that the addition of monoethanolamine acted not only as a clearing agent solubilizing any remaining proteins, but also as an accelerator of the reduction of the phosphomolybdate. The stability of the formed color was not known, and was tested on samples of 0.0, 0.1, 0.2, 0.3 mM P_i by following the change in optical density for 48 minutes after the addition of ethanolamine with a Beckman 24 double-beam spectrophotometer with recorder. The 0 mM phosphate standard was used in the reference cuvette position. The results are shown in Figure 7. The optical densities of the three curves decreased with time. The rates of decrease were -0.00122 A/min for 0.3 mM P_i, -0.000805 A/min for 0.2 mM P_i, and -0.000417 A/min for 0.1 mM P_i. As seen in Figure 8, the rate of decrease was linearly proportional to P_i concentration by least-squares fit. When routinely using the assay, in order to minimize the loss of color (about 1% per minute for an average sample), the absorbance of the samples was read within 2 to 3 minutes after the addition of the monoethanolamine.

Fig. 6.--Estimate of phosphate concentration by determination of reduced phosphomolybdate. Optical density at 580 nm vs. phosphate concentration. Error bars indicate standard deviation of observation. Each point represents the average of 5 determinations. The line was drawn by least squares fit.

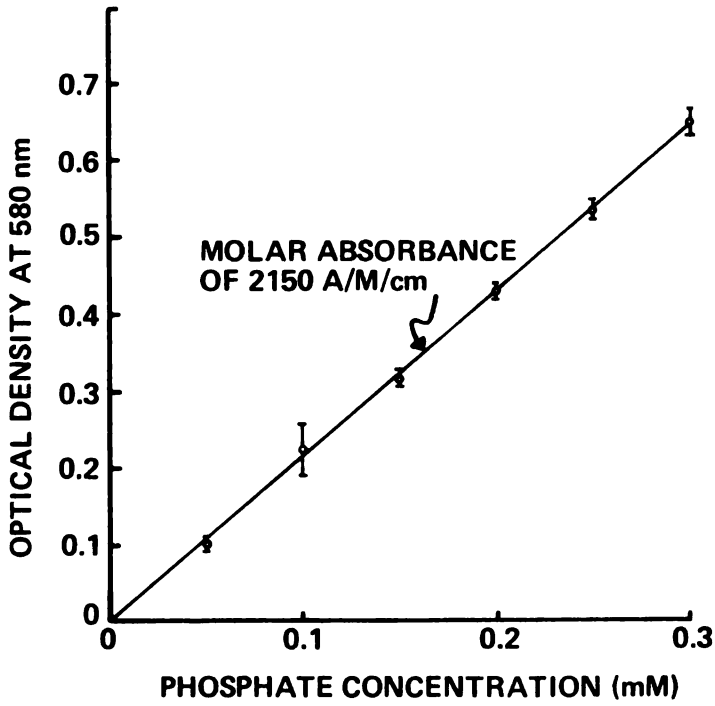

OPTICAL DENSITY AT 580 nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
MOLAR ABSORBANCE
OF 2150 A/M/cm
0
0.1
0.2
0.3
PHOSPHATE CONCENTRATION (mM)

Fig. 7.--Absorbance loss with time. Samples of 0.0, 0.1, 0.2, 0.3 mM P_i were prepared for phosphate determination by the addition of acid molybdate, ascorbic acid, and ethanolamine as indicated in the Methods section. The loss of color was monitored for 48 minutes at 580 nm in a Beckman 24 spectrophotometer.

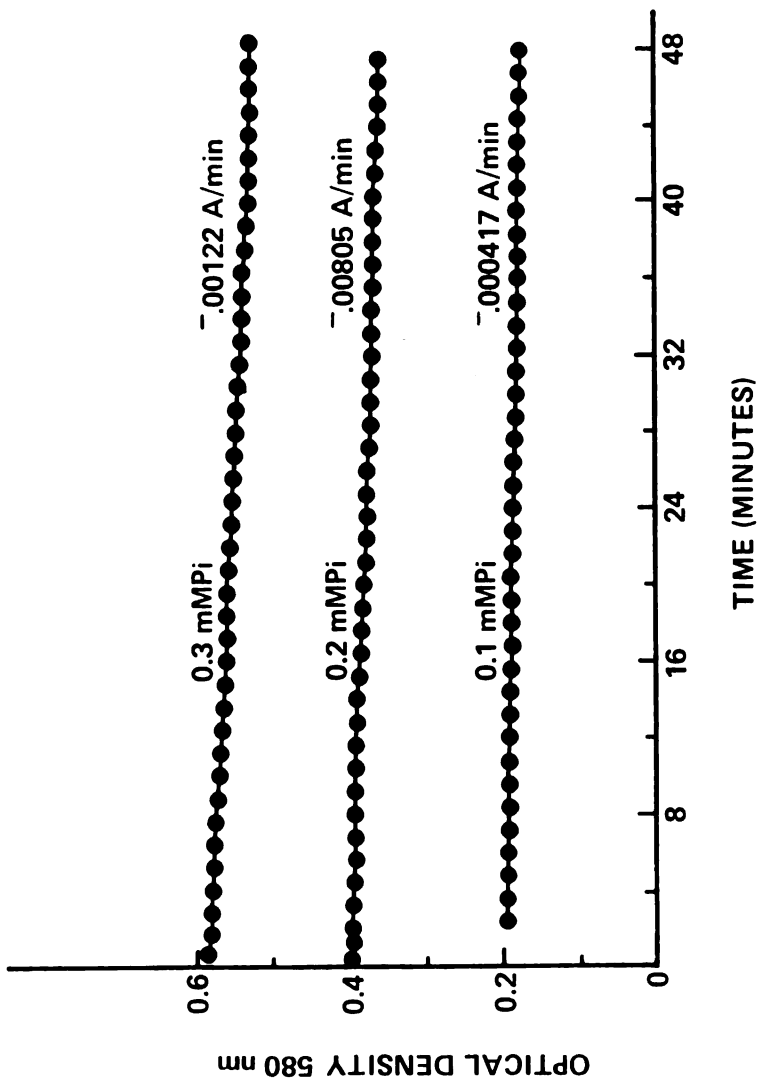
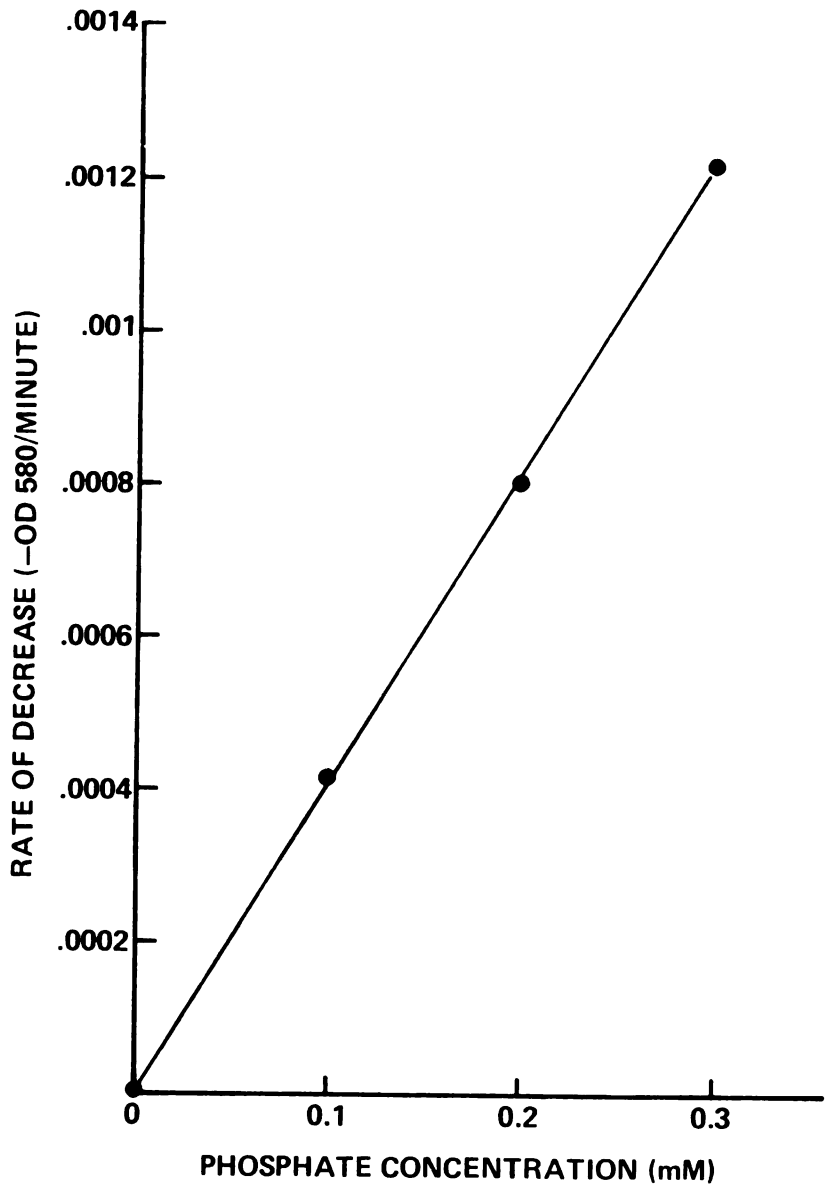

0.3 mMPi
-.00122 A/min
0.2 mMPi
-.00805 A/min
0.1 mMPi
-.000417 A/min
0.6
0.4
0.2
0
8
16
24
32
40
48
TIME (MINUTES)
OPTICAL DENSITY 580 nm

Fig. 8.--Rate of optical density loss vs. phosphate concentration. The slopes (by least-squares fit) of the lines in Figure 7 were plotted against phosphate concentration.


.0014
.0012
.001
.0008
.0006
.0004
.0002
RATE OF DECREASE (–OD 580/MINUTE)
0
0.1
0.2
0.3
PHOSPHATE CONCENTRATION (mM)

In order to determine the wavelength at which reduced phosphomolybdate has its absorbance maximum, a wavelength scan from 350 to 750 nm was performed. Samples of phosphomolybdate initially containing 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mM P_i were prepared according to the Methods section. The results of the wavelength scans are given in Figure 9. The optical densities all increase as the wavelength increases from 500 nm to 750 nm. The results below 500 nm will be considered later. Since a complete absorbance peak was not found in the wavelength range scanned, the wavelength of the absorbance maximum could not be determined, but it was greater than 750 nm.

The 0 mM P_i solution, treated for phosphate determination, was used as a sample for a wavelength scan against a reference sample of water. The scan is shown in Figure 10. There was very little absorbance from 550 to 750 nm; however, from 350 to 550 nm, part of a large absorbance peak was observed.

Results of ATPase Assays

The procedure used for the assay for ATPase activity was given in the Methods section. The assay procedure was checked for validity. The amount of P_i released up to 90 minutes after the initiation of the reaction was tested. The results are given in Figure 11. Four different concentrations of RBC (red blood cell) ghost membranes were tested, and sample reactions were terminated at 10, 20, 30, 50, 70 and 90 minutes. The reactions released P_i linearly with time through 90 minutes. The slopes of the lines were determined by least-squares fits. When the rate of release of P_i was

Fig. 9.--Optical density of reduced phosphomolybdate with ethanolamine at wavelengths from 500 to 750 nm. Samples of 0.0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mM P_i to which acid molybdate, ascorbic acid, and ethanolamine were added as in the Methods section, were scanned from 500 to 750 nm using the 0.0 mM P_i sample as a reference. A Beckman 24 double-beam spectrophotometer was used for the scans.

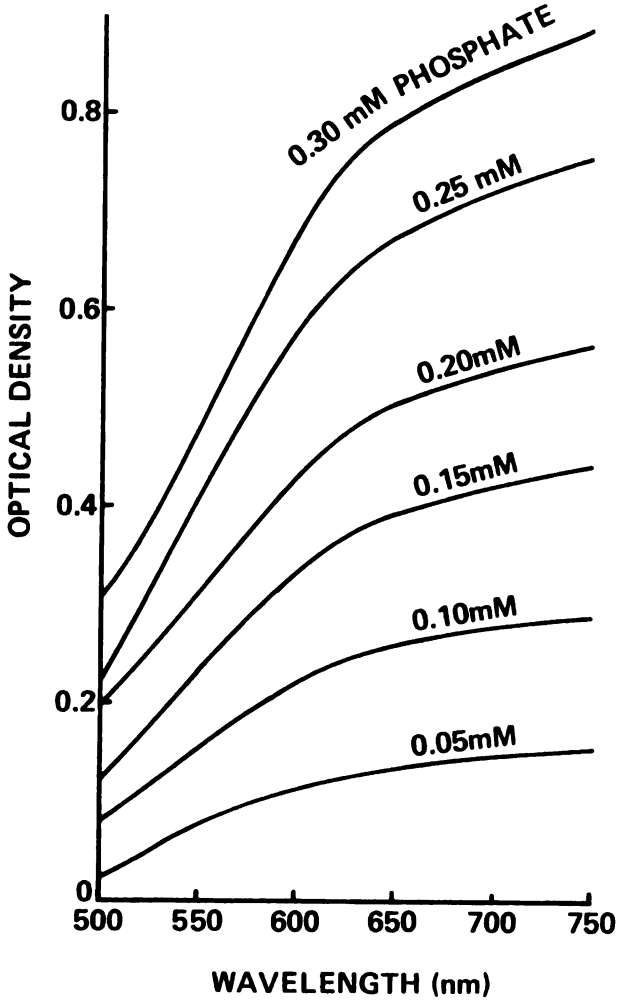

0.30 mM PHOSPHATE
0.25 mM
0.20mM
0.15mM
0.10mM
0.05mM
OPTICAL DENSITY
0
0.2
0.4
0.6
0.8
500
550
600
650
700
750
WAVELENGTH (nm)

Fig. 10.--Optical density of molybdate, ascorbic acid and monoethanolamine compared to distilled water. The 0.0 mM P_i concentration sample prepared for phosphate determination as indicated in the Methods section, was scanned against a reference of distilled water. The wavelength scan was from 750 to 350 nm. The right portion of the curve was scanned for 1.0 A full scale while the left curve was scanned with 2.0 A full scale.

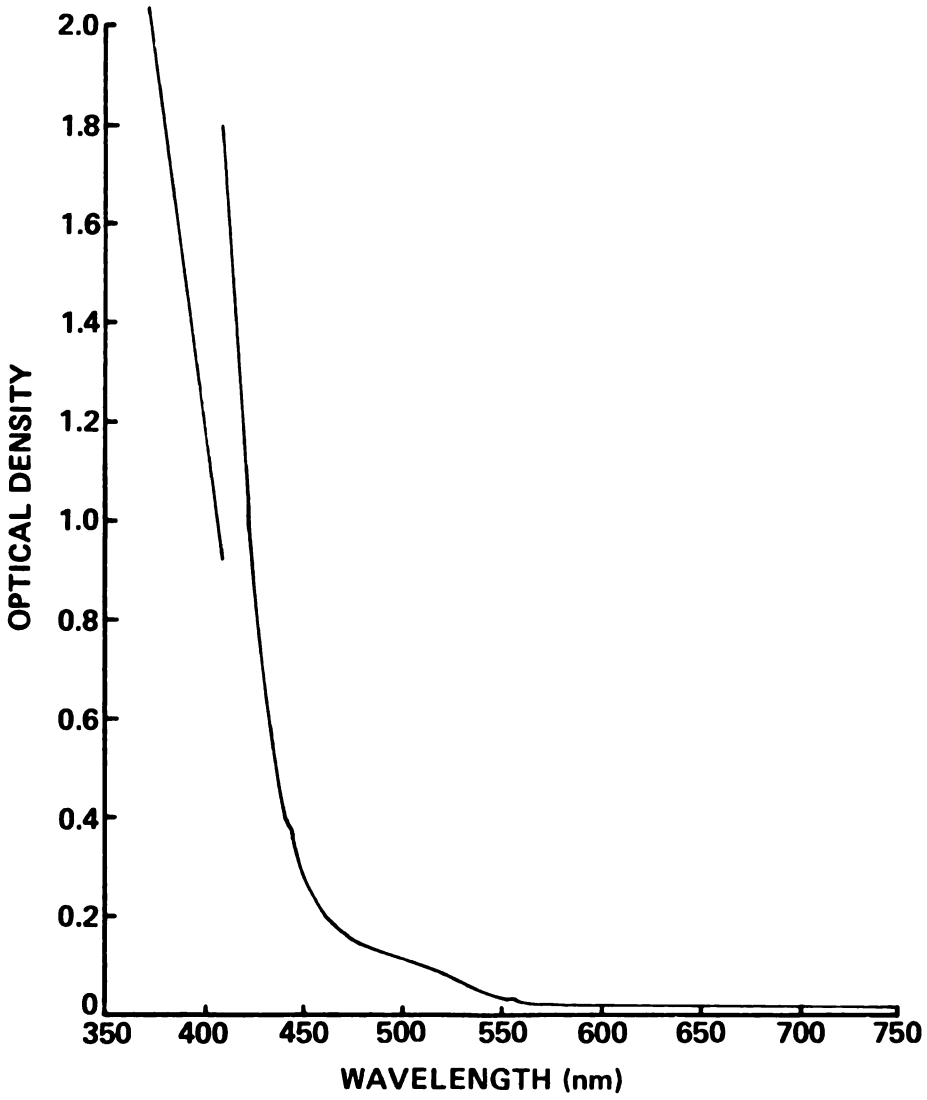
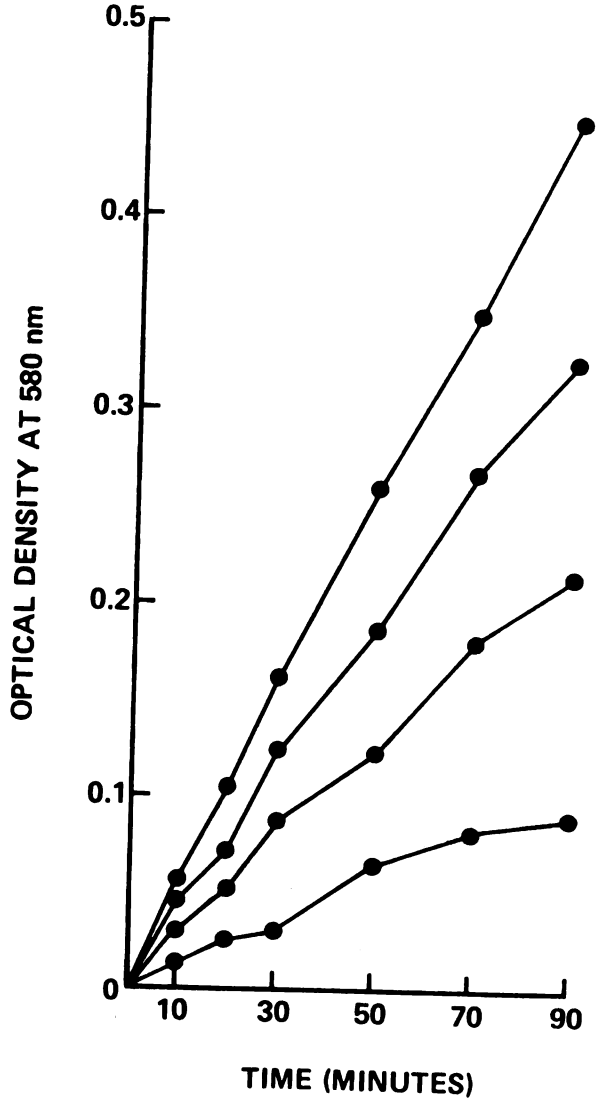

OPTICAL DENSITY
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
350
400
450
500
550
600
650
700
750
WAVELENGTH (nm)

Fig. 11.--ATPase Assay Validation: Linearity of product (P_i) release with time. The curves from highest slope to lowest contain 0.4, 0.3, 0.2, and 0.1 ml RBC ghost membranes in 1.0 ml final volume. The reaction was initiated with addition of enzyme and terminated with addition of 10% (W/V) TCA. Phosphate was determined and plotted as the indicator of activity.


0.5
0.4
0.3
0.2
0.1
0
OPTICAL DENSITY AT 580 nm
10
30
50
70
90
TIME (MINUTES)

plotted against the concentration of RBC ghost membranes (Figure 12), the velocities were linearly proportional to the concentration of ghosts. The line drawn on the figure was by least-squares fit.

Serum Fractions

Pooled serum obtained from Sparrow Hospital in Lansing, Michigan, and University Hospital in Ann Arbor, Michigan, was adjusted to 30% saturated ammonium sulfate and was centrifuged to remove the precipitate. The supernatant was adjusted to 50% saturated ammonium sulfate and the precipitate was recovered by centrifugation. This 30-50% cut was resuspended in 11.1 mM Tris at pH 7.5 and was dialyzed for 48 hours against 4 changes of the 11.1 mM Tris. Three aliquots were tested for P_i concentration, and no P_i was detected. Three other aliquots were tested for ATPase activity in the presence of 100 mM NaCl, 20 mM KCl, 5.0 mM $MgCl_2$, 5.0 mM Na_2 ATP and 20 mM Tris at pH 7.5. No ATPase activity was detected.

The effect of the 30-50% cut was compared to the effect of aliquots of the last change of dialysis buffer on RBC ghost total ATPase activity (in the presence of 100 mM NaCl, 20 mM KCl, 20 mM Tris and equimolar concentrations of $MgCl_2$ and Na_2 ATP).

The results are shown in Figures 13, 14, and 15 in Lineweaver-Burk plots. The K_m's were approximately 0.33 to 0.5 mM for the controls (control = used dialysis buffer). The addition of increasing amounts of the 30-50% cut mainly caused increased ATPase rate. In Figure 16, the Na^+-K^+ stimulated ATPase was shown to exhibit the same sort of effect: the 30-50% cut mostly stimulated activity.

Fig. 12.--ATPase Assay Validation: Linearity of enzyme velocity with enzyme concentration. The slopes of the curves from Figure 9 (obtained by least-squares fits and converted to µMol PO_4/hour) were plotted against enzyme concentration (ml enzyme in 1.0 ml final volume).

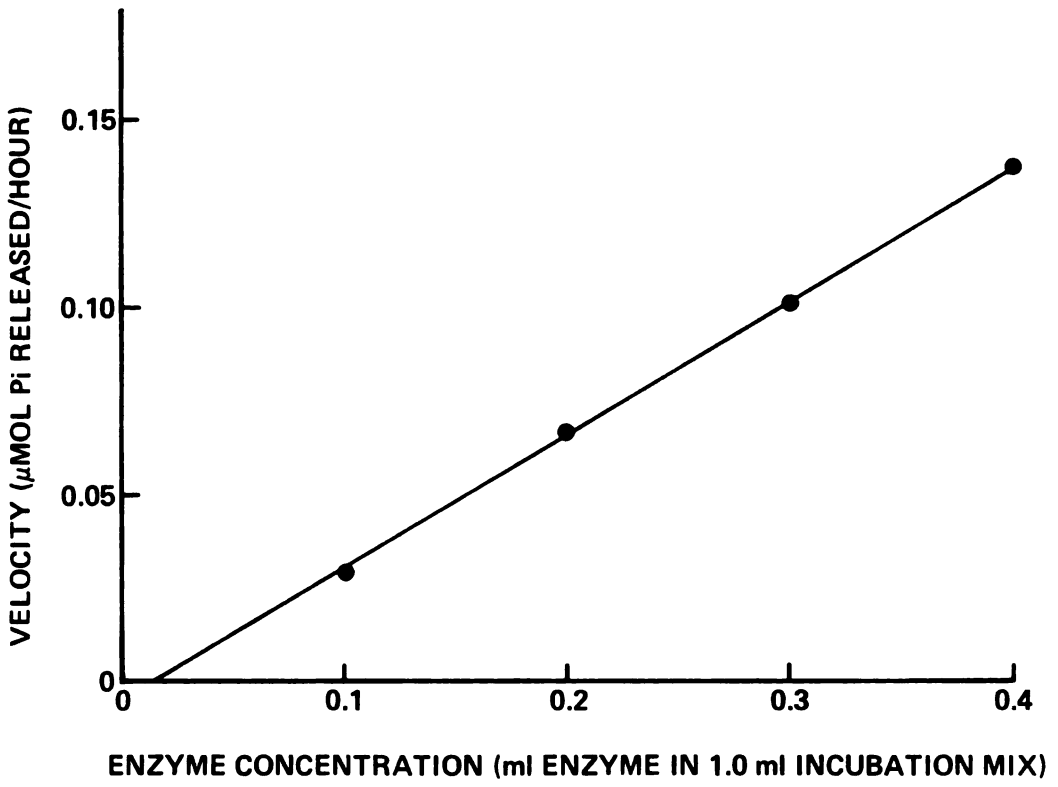

VELOCITY (μMOL Pi RELEASED/HOUR)
0.15
0.10
0.05
0
0
0.1
0.2
0.3
0.4
ENZYME CONCENTRATION (ml ENZYME IN 1.0 ml INCUBATION MIX)

Fig. 13.--Lineweaver-Burk plot: total ATPase of RBC ghosts with varying concentration of 30-50% cut of pooled human serum. The lines are least-squares fit of the data. (o, ———), no 30-50% cut (i.e., no activator); (□, -----), 100 µl 30-50% cut; (Δ, — · —), 200 µl 30-50% cut. Protein concentration was 3.78 mg/ml initial concentration. Final volume was 1.0 ml. Assay run and ghosts prepared according to Methods section. 100 µl (550 µg protein) of RBC ghost preparation #1 were added to the incubation mix to start reaction. Substrate = Mg^{2+} · ATP.

1/VELOCITY (μMOL PO4/HOUR)−1
8.0
6.0
4.0
2.0
0
−1
−2
−3
1
2
3
1/[SUBSTRATE] (1/mM)

Fig. 14.--Lineweaver-Burk plot: total ATPase from RBC ghost membranes with varying concentrations of the 30-50% cut. Symbols, methods, and amounts are as in Figure 13. RBC ghost preparation #2.

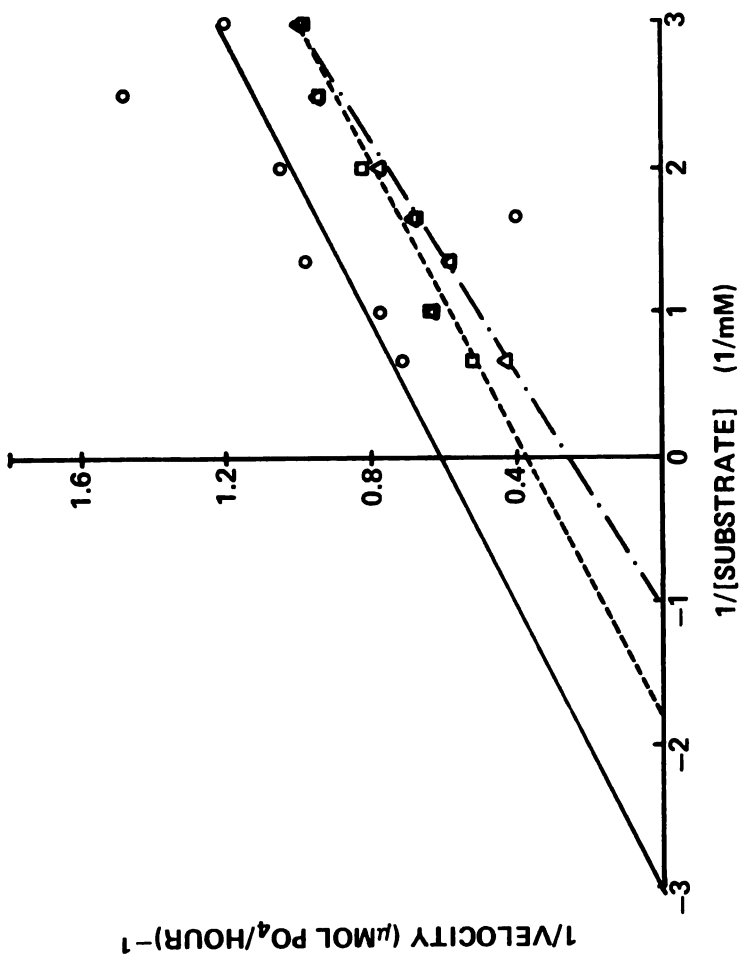

1/VELOCITY (μMOL PO4/HOUR)−1
1.6
1.2
0.8
0.4
0
−1
−2
−3
1
2
3
1/[SUBSTRATE] (1/mM)

Fig. 15.--Lineweaver-Burk plot: total ATPase from RBC ghost membranes with varying concentrations of the 30-50% cut. Symbols: (o,———), no 30-50% cut; (□, -----), 50 µl; (Δ, — – —), 100 µl; (○, — —), 150 µl; (◇, — · —), 200 µl; (▽, — ·· —), 200 µl. RBC ghost preparation #3 was used, other methods and materials are as in Figure 13 legend. Least-squares lines are shown in Figure 15.

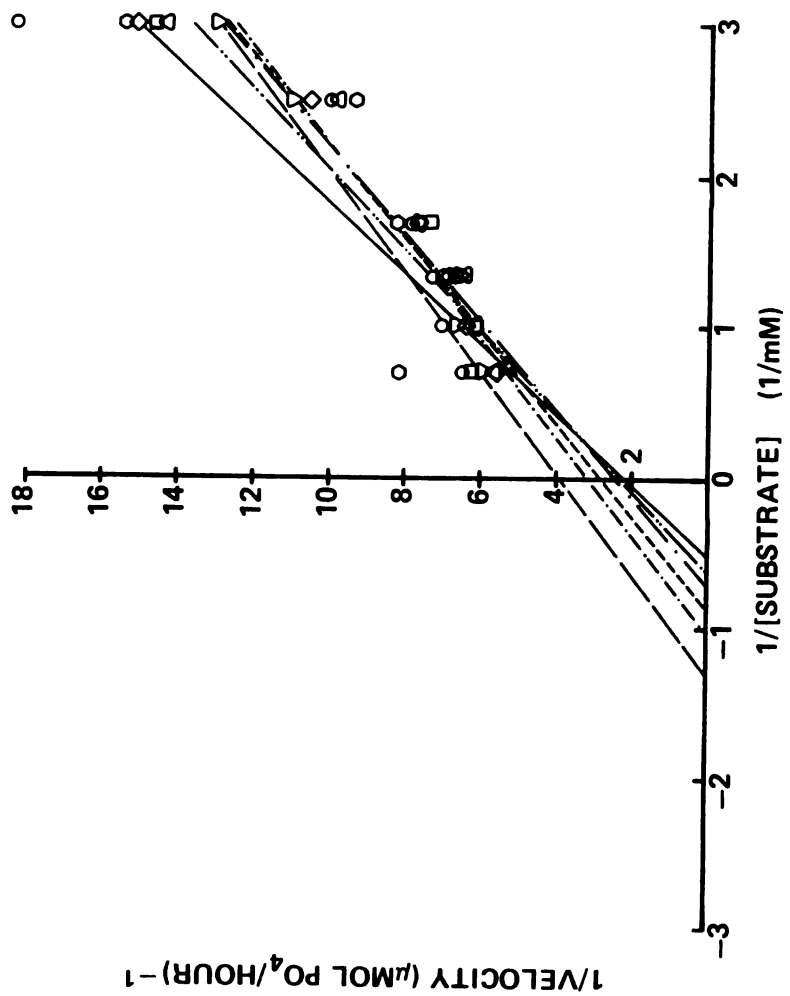
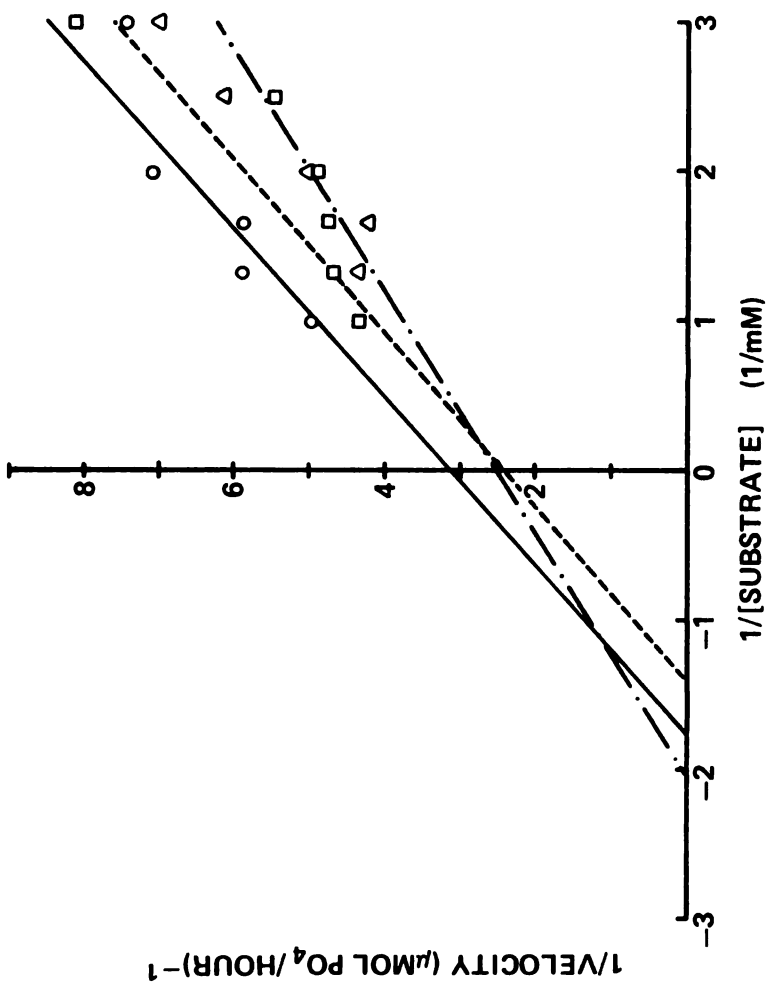

1/VELOCITY (μMOL PO4/HOUR)−1
1/[SUBSTRATE] (1/mM)
18
16
14
12
10
8
6
4
2
0
−1
−2
−3
1
2
3

Fig. 16.--Lineweaver-Burk plot: Na^+/K^+ ATPase from RBC ghost membranes. Symbols are as in the legend for Figure 13. Incubation mix contained 1 mM ouabain in addition to the standard incubation mix. RBC ghost preparation #1 was used. Other conditions were described in the legend for Figure 13. Least-squares fit lines for the data are shown. Na^+/K^+ ATPase activity was defined as the Total ATPase activity minus the ouabain-insensitive ATPase activity.


1/VELOCITY (μMOL PO4/HOUR)-1
8
6
4
2
0
-1
-2
-3
1
2
3
1/[SUBSTRATE] (1/mM)

Ouabain-insensitive ATPase was also stimulated by the 30-50% cut (see Figures 17 and 18).

Brain microsomes were assayed in the presence of the used dialysis buffer and/or the 30-50% cut. The Lineweaver-Burk plot is shown in Figure 19. The 30-50% cut generally stimulated the activity of total ATPase of mouse brain microsomes.

All lines drawn on the Lineweaver-Burk plots were by least-squares fits of the data.

Rabbit Trachea Ciliary Response to Sera

The rabbit trachea ciliary assay was performed as indicated in the Methods section. Six C/F serum samples and 6 control serum samples were tested. Explants from the same rabbit were used. The results were averaged and are shown in Figure 20, which displays the percent of maximum response versus time of exposure to the serum sample. The figure shows that the percent of maximum response of rabbit tracheal ciliated epithelium to C/F sera was greater than the percent of maximum response to control sera. The time required to reach one-half maximal response was less with C/F sera than with control sera. Movement of cilia was significantly different at 20 minutes ($p < 0.1$), 25 minutes ($p < 0.3$), and 30 minutes ($p < 0.1$). Secretion of debris was significantly different at 15 minutes ($p < 0.1$), 25 minutes ($p < 0.01$), and 30 minutes ($p < 0.05$). The test used was a two-tailed Student's t-test without assumption of equal variances.

Fig. 17.--Lineweaver-Burk plot: ouabain-insensitive ATPase from RBC ghost membranes with varying concentrations of 30-50% cut. Symbols are as in the legend for Figure 13. Ouabain (1 mM final concentration) was added to the standard incubation mix. RBC ghost preparation #2 was used. Least-squares fit lines are shown plotted on this figure. Other methods and materials are as in the Figure 13 legend.

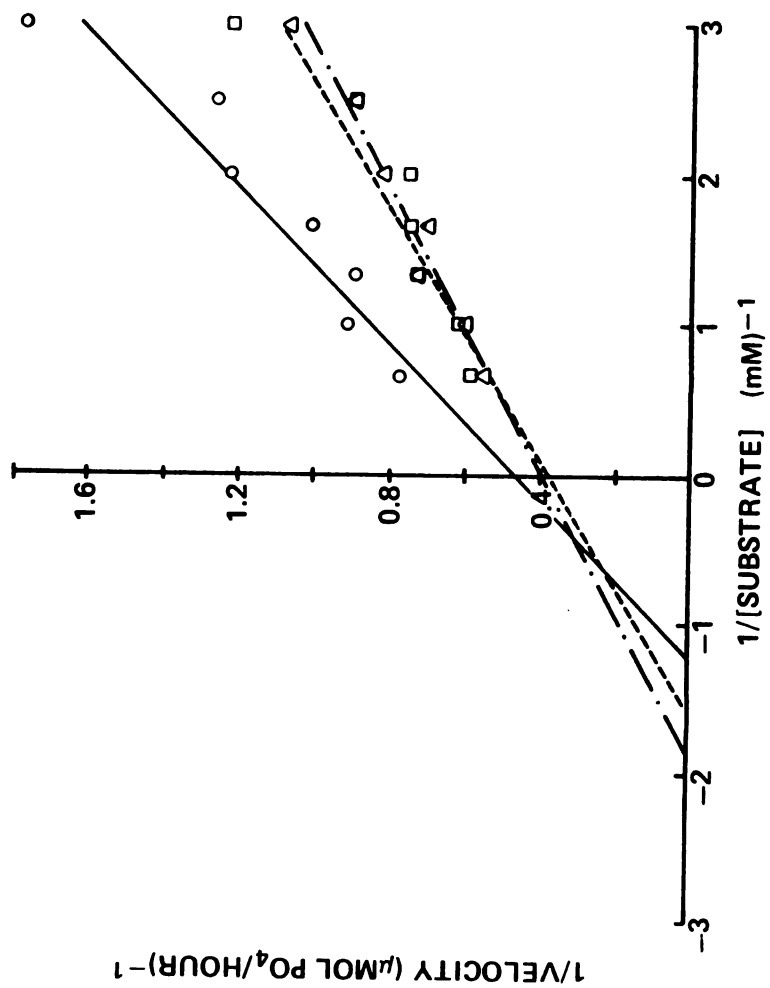

1/VELOCITY (μMOL PO4/HOUR)−1
1.6
1.2
0.8
0.4
0
−1
−2
−3
1
2
3
1/[SUBSTRATE] (mM)−1

Fig. 18.--Lineweaver-Burk plot: ouabain-insensitive ATPase from RBC ghost membranes with varying concentrations of 30-50% cut. Ouabain was added to the standard incubation mix to 1 mM final concentration. Symbols are: (o, ———), no 30-50%; (□, -----), 50 µl; (Δ, — – —), 100 µl; (∇, — —), 150 µl; (◇, — ·· —), 200 µl. RBC ghost preparation #2 was used. Least-squares fit lines are shown. Other materials and methods are as in the legend for Figure 13.

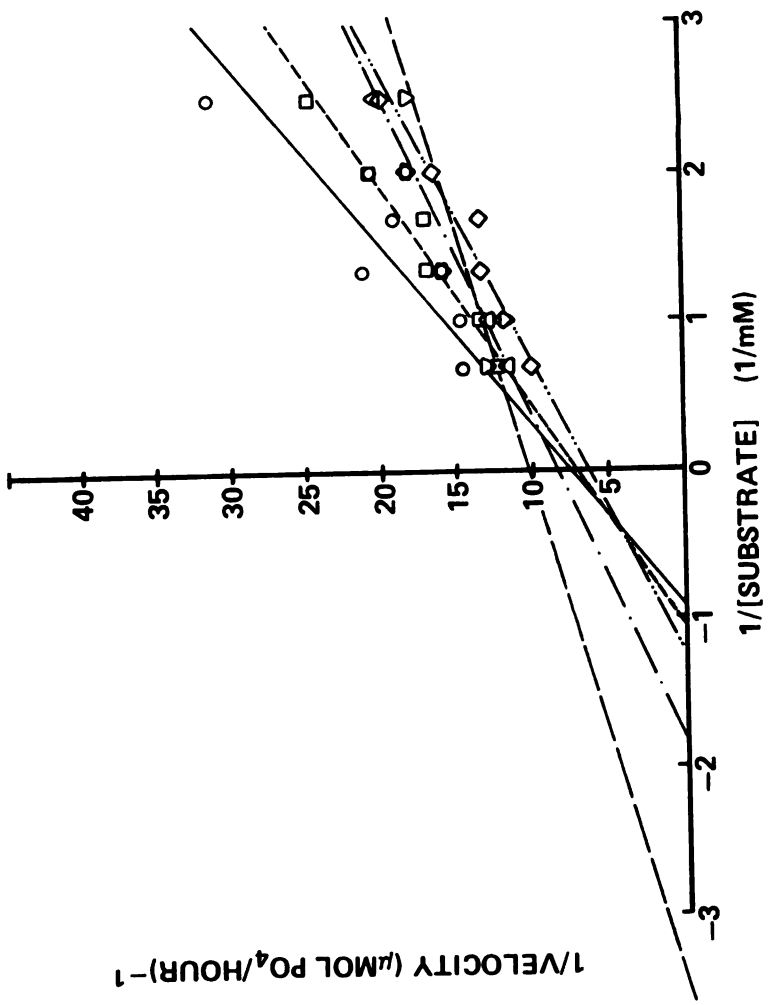

1/VELOCITY (μMOL PO4/HOUR)−1
40
35
30
25
20
15
10
5
0
−1
−2
−3
1
2
3
1/[SUBSTRATE] (1/mM)

Fig. 19.--Lineweaver-Burk plot: total ATPase from mouse brain microsomes with varying concentrations of 30-50% cut. Symbols are as in the Figure 13 legend. The same 30-50% cut concentrations and the same assay conditions as for Figure 13 were used except: 50 µl (33 µg protein) of mouse brain microsomes were used; incubation time was reduced to 20 minutes. Lines were drawn according to least-squares fit of the data.

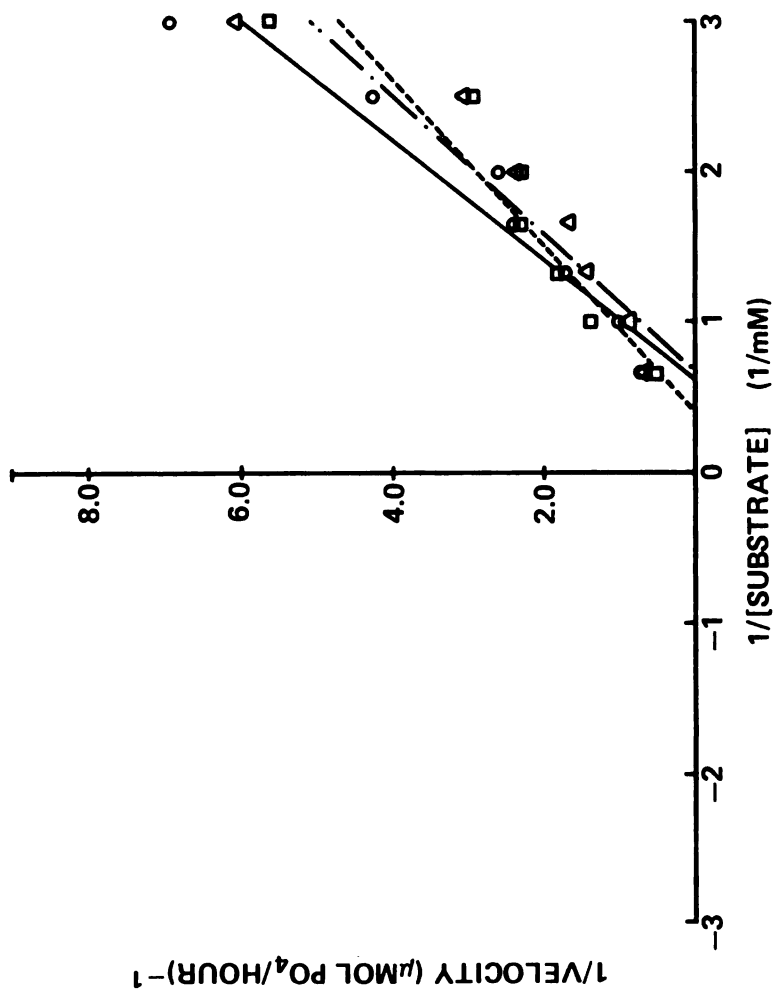
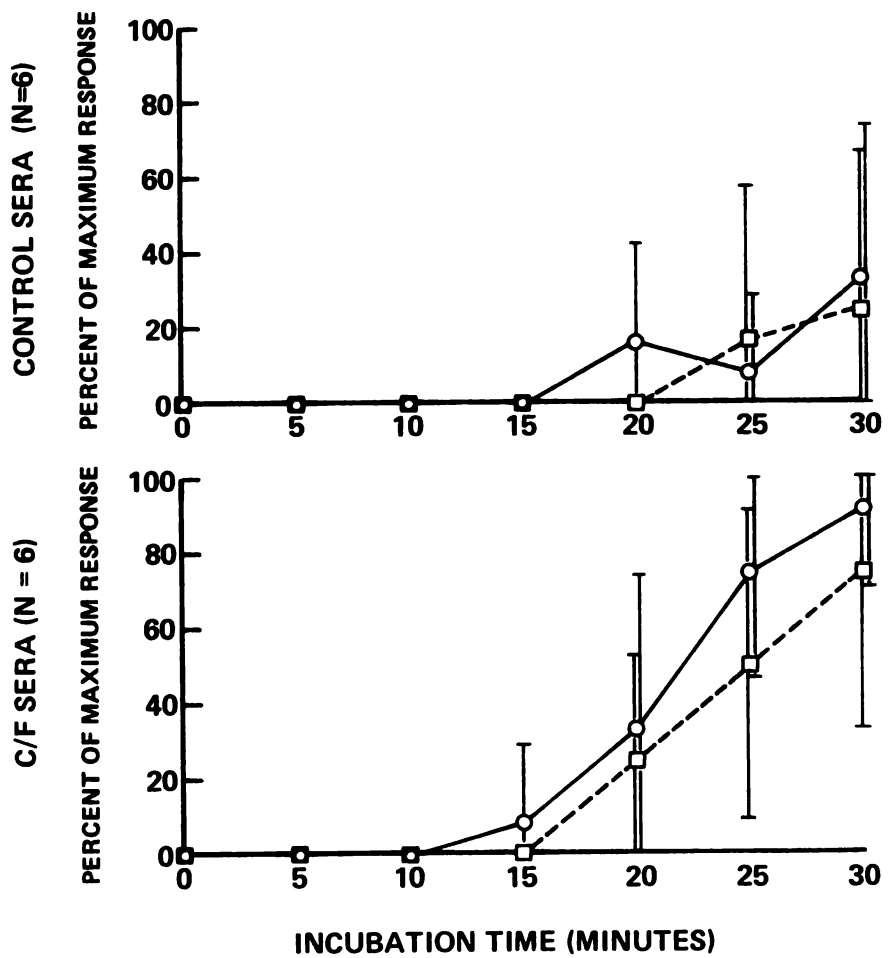

1/VELOCITY (μMOL PO4/HOUR)−1
8.0
6.0
4.0
2.0
0
−1
−2
−3
1
2
3
1/[SUBSTRATE] (1/mM)

Fig. 20.--Expression of secretory and dyskinetic responses of rabbit trachea epithelium to sera with time. The rabbit trachea assay was performed according to the Methods section description. Six control and 6 C/F sera were assayed, and the responses of the ciliated epithelium were evaluated at 5 minute intervals. Responses were graded 0, 50, or 100% of maximal response. The averages and standard deviations were plotted. (o, ———), secretory response; and (□, -----), dyskinetic response.


CONTROL SERA (N=6)
PERCENT OF MAXIMUM RESPONSE
100
80
60
40
20
0
0
5
10
15
20
25
30
C/F SERA (N = 6)
PERCENT OF MAXIMUM RESPONSE
100
80
60
40
20
0
0
5
10
15
20
25
30
INCUBATION TIME (MINUTES)

Incorporation of (^{3}H)-Glucosamine Into Rabbit Trachea in the Presence of Sera (Control, C/F and FCS)

In an attempt to examine rabbit trachea for altered secretory properties generated by treatment with C/F serum, 2 X 8 mm pieces of rabbit trachea epithelium were incubated with 15 μCi (^{3}H-1)-glucosamine and 25% (V/V) sera from controls, C/F subjects and FCS. Following incubation for up to 20 hours, the tissue was put into ice-cold 10% (W/V) TCA (trichloroacetic acid) plus 0.01 M glucosamine and was thoroughly homogenized. The precipitate was collected on a GF/C fiber filter and was washed with 5 ml of 5% (W/V) TCA plus 0.01 M glucosamine 3 times. The filter was put in a scintillation vial with 10 ml of scintillation fluid (1,000 ml toluene, 500 ml Triton X-100, 5 gm PPO, and 0.5 gm dimethyl POPOP) and counted in a Packard scintillation counter. The results are shown in Figures 21 and 22. No significant differences in the incorporation of glucosamine were detected between treatment of the ciliated epithelium with control sera, C/F sera and FCS.

Fig. 21.--Incorporation with time of radioactive glucosamine into TCA precipitable material. The homogenates of rabbit trachea ciliated epithelium were treated with C/F or control sera (to 25% (V/V)) or Medium 199. C.P.M. were normalized to 1 mg protein in the homogenate of the explant. Epithelium was maintained in culture for 2 days before transferring to fresh Medium 199 with 15 μCi (^{3}H-1)-glucosamine and 25% (V/V) C/F of control sera or Medium 199. The tissue incubated for up to 10 hours in 37°C and 5% (V/V) CO_2.

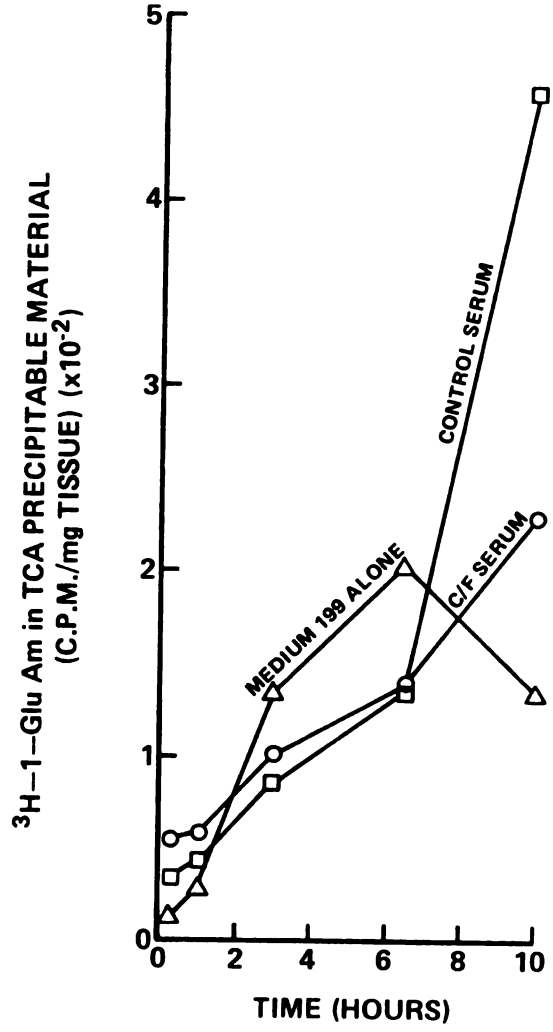
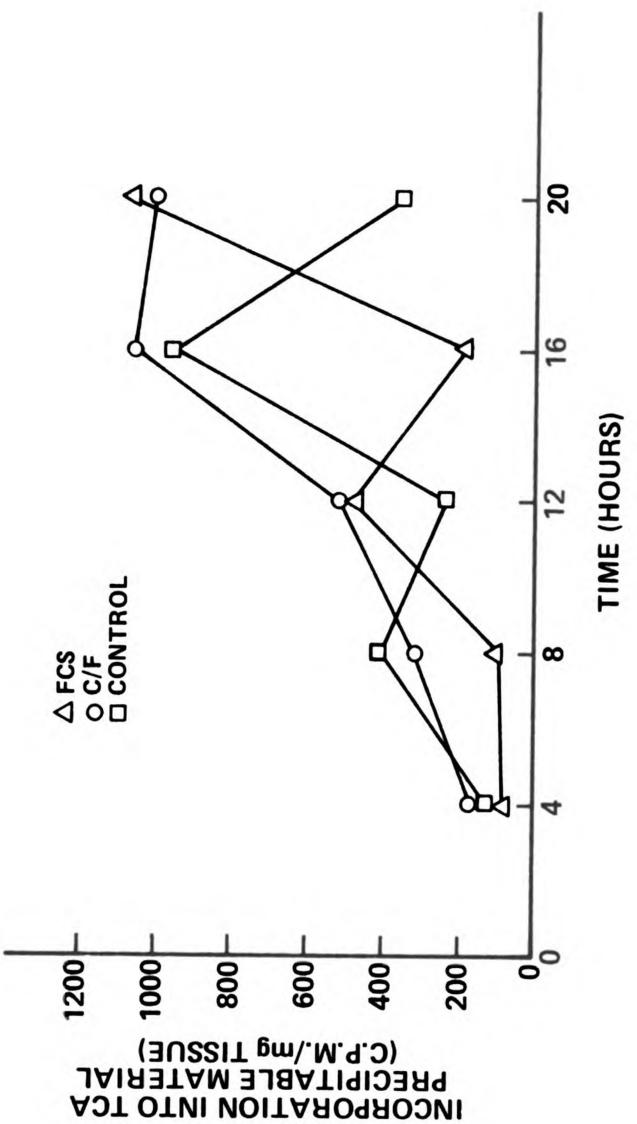

^{3}H–1–Glu Am in TCA PRECIPITABLE MATERIAL
(C.P.M./mg TISSUE) (x10^{-2})
5
4
3
2
1
0
0
2
4
6
8
10
TIME (HOURS)
CONTROL SERUM
C/F SERUM
MEDIUM 199 ALONE

Fig. 22.--Incorporation with time of radioactive glucosamine into TCA precipitable material. Homogenates of rabbit trachea epithelium were treated with C/F or control sera or FCS. Incorporations of radioactivity were normalized to 1 mg protein of homogenate. See Figure 21 for experimental details, except that: one set of samples was treated with 25% (V/V) FCS instead of Medium 199 alone and samples were incubated for up to 20 hours instead of 10 hours.


INCORPORATION INTO TCA PRECIPITABLE MATERIAL (C.P.M./mg TISSUE)
1200
1000
800
600
400
200
0
0
4
8
12
16
20
TIME (HOURS)
△ FCS
○ C/F
□ CONTROL

DISCUSSION

The basic defect in C/F is not known at this time, and researchers have little idea of the nature of the basic defect due to a lack of understanding of the processes differing in C/F and control individuals (i.e., the pathology of C/F is only understood at the clinical level). However, one generalization can be made about the pathogenesis in C/F: some of the functional activities of the cell coat and/or the cell membrane of C/F tissue or C/F treated normal tissue are altered when compared to controls. The research presented here has been on three materials on or in contact with the cell coat and cell membrane: CBLA, ATPase, and the tracheal epithelium ciliary border. Each topic will be discussed below.

CBLA: Introduction

CBLA was implicated in C/F as the basic defect and was thought to be deficient in C/F sera. Lieberman (1975) and Corbin et al. (1976) showed that CBLA was not significantly lower in C/F; moreover, in certain C/F patient groups it was elevated. The question of the existence of an isozyme of CBLA in serum has been raised and will be discussed later. The significance of an isozyme of CBLA to C/F research may not be great since Bowman et al. (1975) presented evidence that the ciliary inhibitor does not cross-react with mono-specific anti-C3a. (Conover et al. (1974) had suggested that C3a

was the ciliary inhibitor, which they hypothesized to be in excess in C/F because anaphylatoxin inhibitor (CBLA) failed to inactivate it.)

CBLA Assay and Purification

The CBLA assay was shown to be valid by its meeting the two criteria that: product formed (hippuric acid) is released linearly with time; and that enzyme velocity is linear with enzyme concentration. See Figures 1 and 2, respectively, which show that the assay met the two above criteria.

Cohn Fraction IV-1 was used as a source from which CBLA was purified. At pH 8.0 in 50 mM potassium phosphate buffer CBLA was capable of binding to TEAE cellulose; but when the ionic strength was raised using a linear 0 to 0.3 M NaCl gradient, CBLA was eluted from the TEAE cellulose. As seen in Figure 3, the activity was eluted in two peaks. The elution of activity in two peaks suggested that either isozymes of CBLA exist or that part of the activity was bound to a second protein while the remainder of the activity did not bind to that second protein, giving two peaks of activity eluting at different ionic strengths. The CBLA peak that eluted first coincided with the elution of the peak of protein concentration. This fraction of activity was not further purified. However, if it were purified, it would be interesting to compare it with the subunit structure of the second activity peak on denaturation with urea or guanidinium HCl and analytical centrifugation.

The first activity peak was not included in Table 2, the Enzyme Purification Summary Sheet, and that loss is reflected in the drop in yields from 85.2% to 21.6% recovery between Steps 2 and 3

(i.e., before application to the 5 x 80 cm TEAE column to after its elution). The fractions making up the second activity peak were pooled, concentrated, and applied to Sephadex G-200. The elution profile (Figure 4) showed the separation of CBLA from a higher molecular weight protein peak. The fractions containing activity that were pooled were selected in a narrow range. This procedure sacrificed activity, as indicated by the decrease in yield from 21.6% to 4.46%, but it also removed a large amount of extraneous protein. On the second G-200 column little activity was lost: the yield was reduced from 4.46% to 3.99%, but total protein was reduced by about a factor of 5. The fold purification of all the procedures was 86.5. This value compares with a 14% yield with a 252 fold purification from serum by Erdös et al. (1967) and with the purification of Bokisch and Müller-Eberhard (1970) of anaphylatoxin inhibitor from pseudoglobulin who obtained a preparation of 280 μMol hippuric acid released per minute per mg protein with a yield of 6%.

The CBLA purified as indicated in the Methods and Results sections was undoubtedly not purified to homogeneity. If a very high specific activity preparation were needed, additional methods to be used in purification would include affinity chromatography. The method of Sokolovsky (1974) could be used to prepare a suitable column. He bound EACA to CNB_r-activated Sepharose and, using N, N' dicyclohexylcarbodiimide, linked the EACA spacer to arginine.

A precaution which could not be taken during purification was to run all purification steps at 4°C instead of 23°C. While this purification was being done, there was no access to a cold room.

Since columns usually took 24 or more hours to develop, the maintenance of the higher temperature probably contributed to a substantial loss in yield. If columns were run at the lower temperature, the peptidase activity would have been about 4-fold lower (assuming $Q_{10} \simeq 2.0$), and considerably less enzyme would have been self-destructed. Further recommendations for CBLA purification include: in place of the second G-200 column, run a column of Sepharose 6B; run a 5 to 20% (W/V) sucrose gradient centrifugation to separate the CBLA based on sedimentation coefficient as opposed to separation based on charge properties and Stoke's radius as in previous steps; and run preparative isoelectric focusing.

Phosphate Determination

The technique used for phosphate determination described in the Methods section was a modification of the techniques of Fisk and Subbarow (1925), Ames (1966), and Drewes (1972). All of the above techniques required incubation periods before the optical density was measured. The modification of the above techniques, as presented in the Methods section, eliminated the incubation period. The method was nearly as sensitive as the method of Ames who reported a molar absorbance of 2600 A/M/cm at 820 nm, and was more sensitive than the method of Drewes who reported a molar absorbance of 732 A/M/cm.

The color stability of the technique was reported in the Results section, and as indicated in Figures 7 and 8, the color diminished slowly and the rate of loss was linear with the concentration of phosphate. However, the fastest rate of color loss was -0.00122 A/min. Consequently, optical densities were measures within

2 to 3 minutes after the addition of ethanolamine so that the amount of color change was no more than the size of the measurement reliability of the spectrophotometer.

The wavelength scan results shown in Figure 9 indicated that the absorbance peak corresponding to the reduced phosphomolybdate began at 500 nm and increased through the end of the scan at 750 nm. A point of inflection in the curve was located at 550 nm, and the shape of the curve indicated that the wavelength of the maximum was roughly 800 to 850 nm. This estimate corresponded to the value obtained by Ames of 820 nm.

The optical density routinely was measured at 580 nm due to the inaccessibility of a spectrophotometer capable of reading at a higher wavelength. The suitability of reading at that wavelength, given that no higher wavelength could be used, is justified, inasmuch as the value at 580 nm was about 67% of the maximum value at 750 nm and about 80% of the molar absorbance of Ames. It also was shown that any wavelength below 550 nm was unsuitable. Below 550 nm results were inconsistent due to the low molar absorbance of reduced phosphomolybdate at those wavelengths and partly due to the rise in optical density of the blank. Figure 10 illustrated the magnitude of the increase in optical density with decreasing wavelength when no phosphate was present in the sample. The reference to which the sample was compared contained only distilled water.

ATPase Assay Validation

The ATPase assay as described in the Methods section was shown to meet the two criteria for assay validation: first, product

formed (P_i) was shown to be produced linearly with time (up to 90 minutes), and second, the rate of P_i formation was shown to increase linearly with enzyme concentration. See Figures 11 and 12, respectively.

The Effect of Serum Proteins on ATPase Activities

As seen in Figures 13, 14, and 15 the addition of 30-50% cut of pooled human serum caused an increase in total ATPase activity in human RBC ghost membranes. This increase in activity was also shown with Na^+/K^+ ATPase (Figure 16) and ouabain-insensitive ATPase (Figures 17 and 18) of RBC ghost membranes and total ATPase of mouse brain microsomes (Figure 19). The stimulation of RBC ATPase activity by immunoglobulins was reported by Bader (1971) as preliminary data, and Schmoyer and Baglia (1974) reported that ATPase activity from RBC ghosts was increased by the addition of dialyzed unused media supplemented with FCS added to the assay mix. Schmoyer and Baglia used 0.02 M TES pH 7.5 as a control against which ATPase treated with media was compared.

The results reported on the Lineweaver-Burk plots indicated that there was activation by nondialyzable material and that the amount of activation was dependent on the concentration of the 30-50% cut added. In addition, most of the least-squares fit lines ran roughly parallel to each other. For these reasons, the kinetics did not rule out the possibilities that the 30-50% cut affected the ATP-enzyme complex, that the 30-50% cut supplied one of the substrates, or that the 30-50% cut acted to aid the enzyme in binding one of the substrates. Alternatively, the kinetics did not rule out the possibility that the 30-50% cut contained a molecule (a subunit of

the enzymes) that was bound to the ATPases in vitro but that was lost during the preparation of the enzymes.

Future tests of the effect of 30-50% cut on ATPases include: the effect of heat-inactivated 30-50% cut, the effect of 30-50% cut which was heavily dialyzed against chelating agents, the effect of DEAE and CM cellulose fractionated 30-50% cut, and the effect of 30-50% cut fractionated by gel filtration. Further kinetic experiments using wider ranges of 30-50% cut concentrations are also planned.

The continued investigation of the activation of RBC ghost and brain ATPases will hopefully allow the determination of how this apparently normal physiological activation occurs. Knowledge of the mechanism and functional components involved in thos process will aid in the interpretation of the results of Schmoyer and Baglia (1974), Cole and Dirks (1972), and Cole and Sella (1975). They showed that C/F serum, saliva, and used medium from fibroblasts contain materials which inhibit ATPases or activate ATPases less than the corresponding control material. It was hoped that the data reported here would serve as a baseline from which the normal physiological and biochemical situation could be understood; and having evaluated the normal condition, a comparison of the effect of C/F and control materials on ATPases with an interpretation of the differences could be made.

Rabbit Trachea Ciliary Bioassay

The rabbit trachea ciliary bioassay has provided a great deal of information on the differences between C/F, heterozygote, and

control materials (either serum or used medium from cultured cells). See Conover et al. (1973a, 1974), Yeates et al. (1976), and Spock et al. (1967). However, Wood and di Sant'Agnese (1973) pointed out that the bioassay was unreliable as a diagnostic test in differentiating C/F homozygote and heterozygote sera from control sera.

Results from this laboratory indicate that one cause for the unreliability of the bioassay was the need for an observer to evaluate the state of the tissue. A second drawback was the variability of responses to the same test sample from rabbit to rabbit and to a lesser extent from explant to explant. Other assay deficiencies were that the threshold level for response was high (preventing the assay of diluted material), and the magnitude of response was not great. Moreover, only a very few samples could be observed during one run which took 30 to 35 minutes; consequently, the bioassay was tiresome and difficult to perform. Despite the multitude of disadvantages, data were obtained following the procedure described in the Methods section. As shown in the Results section the magnitude of the secretory response of the tissue to C/F sera was significantly greater beyond 20 minutes exposure to C/F serum, as compared to control sera.

Other observers have reported a wide variety of incubation times prior to the initiation of a CD+ response to C/F serum. Conover et al. (1973a) reported CD+ response times of 3 to 6 minutes, Sturgess (personal communication) reported 10 to 15 minutes, and Cheung and Jahn (1976b) reported 1 to 2 hours. These variations in

response time were undoubtedly dependent on technique and animal variation.

Future Experiments Using the Ciliary Bioassay

One problem in quantifying the ciliary bioassay is that the mechanism of the secretory and dyskinetic response of rabbit tracheal epithelium has not been characterized. A proposal to investigate the mode of the epithelial cells' response is to examine the necessity or lack of necessity for microfilament and microtubule function in the ciliated cells in order to elicit a CD+ response on treatment with C/F serum. Another subcellular requisite to elicit a CD+ response on treatment with C/F is hypothesized to be ATP. The pre-treatment of explants with uncouplers would lead to intracellular ATP depletion and might eliminate CD+ response capability. Another hypothesis is that C/F serum causes the lysis and death of the ciliated epithelial cells; this hypothesis is being tested by using time-lapse microcinematography of the epithelium in the presence of C/F or control serum. Lysis and death of the ciliated cells will be detected by vital stains. Suitable stains include trypan blue, eosin Y, and erythrosin B, all of which are excluded by viable cells, and accepted by lysed cells.

Incorporation Experiments

The incorporation of (^{3}H-1)-glucosamine into rabbit trachea epithelium treated with C/F and control sera was tested as a possible alternative to the ciliary bioassay. The results indicated that there were no significant differences in the amounts of incorporation

of tritiated glucosamine into the TCA precipitable material in the homogenized explants. Although this particular method did not provide a quantified assay to replace the ciliary bioassay, it did indicate that C/F serum had no toxic or stimulatory effect on the incorporation of glucosamine into the TCA precipitable material of the explant. However, the explant contained a mixture of cell types, and it could be argued that only the ciliated epithelial cells were affected, while none of the other cell types were affected, masking the alteration in epithelial cell function by the C/F serum.

It should be pointed out that the experiment described here used a crude and preliminary method. Effects possibly contributing to the lack of sensitivity of this technique are:

(1) entry of glucosamine into the cell could be affected by C/F and control sera

(2) C/F and control sera could affect the pool size of glucosamine in the ciliated cells

(3) the possibility exists that only mucopolysaccharide, glycosphingolipid, or glycoprotein synthesis alone is affected by C/F or control serum, and the other two synthesis rates are similar

(4) C/F sera might increase synthesis of a particular glucosamine incorporating product and decrease synthesis of other products, giving roughly equal amounts of glucosamine incorporation into TCA precipitable material

(5) the steady state amount of incorporated glucosamine with C/F and control sera treatment might increase at the same rate;

while the rate of secretion of incorporated glucosamine might be very different

(6) more than one of the above effects may occur when ciliated epithelium is treated with C/F serum.

Further experiments include the separation of glycosphingolipids from glycoproteins and acid mucopolysaccharides of the ciliated epithelium which was incubated with radioactive glucosamine and C/F or control sera. The lipid material (containing the glycosphingolipids) will be extracted from the homogenized material with chloroform: methanol (2:1). The examination for possible differences in the levels of glycosphingolipids between C/F and control sera treatment can be checked in this manner.

Changes in the level of synthesis of particular glycoproteins and mucins labelled with radioactive glucosamine can be monitored by polyacrylamide gel electrophoresis of the homogenate of the ciliated epithelium treated with C/F or control serum and radioactive glucosamine. The incorporation into specific fractions could be tested by slicing the gels into thin discs and counting the slices in a scintillation spectrophotometer.

Ongoing experimentation using incorporation of tritiated glucosamine as an indicator of glycoprotein and mucin synthesis include:

(1) A test of the levels of nondialyzable radioactivity in the medium of rabbit trachea epithelium incubated with C/F, control, and FCS and tritiated glucosamine. This experiment

will indicate the effect of the sera on the production of secreted soluble, nondialyzable glycoproteins and/or mucins.

(2) A pulse and chase experiment--pulse label the ciliated epithelium with tritiated glucosamine and chase with non-radioactive glucosamine with C/F, control, or FCS. Tissue explants are examined in histologic sections via autoradiography. The purpose of the experiment is to examine for heightened or decreased synthesis of glycoproteins caused by the C/F sera.

(3) Injection experiment--young mice are injected with tritiated glucosamine and later with C/F or control sera subcutaneously in the neck region. Thirty minutes later the mice are sacrificed. Incorporation of glucosamine into the salivary glands and trachea are assessed in histologic sections by autoradiography. This experiment has the advantage of determining the effect of C/F and control sera in an in vivo model system.

BIBLIOGRAPHY

BIBLIOGRAPHY

Ames, B. N. 1966. Assay of Inorganic Phosphate, Total Phosphate and Phosphates. Meth. in Enzymol. 8:115-118.

Araki, H., M. Field and H. Schwachman. 1975. A New Assay for Cystic Fibrosis Factor: Effects of Sera from Patients with Cystic Fibrosis in the in Vitro Electrical Properties of Rat Jejunum. Pediat. Res. 9:932-934.

Akera, T., and T. M. Brody. 1968. Inhibition of Brain Sodium- and Potassium-Stimulated Adenosine Triphosphate Activity by Chlorpromazine Free Radical. Mol. Pharmacol. 4:600-612.

Akera, T., and T. M. Brody. 1969. The Interaction between Chlorpromazine Free Radical and Microsomal Sodium- and Potassium-Activated Adenosine. Triphosphatase from Rat Brain. Mol. Pharmacol. 5:605-614.

Bader, H. 1971. In "GAP" Conference Reports," Cystic Fibrosis Factor," February 19-20, 1971. Galveston, Texas.

Barnett, D. R., A. Kurosky, B. H. Bowman, H. T. Hutchinson, I. Schmoyer, and S. D. Carson. 1973a. Cystic Fibrosis: Molecular Weight Estimation of the Ciliary Inhibitor. Tex. Rep. Biol. Med. 31:703-708.

Barnett, D. R., A. Kurosky, B. H. Bowman, and S. C. Barranco. 1973b. Loss of Ciliary Inhibitory Effect of the Cystic Fibrosis Factor Following Proteolytic Digestion and Heat Denaturation. Tex. Rep. Biol. Med. 31:697-701.

Barnett, D. R., M. S. Schanfield, M. C. McCombs, and B. H. Bowman. 1973c. Isoelectric Focusing and IgG Allotyping of the Serum Fraction Containing the Cystic Fibrosis Ciliary Inhibitor. Tex. Rep. Biol. Med. 31:709-714.

Barnett, D. R., S. C. Barranco, L. H. Lockhart, B. J. Lankford, J. Meyne, and B. H. Bowman. 1973d. Cystic Fibrosis: Growth Kinetics and Production of the Ciliary Inhibitor by Cultured Fibroblasts. Tex. Rep. Biol. Med. 31:691-696.

Barranco, S. C., W. E. Bolton, B. R. Haenelt, and C. W. Abell. 1976. Differences in the Incorporation of Thymidine into DNA of Normal and Cystic Fibrosis Fibroblasts in Vitro. Jour. Cell Physiol. 88:33-41.

Beratis, N. G., J. H. Conover, E. J. Conod, E. J. Bonforte, and K. Hirschhorn. 1973. Studies on Ciliary Dyskinesia Factor in Cystic Fibrosis. III. Skin Fibroblasts and Cultured Amniotic Fluid Cells. Pediat. Res. 7:958-964.

Bowman, B. H., L. H. Lockhart, and M. L. McCombs. 1969. Oyster Ciliary Inhibition by Cystic Fibrosis Factor. Sci. 164: 325-326.

Bowman, B. H., M. L. McCombs, and L. H. Lockhart. 1970. Cystic Fibrosis: Characterization of the Inhibitor to Ciliary Action in Oyster Gills. Sci. 167:871-873.

Bowman, B. H., D. R. Barnett, R. Matalon, B. S. Danes, and A. G. Bearn. 1973. Cystic Fibrosis: Fractionation of Fibroblast Media Demonstrating Ciliary Inhibition. Proc. Nat. Acad. Sci. USA. 70:548-551.

Bowman, B. H., B. J. Lankford, G. M. Fuller, S. D. Carson, A.Kurosky, and D. R. Barnett. 1975. Cystic Fibrosis: The Ciliary Inhibitor is a Small Polypeptide Associated with Immunoglobulin G. Biochem. Biophys. Res. Comm. 64:1310-1315.

Blomfield, J., A. R. Rush, H. M. Allars, and J. M. Brown. 1976. Parotid Gland Function in Children with Cystic Fibrosis and Child Control Subjects. Pediat. Res. 10:574-578.

Cheung, A. W., and T. L. Jahn. 1976a. High Speed Cinemicrographic Studies on Rabbit Tracheal (Ciliated) Epithelia: Determination of the Beat Pattern of Tracheal Cilia. Pediat. Res. 10: 140-144.

Cheung, A. W., and T. L. Jahn. 1976b. High Speed Cinemicrographic Studies on Rabbit Tracheal (Ciliated) Epithelia: Cytolytic Effect of Cystic Fibrosis Serum on Tracheal Epithelial Cells. Pediat. Res. 10:144-147.

Cole, C. H., and J. H. Dirks. 1972. Changes in Erythrocyte Membrane ATPase in Patients with Cystic Fibrosis of the Pancreas. Pediat. Res. 6:616-621.

Cole, C. H., and G. Sella. 1975. Inhibition of Ouabain-Sensitive ATPase by Saliva of Patients with Cystic Fibrosis of the Pancreas. Pediat. Res. 9:763-766.

Conod, E. J., J. H. Conover, and K. Hirschhorn. 1975. Demonstration of Human Leukocyte Degranulation Induced by Sera from Homozygotes and Heterozygotes for Cystic Fibrosis. Pediat. Res. 9:724-729.

Conover, J. H., R. J. Bonforte, P. Hathaway, S. Pacuic, E. J. Conod, K. Hirschhorn, and F. B. Koppel. 1973a. Studies on Ciliary Dyskinesia Factor in Cystic Fibrosis. I. Bioassay and Heterozygote Detection in Serum. Pediat. Res. 7:220-223.

Conover, J. H., N. G. Beratis, E. J. Conod, E. Ainbender, and K. Hirschhorn. 1973b. Studies on Ciliary Dyskinesia Factor in Cystic Fibrosis. II. Short Term Leukocyte Cultures and Long Term Lymphoid Lines. Pediat. Res. 7:224-228.

Conover, J. H., E. J. Conod, and K. Hirschhorn. 1973c. Complement Components in Cystic Fibrosis. Lancet iii:1501.

Conover, J. H., E. J. Conod, and K. Hirschhorn. 1974. Studies on Ciliary Dyskinesia Factor in Cystic Fibrosis. IV. Its Possible Identification as Anaphylatoxin (C3a)-IgG Complex Life. Sci. 14:253-266.

Corbin, N. C., T. E. Hugli, and H. J. Müller-Eberhard. 1976. Serum Carboxypeptidase B: A Spectrophotometric Assay Using Protamine as Substrate. Anal. Biochem. 73:41-51.

Danes, B. S., S. D. Litwin, F. H. Hutteroth, H. Cleve, and A. G. Bearn. 1973. Characterization of Cystic Fibrosis Factor and its Interaction with Human Immunoglobin. Jour. Exp. Med. 137:1538-1543.

di Sant'Agnese, P. A., R. C. Darling, G. A. Perera, and E. Shea. 1953. Sweat Electrolyte Disturbances Associated with Childhood Pancreatic Disease. Amer. Jour. Med. 15:777-784.

di Sant'Agnese, P. A., and R. C. Talamo. 1967. Pathogenesis and Pathophysiology of Cystic Fibrosis of the Pancreas. New Eng. Jour. Med. 277:1287-1294; 1344-1352; and 1399-1408.

Dische, Z., P. A. di Sant'Agnese, C. Pallivicini, and J. Youlos. 1959. Composition of Mucoprotein Fractions From Duodenal Fluid of Patients with Cystic Fibrosis of the Pancreas and from Controls. Pediat. 27:74-91.

Doggett, R. G., and G. M. Harrison. 1973. Cystic Fibrosis: *In vitro* Reversal of the Ciliostatic Character of Serum and Salivary Secretions by Heparin. N.N.B. 243:251-252.

Drewes, P. A. 1972. Direct Colorimetric Determination of Phosphorus in Serum and Urine. Clin. Chim. Acta. 39:81-88.

Duffy, M. J., D. Comer, and V. Schwarz. 1973. Serum Binding of Ca^{2+} and Cystic Fibrosis. Nature New Biol. 246:151.

Erdös, E. G., E. M. Sloane, and I. M. Wohler. 1964. Carboxypeptidase in Blood and Other Fluids-I. Properties, Distribution and Partial Purification of the Enzyme. Biochem. Pharmacol. 13: 893-905.

Erdös, E. G., H. Y. T. Yang, L. L. Tague and N. Manning. 1967. Carboxypeptidase in Blood and Other Fluids - III. The Esterase Activity of the Enzyme. Biochem. Pharmacol. 16:1287-1297.

Fisk, C. H., and Y. Subbarow. 1925. A Colorimetric Method for the Determination of Phosphorus. J. Biol. Chem. 66:375-400.

Gugler, E., C. J. Pallivicini, H. Swerdlow, and P. A. di Sant'Agnese. 1967. The Role of Calcium in Submaxilliary Saliva of Patients with Cystic Fibrosis. Jour. Pediat. 71:585-588.

Hanahan, D. J., and J. E. Ekholm. 1974. The Preparation of Red Cell Ghosts (Membranes). Meth. in Enzymol. 31:168-171.

Herzberg, V., L. Calvert and B. H. Bowman. 1973. Evidence that the Ciliary Inhibitor of Cystic Fibrosis is not an Antibody. Jour. Clin. Invest. 52:2732-2736.

Lieberman, J. 1974. Complement Components in Cystic Fibrosis. Lancet ii:1230.

Lieberman, J. 1975. Carboxypeptidase B-like activity and C3 in Cystic Fibrosis. Am. Rev. Resp. Dis. 111:100-102.

Mangos, J. A., N. R. McSherry, and P. J. Benke. 1967. A Sodium Transport Inhibitory Factor in the Saliva of Patients with Cystic Fibrosis of the Pancreas. Pediat. Res. 1:436-442.

Rennert, O. M. 1976. RNA Methylation and Polyamine Metabolism in Cystic Fibrosis. Miner. Pediat. 28:62-68.

Satir, P. 1975. Ionophore-Mediated Calcium Entry Induces Mussel Gill Ciliary Arrest. Sci. 190:586-588.

Schmoyer, I. R., S. P. Brooks, and J. F. Fischer. 1972. Isolation and Characterization of a Ciliary Dyskinetic Factor from Cystic Fibrosis Heterozygotes Serum. Life Sci. 11:1037-1044.

Schmoyer, I. R., and F. A. Baglia. 1974. Cystic Fibrosis: Effect of Media From Cultured Cystic Fibrosis Fibroblasts on ATPase Activity. Biochem. Biophys. Res. Comm. 58:1006-1070.

Sokolovsky, M. 1974. Carboxypeptidase B. Meth. in Enzymol. 34: 411-414.

Spock, A., H. M. G. Heick, H. Cress, and W. S. Logan. 1967. Abnormal Serum Factor in Patients with Cystic Fibrosis of the Pancreas. Pediat. Res. 1:173-177.

Warwick, W. J., R. E. Pogue, H. U. Gerber, and C. J. Nesbitt. 1975. Survival Patterns in Cystic Fibrosis. Jour. Chron. Dis. 28:609-622.

Wilson, G. B., and H. H. Fudenberg. 1975. Studies on Cystic Fibrosis Using Isoelectric Focusing. I. An Assay for Detection of Cystic Fibrosis Homozygotes and Heterozygote Carriers from Serum. Pediat. Res. 9:635-640.

Wood, R. E., and P. A. di Sant'Agnese. 1973. Bioassays of Cystic Fibrosis Factor. Lancet iii:1452-1453.

Yeates, D. B., J. M. Sturgess, S. R. Khan, H. Levison, and N. Aspin. 1975. Mucociliary Transport in the Trachea of Patients with Cystic Fibrosis. Arch. Dis. Child. 51:28-33.