Yale University
EliScholar – A Digital Platform for Scholarly Publishing at Yale

Yale Medicine Thesis Digital Library School of Medicine

1976

Alpha$_1$-fetoproteins and cystic fibrosis

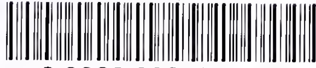
Paul R. Ramirez
Yale University

Follow this and additional works at: http://elischolar.library.yale.edu/ymtdl

Recommended Citation

Ramirez, Paul R., "Alpha$_1$-fetoproteins and cystic fibrosis" (1976). *Yale Medicine Thesis Digital Library*. 3050.
http://elischolar.library.yale.edu/ymtdl/3050

This Open Access Thesis is brought to you for free and open access by the School of Medicine at EliScholar – A Digital Platform for Scholarly Publishing at Yale. It has been accepted for inclusion in Yale Medicine Thesis Digital Library by an authorized administrator of EliScholar – A Digital Platform for Scholarly Publishing at Yale. For more information, please contact elischolar@yale.edu.


YALE MEDICAL LIBRARY
3 9002 08676 3126

ALPHA$_1$-FETOPROTEINS AND CYSTIC FIBROSIS

PAUL R. RAMIREZ

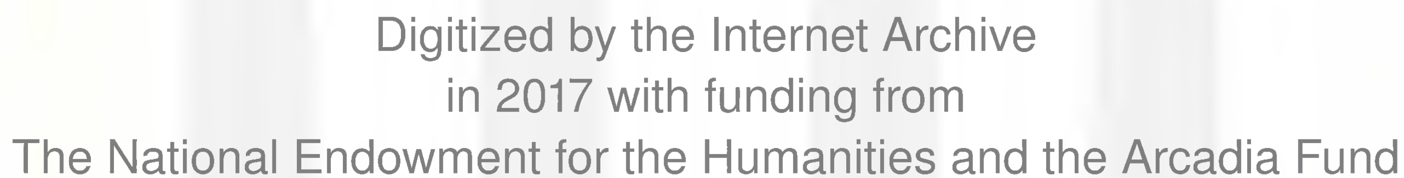
1976

YALE

MEDICAL LIBRARY

YALE

MEDICAL LIBRARY


Digitized by the Internet Archive
in 2017 with funding from
The National Endowment for the Humanities and the Arcadia Fund

https://archive.org/details/alphafetoprotein00rami

ALPHA$_1$-FETOPROTEINS and CYSTIC FIBROSIS

A Thesis Presented In Partial Fulfillment
of the Requirements for the Degree of
Doctor of Medicine

by

Paul R. Ramirez

February 1976

DEDICATION

TO my wife, Carrie, and to my parents.

ACKNOWLEDGEMENTS

I would like to offer thanks to Dr. Thomas Dolan and a special thanks to Dr. Alexander Baumgarten for the invaluable assistance and encouragement which they offered in acting as my advisors for this project.

I would also like to offer a very special thanks to my wife, Carrie, for her assistance, encouragement, patience, and love.

A final thanks to my parents for all that they sacrificed in my behalf.

TABLE OF CONTENTS

I.	Introduction	1
II.	Review of the Literature	2
	Cystic Fibrosis	2
	Alpha$_1$-Fetoprotein (AFP)	14
III.	Materials and Methods	30
	Subjects	30
	Collection of Specimens	31
	Isolation of AFP	31
	Production of Anti-Serum	34
	Counterimmunoelectrophoresis	35
	Radioiodination of AFP	36
	Immunoautoradiography	38
	Radioimmunoassay	41
IV.	Results	46
	Counterimmunoelectrophoresis	46
	Immunoautoradiography	46
	Radioimmunoassay	49
	Standard Inhibition Curve	49
	Serum Samples	49
V.	Discussion	52
VI.	Illustrations	
	Figure I. Purity of Isolated AFP	33
	Figure II. Specificity of Rabbit Anti-AFP Sera	35
	Figure III. Antiserum Dilutions	43
	Figure IV. Immunoautoradiography Standard Curve	47
	Figure V. Immunoautoradiography Samples	48
	Figure VI. Radioimmunoassay Standard Curve	50
	Table I. Immunologic Techniques for AFP measurement.	15
	Table II. Radioimmunoassay Results	51

INTRODUCTION

Cystic Fibrosis (CF), with an incidence of approximately 1 in 2000[1] and a gene carrier rate of between 3 and 5 percent,[2] is the most frequently occurring lethal hereditary disease of Caucasians in this country. Rare among non-Whites, the CF gene is five times more common in the general U.S. population than the gene for the next most frequent lethal hereditary disease, sickle cell anemia.

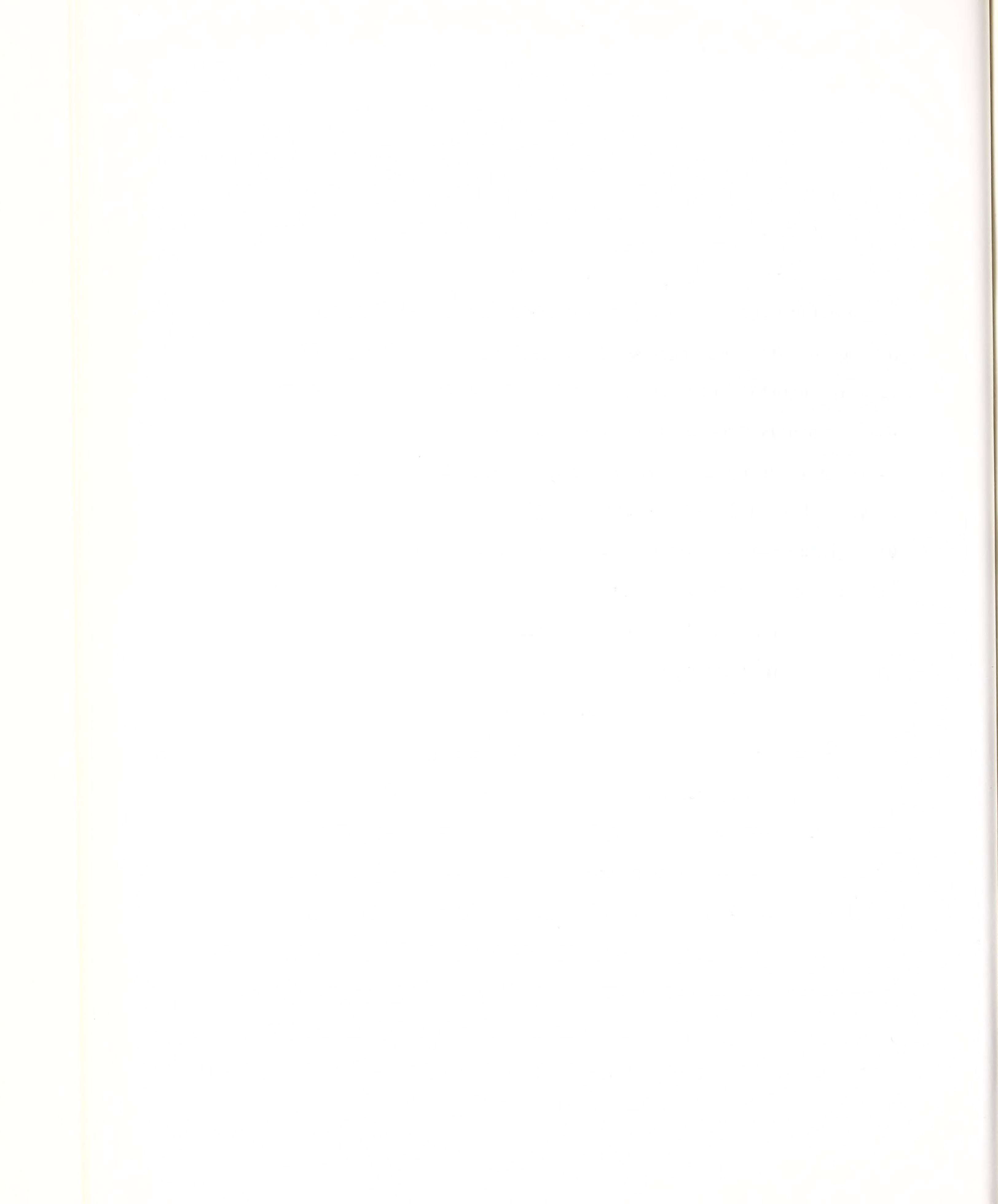
While a definitive method for diagnosis of the disease, the sweat test, has been available since 1953,[1] there has existed no such method for reliably identifying those heterozygous for the CF Gene. In March, 1975, Chandra et al.,[3] described a study in which, by radioimmunoassay, they were able to detect differences in the levels of serum $Alpha_1$-Fetoprotein (AFP) between normals, those heterozygous for the CF Gene, and those homozygous for the same Gene. This, if reliably reproducible, would present a means for early identification of those at risk for producing a child with CF, and would provide the opportunity for adequate genetic counselling. Because of this, our interest in this means of heterozygote identification was greatly aroused and so we attempted in this study to determine if the population of heterozygotes having contact with Yale-New Haven Hospital (YNHH) could be similarly identified.

REVIEW OF THE LITERATURE

Cystic Fibrosis

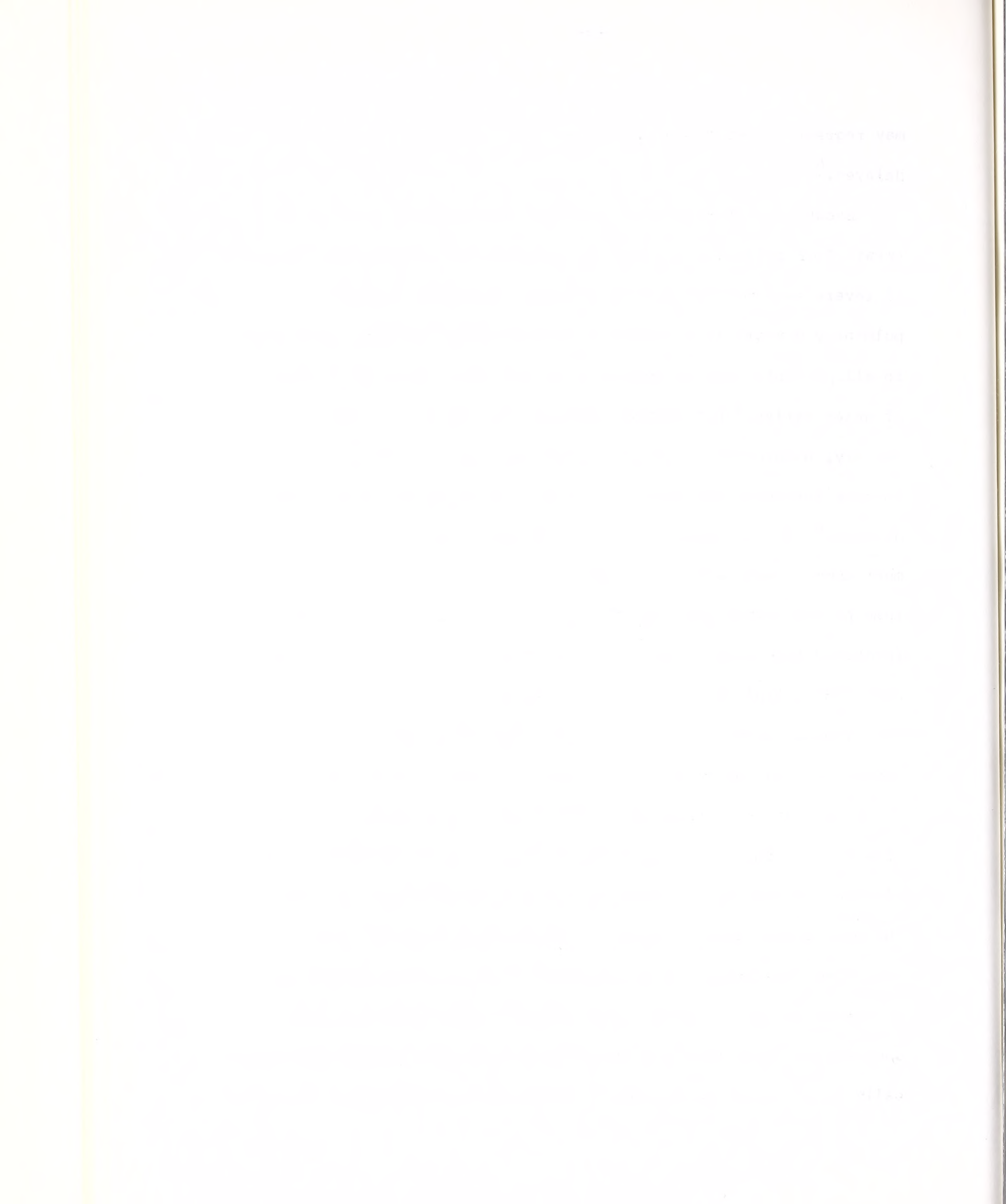
Cystic Fibrosis is a truly protean disease, classically presenting with the triad of chronic pulmonary disease, pancreatic insufficiency and abnormally elevated sweat electrolytes, but frequently manifested as well by such entities as hepatic cirrhosis, rectal prolapse, intestinal obstruction, and nasal polyps. Meconium ileus, virtually pathognomic of CF, is present at birth in 10-15% of children with the disease[4] and is often complicated by meconium peritonitis, volvulus, and/or atresia.[5,4] Patients beyond the newborn period may present with the syndrome of meconium ileus equivalent marked by intestinal obstruction due to tenacious impacted stools.

If untreated, children with CF typically exhibit failure to thrive despite ravenous appetites, foul-smelling stools, and abdominal distension. Steatorrhea, due to the malabsorption caused by pancreatic insufficiency, can be quite severe, with fecal fat content averaging 4-5 times normal.[2] The children tend to be small, with below average body weights, and appear malnourished. Liposoluble vitamin deficiencies (especially Vitamins A and K) may occur. Early diagnosis and symptomatic therapy may promote normal growth rates and physical appearance. Despite the fact that symptoms



may regress during the period of adolescence, puberty may be delayed.[6]

Because of the elevated levels of electrolytes found in the sweat of CF patients, hot weather presents the considerable threat of severe sodium depletion and vascular collapse. Chronic pulmonary disease is a prominent feature of CF, eventually develops in all patients, and is generally severe and progressive. Time of onset varies,[7] but initial features usually include wheezing and dry, nonproductive cough. Atalectasis is not uncommon, and in some instances may present as the first manifestation of the disease.[8] Mucoid impaction of the bronchi presents as one of the most common complications. Episodes of pneumonia are frequent in some patients with Staphlococcus aureus and Pseudomonas aeruginosa (probably secondary to antibiotic therapy) being the most common infecting organisms.[9] As pulmonary involvement becomes chronic and symptoms progress, chronic productive cough, wheezing and tachypnea, and cor pulmonale may become severe. Pulmonary function tests show decreased vital capacity and an increase in airway resistance proportional to the severity of the obstructive disease. Barrel-chest deformity and digital clubbing are common. The most common cause of death is acute respiratory distress resulting from pneumonia and anoxia.[2] The liver involvement in CF occurs in approximately 2% of cases.[7] These patients, most commonly in their teens, develop clinical liver cirrhosis (pathologically focal bilary cirrhosis).[10] Increased consistency of the organ



and a nodular surface are noted. Elevations of serum ceruloplasmin, low serum cholinesterase, elevated alkaline phosphatase, and abnormal BSP retention are confirmatory tests of this complication.[8] Patients dying early in life commonly show small scattered areas of hepatic abnormality at autopsy. Plugs of inspissated material are seen in some of the small bile ducts, suggestive of stagnation of secretions. Early fibrosis around the distended ducts and biliary ductule multiplication in the portal tract may be present.[11] Patients with cirrhosis may develop portal hypertension, hypersplenism, and bleeding esophageal varices.

While the clinical manifestations of CF may vary, it seems clear that the disease is transmitted as an autosomal recessive trait.[12] In addition, Danks et al.[12] suggests that it is quite likely that the disease may be caused by a single mutant allele at the locus concerned. There currently exists no adequate explanation for the high carrier frequency for CF observed in the Caucasian population. A high mutation rate, unlike that observed for other recessive traits, would be required in order to maintain the observed incidence by mutation alone.[2] In attempting to explain this, Anderson et al.[13] have postulated a heterozygote advantage of increased fertility of female CF carriers. While such an advantage is quite possible, McCombs[2] describes it to have been calculated to be about 2% and therefore difficult to detect.

Several theories exist as to the pathogenesis of CF, each attempting to explain the myriad of symptoms characteristic of

the disease in terms of a single basic defect. These theories have been well described by numerous authors.[1,11,14,2,15] Among the most popularly accepted are four which attribute the basic pathogenesis of CF to ion transport dysfunction, abnormal secretions, autonomic dysfunction, and abnormal serum factors respectively.

The abnormally elevated level of sodium and chloride (and to some extent potassium) in the sweat of CF patients stands as the most constant feature of the disease,[16] and as such lends great reliability to the sweat test used for its diagnosis. The reason for these observed elevations, however, remains obscure. CF is marked by no morphologic differences in sweat glands,[17] rates of sweating,[18] or in the concentration of the organic constituents of sweat.[19] Schulz,[20] however, demonstrated by micropuncture techniques a failure in the reabsorption of sodium from the fluid secreted into the sweat gland duct. Mangos,[21] on the basis of microperfusion studies, concluded that this failure in reabsorption was due to an inhibitory effect exerted intraluminally by the sweat of affected persons. In 1967, Mangos and McSherry[22] isolated a nondialyzable heat labile factor from CF sweat and saliva which inhibited sodium reabsorption in the parotid gland of rats and in the normal sweat gland.[23] The sweat glands in CF appear to be less sensitive than normal to the effects of salt-retaining steroids.[16]

Balfe et al.[24] examined active transport of sodium in the erythrocytes of CF patients. They reported both sodium pumps I and II to be decreased in activity in the red cells of children with CF.

While pump I appeared normal in parents of CF patients, th was a notable decrease in pump II (ethacrynic acid-sensitive) ivity in their red cells as compared with those of normal contr s. Other investigators have, however, been unable to support these observations.[25]

Gibson et al.[26] have attempted to explain the basic defect of CF in terms of the abnormally high calcium content observed in all glycoprotein-rich secretions in CF (e.g., those elaborated by exocrine glands). Calcium levels in these secretions are elevated despite the presence of normal serum calcium, phosphorous,[1] and probably sweat calcium levels.[27] Gibson et al.[26] report the presence of a factor in CF serum which produces calcium hyper-secretion, while Horton et al.,[28] based on their findings of reduced calcium-dependent red cell ATP-ase in CF erythrocyte ghosts, propose a defective resorptive mechanism as the reason for calcium elevations. Hadden et al.,[25] however, failed to demonstrate a defect in CF patients of the calcium active transport system, which requires calcium-dependent ATP-ase. Another source[15] attributed the high concentrations of calcium in exocrine secretions to the inclusion of excessive amounts of calcium in secretory granules. Gibson[26] suggests that excess calcium secretion by exocrine glands results in the hyperpermeability of mucus which is therefore unable to inhibit the passive flow of water and small ions. This would result in the elaboration of abnormally highly concentrated and hyperviscous secretions which Johansen et al.[11] claim might result in direct tissue damage and duct obstruction, eventually leading to

the glandular lesions (lungs, pancreas, liver, intestine) characteristic of CF. Di Sant'Agnese[1] has alternatively suggested that this hyperviscosity might be due to the formation of a calcium-glycoprotein complex. Glycoprotein quantities in CF secretory glands are said to be normal, but they have been shown to have an abnormal composition with an increased fucose to sialic acid ratio.[29,30] Saliva elaborated by the submaxillary gland in CF is rich in glycoproteins and abnormal in its make-up and appearance.[31,29] CF saliva contains elevated amounts of calcium, fucose, sialic acid, hexose, and total protein.[31] It is turbid in appearance, but can be cleared to resemble normal saliva by the addition of chealating agents. The addition of calcium to normal saliva results in increased turbidity.[32] As in sweat, sodium and chloride concentrations have been noted to be elevated in CF saliva.[33]

In 1959, Roberts[34] postulated that the basic defect in CF might be due to a chronic excess of parasympathetic stimulation of the body's cholinergic glands, including Brunner's glands, sweat glands, pancreatic, bronchial, and salivary glands. The histologic changes seen in the disease were, he proposed, due to the exhaustion of these glands. This hypothesis has long been a popular one since the autonomic nervous system innervates and controls the secretory function of the very same exocrine glands which are most notably affected in CF. Chernick et al.[31] demonstrated that parasympathomimetic stimulation in normal children (but not in adults) resulted in the production by them of saliva similar in composition and appearance to

that of CF patients. They also showed that CF saliva became clear and normal in the concentrations of its constituents (except for calcium which remained elevated) after injection with the adrenergic blocking agent, guanethidine.[35] An examination of the adrenal medullas of CF patients at autopsy has revealed the presence of elevated levels of catecholamines.[36] The observation of elevated urinary Catecholamine metabolites in CF was made in 1967 (as recorded by di Sant'Agnese[1]), but others found these levels to be normal.[37] Rubin et al.[38] concluded that a generalized autonomic dysfunction existed in CF on the basis of differences they observed between the pupillary reactivity of individuals with CF and normals under conditions of rest and stress, and after termination of painful stimuli. They concluded that the CF patient suffered from adrenergic underactivity. Spock,[14] however, suggests that rather than being a generalized autonomic disturbance, the site of such a defect in CF would more likely be at the neuroeffector junction or within the affected secretory gland itself. Working on isolated dog salivary glands, Dische et al.[39] reported an alteration in glycoprotein composition reminiscent of that seen in CF (increase in fucose and decrease in sialic acid) after pilocarpine stimulation. On the other hand, chronic pilocarpine[40] or atropine[41] stimulation has failed to reproduce the CF complex in experimental animals. An attempt to alleviate or reverse symptoms in CF patients by sympathetic blockade with cocaine, followed by a right-sided complete splanchnicectomy

has proved ineffective.[42]

Spock et al.[43] demonstrated in 1967 the presence of a heat labile, nondialyzable factor in the sera of children with CF which disorganized the regular ciliary rhythm in rabbit tracheal explants. This ciliary dyskinesia factor (CDF), was detected in the sera of obligate heterozygotes for the CF Gene, though in lower concentration. While initially encouraging as a possible means for heterozygote detection, this technique is based on a subjective judgment and has proved unreliable, as normal sera have been observed to produce the CDF effect.[44] In 1969, Bowman et al.[45] introduced a modification of Spock's technique which employed oyster gill cilia in place of rabbit tracheal explants, and which was more quantitative in that the time required for cessation of all ciliary motion was the recorded value for each serum tested. The group found CDF effects in the same settings as did Spock. The bulk of the work which has been done on CDF has employed the oyster gill technique since the time of its introduction. Much work has been done in attempting to determine the exact identity of CDF. In 1970, Bowman et al.[46] determined that CDF migrates in the γ-globulin fraction of serum, raising the possibility that it might be a specific antibody. Herzberg et al.,[47] however, determined that while associated with the IgG fraction, CDF does not interact with oyster cilia in a typical antigen-antibody reaction. Danes et al.[48] have provided evidence to show that CDF is probably simply bound to certain γ-globulins. It was shown in 1972[49] that the CDF effect could be

produced by the medium from in vitro cultures of skin fibroblasts from those with CF, but not by fibroblast from normal individuals. Conover et al.[50] have presented evidence that anaphylotoxin (C3a) is a pre-CDF which, both at the cell surface and bound to IgG (C3a-IgG complex is CDF), is the prime mediator of the various pathophysiologic conditions seen in CF. They suggest that C3a may act at the secretory cell surface to facilitate extrusion of granules or other materials reflecting the cell's function (e.g., digestive enzymes from salivary cells). This secretion, rapidly terminated in the normal state by a degradative enzyme (anaphylotoxin inhibitor), would continue if such enzyme were deficient or absent, resulting in the accumulation or secretory products leading to the classic picture of organ involvement seen in CF. Thus, they suggest a defect in or deficiency of anaphylotoxin inhibitor would be the primary defect in cystic fibrosis. Polley and Bearn[51] in 1971 also suggested the possible relation of CDF to the complement system. While this appears as one of the more promising developments in the research on CDF, factors such as the lack of clear definition of CDF as central to the pathogenesis of CF, or byproduct of the disease; the report by Wood and di Sant' Agnese[52] suggesting that CDF activity may be present in normal serum even with the oyster assay; and the alternate claim of identification of CDF as amylase by Doggett et al.,[53,54] must be resolved.

Another long recognized defect in CF is the observation made by Mangos and McSherry[22] that sweat and saliva from affected

individuals markedly inhibit the reabsorption of sodium when infused into rat parotid ducts. Whether the factor responsible for this effect and CDF are the same is not known. Murakami and Eckert[55] have shown that the regular, well coordinated beating of cilia is dependent on normal calcium transport across cell membranes. It is indeed possible that the CDF may produce its effect by causing abnormal calcium flux or abnormal calcium secretion by exocrine glands as Gibson postulated.[26] Spock et al.[43] speculated that the serum factor he described might alter the transport of electrolytes, thereby producing changes in the action potential within the cell and resulting in the observed ciliary dyskinesia. Mangos and McSherry,[22] observing that positively charged molecules acted in the same way as their CF factor to inhibit sodium transport in the parotids of rats, suggested that this factor might be such a positively charged molecule, whose effect was the result of an alteration in membrane permeability. Interested in this possibility, they added heparin, a negatively charged molecule which has been shown to form complexes with positively charged molecules, to the sera of individuals with CF. Upon doing so they noted an apparent negation of the inhibitory effects originally exhibited by these sera. CDF has been shown to bear a positive charge,[56] and Bowman et al.[45] observed that positively charged molecules, other than CDF, could produce the same effect on oyster cilia. Finally, Doggett et al.[53,54,57] demonstrated that heparin could reverse the dyskinetic effects of CF serum and saliva upon oyster cilia, both

in vivo and in vitro.

Danes and Bearn[58] have demonstrated that fibroblasts of CF patients contain an abnormal cytoplasmic substance which causes them to stain metachromatically. They also noted that the fibroblasts of heterozygous relatives showed the same metachromasia which was absent from the stained fibroblasts of normal subjects. Two different patterns of metachromatic staining were identified. These are genetically consistent (CF patients and parents always show the same type) and may therefore be demonstrating heterogeneity of the CF locus. The observed staining is thought to represent accumulated acid mucopolysaccharides whose presence, Spock[14] has speculated, may be due to a cell membrane defect, a defect in the lysozymes or a specific enzyme within the lysozyme which normally breaks down mucopolysaccharides, or an increased synthesis due to lack of an inhibitor. The exact significance of the observed staining characteristics of CF fibroblasts remains uncertain.

With regard to heterozygote detection, the only test available in the recent past upon which to base genetic counseling of those at risk has been the oyster gill bioassay for CDF. This test, however, by the condemnation of those rejecting it,[52] and the admission of some of those employing it,[51] is unreliable and subject to inherent variability due to the fact that it is a bioassay. It can also be expensive and difficult to set up--all in all unsuitable for mass screening for detection of heterozygotes. In attempting to isolate and identify CDF by an alternate means,

Atland et al.[59] employed isoelectric focusing and disc electrophoresis. They were successful in isolating a factor from the serum of CF patients and obligate heterozygotes which they felt to be similar to, if not identical with, CDF. Wilson and Fudenberg[60] also employed isoelectric focusing to obtain similar results in successfully identifying CF homozygotes and heterozygotes. Swachman et al.[61] were able to distinguish all homozygotes and 90% of the heterozygotes in their study from normal subjects. They did this by utilizing the Ussing chamber technique to measure differences in the electrical properties (potential difference and resistance) of rat jejunum in the presence of the sera to be tested. Finally, as mentioned in the introduction, Chandra et al.[3] have described the ability to distinguish CF patients and obligate heterozygotes from normal subjects on the basis of their serum Alpha$_1$-fetoprotein levels. This work, forming the basis for the current study, will be more fully discussed in the next section of this paper ("Alpha$_1$-Fetoprotein").

What occurs, then, from a review of the evidence for each of the four major theories of CF pathogenesis is a multiplicity of results, some positive, some negative, from which no clear cut conclusions can as yet be drawn. The pleiotropic effects of the CF Gene will continue to mask the identity of the basic defect in this very common disease until we are able to develop a better working knowledge of the basic principles behind the pathologic features of each of the systems involved. Only then can we hope to recognize the

set of generalized principles at work in all of these systems which produces the entity we call cystic fibrosis.

Alpha$_1$-Fetoprotein (AFP)

In the progression from fetus to adult, there is presumably a complex sequence of biochemical "on-off" switches which controls the normal pattern of growth. It is possible that the relatively uninhibited growth of malignant cells is somehow representative of a de-differentiation process causing the cells to revert to embryonic growth patterns. The first immunological evidence that embryonic phase-specific antigens (PSA) might be related to tumor antigens appeared in 1932 and, since that time, numerous investigators [65,66,67,62,63,64] have examined this possibility. Among the more intriguing and useful applications of this concept has been the measurement of elevated levels of embryonic PSA in adults, and the use of such measurement both to indicate the presence of neoplastic disease and to monitor its course and response to treatment.[68] In recent years, the presence of elevated levels of a protein which normally occurs in the sera of human fetuses (variously known as fetoprotein, postalbumin, α_1-fetoprotein, and α_1-globulin) has been demonstrated in a large number of patients with primary tumors of the liver or with teratoblastomas. Its reappearance in large quantity in the serum of adults has resulted in the generalized acceptance of AFP measurement as a diagnostic test for the presence

of these neoplasms.[67,68]

AFP was first identified by Pedersen in 1944[69] in sera from the newborn calf and was named "fetuin" at that time. After detecting a trace of AFP in human cord blood, Bergstrand and Czar[70] extended this observation and later reported the detection of AFP in the serum of human fetuses less than 5 months old.[71,72] The new protein was found to migrate between albumin and α_1-globulin on paper electrophoresis. It was further established to have a molecular weight between 64000[73] and 70000[72,74] and to be formed by a single polypeptide chain.[75,74] Reliable detection and measurement initially presented formidable technical problems,[76] but with the development of more sophisticated immunological techniques [72,77] these problems were overcome, setting the scene for more detailed inquiries. A summary of immunological techniques for AFP detection, along with their sensitivities and applications is provided in Table I from Adinolfi et al.[78]

TABLE I

SOME IMMUNOLOGICAL METHODS FOR THE DETECTION AND ESTIMATION OF AFP*

Methods	Sensitivity†	Application
Double diffusion in agar gel	2–5 μg/ml	Detection
Counter-current electrophoresis	0.25–0.5 μg/ml	Detection; semi-quantitative
Single radial diffusion	2.5 μg/ml	Quantitative
Electroimmunodiffusion (rocket)	0.5–1.0 μg/ml (50 ng/ml)‡	Quantitative
Radioimmunoassay	2–5 ng/ml	Quantitative
Enzyme-linked immunoassay	5 ng/ml	Quantitative
Reverse agglutination	5 ng/ml	Quantitative

* For references see text.
† Lowest sensitivity using conventional technique.
‡ The sensitivity is increased using an immunoradioassay or an immunoperoxidase test.

(Adinolfi, A., et al., Alpha-feto-protein during development and in disease. J. Med. Gen., 12:138-151, 1975.)

Recent results with radioimmunoassays for AFP have demonstrated even greater sensitivities than those quoted by Adinolfi, with Ruoslahti and Seppala[74] reporting a sensitivity threshold of 250 picograms per milliliter. Other workers[139] have reported sensitivities down to 165 ng/ml for the single radial diffusion technique. Sizaret[143] has utilized the technique of radioelectro-complexing (a radioimmunoassay which employs electric current first to bring together the immunoreagents, and then to separate the bound from the unbound radiolabelled antigen) to reach sensitivities between 200 and 400 picograms per milliliter of AFP.

By means of the immunodiffusion techniques, it has been established that AFP is normally present in human fetal serum, making its appearance in measurable quantities by the 4th week of development. The levels of the protein rise steadily over the next 1-3 months, reaching their peak values of 200-400 milligrams per 100 milliliters by 12 to 16 weeks of gestation.[72,79] At this time AFP may comprise one-tenth of the total fetal proteins.[74] Beyond this period, AFP levels fall rapidly, until at birth concentrations of 2-17 milligrams per 100 milliliters[72,75] or less[68] are present. In the period immediately after birth, this level decreases ($T\frac{1}{2}$ = 3-5 days[68]) to levels detectable only by radioimmunoassay. Any form of acute liver disease during this period of neonatal decrease can result in transient elevations of serum AFP.[80] Increased levels of serum AFP (44-2800 micrograms/milliliter) were found in children with ataxia-telangiectasia, but were not detected in those with various

other immune deficiency diseases.[81] The persistence of high AFP levels in these patients is associated with the hypothesis that the primary defect is an abnormality of tissue differentiation. The generally accepted range of AFP levels measurable in normal subjects by radioimmunoassay is 1-25 ng/ml,[74,141] although levels as high as 50 ng/ml have been reported in apparently normal subjects free from detectable disease.[141]

Noting the decrease in fetal serum AFP levels during the last trimester, Bergstrand et al.[82] have attempted to correlate AFP levels with fetal maturity. Their results indicated that AFP levels in premature infants' sera were generally higher than those in full-term infants. They also found AFP levels to be more closely correlated with gestational age than either birth weight, total serum protein level, or serum albumin.

Seppälä and Ruoslahti[142] also found a correlation between amniotic fluid AFP levels and gestational age, with AFP levels decreasing with advancing gestation during the last trimester. By employing radioimmunoassay, these experimenters were able to detect AFP in amniotic fluid of normal fetuses from all three trimesters. Prior to this, immunodiffusion techniques had only been able to detect amniotic AFP during the first 22 weeks of gestation.[72] Amniotic AFP levels decreased from levels of 2600-2800 nanograms per milliliter during the second trimester, to levels of 15-535 nanograms per milliliter during the third trimester. AFP is known to be produced by the fetal liver and

yolk sac.[68] Seppälä and Ruoslahti[83] have suggested that the high amniotic component of AFP seen early in gestation may be derived from urinary AFP. This suggestion was made on the basis of their measurements of early fetal urinary and amniotic AFP as well as their observations of elevated amniotic AFP in the case of fetuses with congenital nephrosis.

Abnormally elevated amniotic AFP levels have been used at various times of gestation as a marker for an affected fetus. Thus, between 18 and 36 weeks of gestation, elevated amniotic AFP levels have been correlated with and successfully diagnosed anencephaly and spina bifida in utero.[84,85] Seller and Adinolfi[86] have suggested that the high levels of amniotic AFP in the case of open neural-tube defects is due to the transfer of AFP from the CSF. They detected AFP levels of 52 to 1220 micrograms per milliliter in the CSF of normal fetuses from 16 to 25 weeks of gestation. In a prospective study, Harris et al.[85] concluded that most cases of spina bifida and anencephaly were detectable by a combination of amniotic AFP measurement and ultrasound before 20 weeks of gestation, providing the opportunity for selective abortion. They found closed neural-tube defects to be associated with normal amniotic AFP levels while open neural-tube defects showed low AFP levels if measured during the last trimester when only a small amount of the protein is synthesized by the fetus.

Seppälä[87] noted elevated AFP levels in both amniotic fluid and in maternal serum in the case of intrauterine death. Adinolfi

et al.[78] have suggested that these elevations may be due to transendation of AFP in the amniotic fluid and transfer of the protein across the placenta associated with fetal death. This mechanism may also explain elevated maternal serum AFP levels which have been noted in the event of severe distress.[88] Elevated amniotic AFP levels in cases of severe Rh incompatability have been attributed to increased synthesis of AFP.[89,90]

Hirose[91] has well summarized the implication of abnormally high AFP levels in pregnancy.

> (1) fetal malformation: when maternal serum-AFP level is within the normal range but the amniotic fluid AFP concentration is markedly elevated; (2) placental dysfunction: when the maternal AFP level is markedly elevated irrespective of the level of AFP in amniotic fluid; and (3) placental dysfunction combined with fetal malformation: when both maternal serum-and amniotic-AFP levels are extraordinarily elevated.

Seppälä and Ruoslahti[88] have reported, by measurement with radioimmunoassay, high levels of AFP in maternal sera throughout pregnancy. They found levels of 18-119 nanograms per milliliter during the first trimester, 96-302 nanograms per milliliter during the second trimester, and 160-550 nanograms per milliliter during the third trimester. At labor these levels were between 103 and 400 nanograms per milliliter. The facts that the maternal and fetal serum AFP levels were significantly correlated (the highest maternal AFP levels were measured in those whose fetuses had highest levels, though maternal peak levels lagged behind fetal peak levels) and that maternal AFP levels fell rapidly during the post partum

period ($T\frac{1}{2}$ = 5 days as in infant) suggested to these investigators that the fetus is the source of maternal AFP elevation. The alternative possibility that the hormonal changes of pregnancy might temporarily de-repress AFP synthesis was considered by Seppälä.[92] This investigator found no rise in AFP following the hormonal changes induced by oral contraceptives. Seppälä and Ruoslahti[88] were unable to demonstrate the presence of maternal antibodies to AFP. The mean levels of maternal serum AFP have been found to be higher for multiple pregnancies than for single ones.[93]

The exact biological function of AFP in humans remains unknown. Uriel et al.[94] found that the hormones estrone, estradiol, estriol, and diethylstilbestrol are bound by AFP in the rat, and suggest that AFP may play a role in hormonal transport and in the feto-maternal relationship across the placental barrier in this animal. The fact that estrogen binding in humans is known to be associated with albumin and a β-globulin and the fact that Swarty et al.[95] were unable to demonstrate estrogen-binding by human AFP suggest that AFP probably plays a different role in man. Observations on the part of Murgita and Tomasi[96] on the effects of AFP on 1° and 2° antibody responses in mice has led them to suggest a possible immunoregulatory function for AFP. They postulate that AFP may in normal development protect the fetal mouse against immunologic attack by the mother. Again these results are not necessarily applicable to humans.

Abelev et al.[65] first reported that transplantable mouse

hepatomas synthesized and secreted into the blood an α-globulin immunologically identical to AFP, in 1963. In 1964, Tatarinov extended this observation to human patients with hepatocellular carcinoma.[66] Since that time, numerous studies examining the relationship between AFP, hepatoma, and liver disease in general have appeared.[80,98,99,100]

Employing double-gel diffusion, AFP is detectable in the sera of some 30% of Caucasian hepatoma patients in the U.S.[68] This figure is increased to between 50%[68] and 60%[101] of Caucasian hepatoma patients and a higher percentage of other racial groups if the more sensitive counterelectrophoresis and electroimmunodiffusion techniques are employed. Between 75% and 90% of hepatoma patients in Africa have been found to be AFP-positive by these techniques.[102] In addition to geographic variations, Bagshawe and Parker[103] have observed variation in the detectability of AFP in the sera of hepatoma patients of the same ethnic background who differed in age. Elevated AFP levels were detected significantly more frequently in hepatoma patients less than 30 years of age than in those over 60. In view of these variations, it has been suggested that the fetal gene for AFP production may undergo reactivation in the adult more readily under the influence of certain genetic or etiological factors and at younger ages.[78] Abelev[104] has suggested that the findings of elevated AFP levels in apparently healthy adults from "high risk" regions (Senegal and Congo)[105,106] may be the evidence of an enlarged initial pool of the "precursor cells"

(oval cells whose conversion into small hepatocytes during liver regeneration in the presence of carcinogens has been correlated with maximal AFP production)[107] or of an easiness of their activation. This, he believes, might be a basic reason for the comparatively high frequency of primary hepatocellular carcinoma among certain human populations.

Partial hepatectomy in humans has been reportedly associated with AFP synthesis,[108,109] but such reports have been episodical and often contradictory.[110] This has tended to support the conclusion that human AFP cannot be simply a product of regenerating hepatocytes.[68] Abelev[104] has chosen to differentiate between the type of hepatic regeneration seen after hepatectomy and that observed during the acute phase of chemical carcinogenesis. Watabe[111] has demonstrated that AFP synthesis during the acute phase of chemical carcinogenesis in the liver takes the form of a steep wave which drops off until the actual appearance of hepatoma, but rises sharply again with tumor development. Abelev explains that the basic process of regeneration after hepatectomy involves proliferation of pre-existing hepatocytes as shown by Leduc.[112] During the poisoning of liver cells by a carcinogen and liver regeneration in its presence, however, new hepatocyte formation takes place from the precursor cells with complete renewal of the hepatocyte population.[113] This basic difference, suggests Abelev, may well be the explanation for inconsistent findings of AFP synthesis after hepatectomy as compared to the well documented

AFP rise which accompanies chemical carcinogenesis.

Abelev[104] believes that his "precursor cells" are at greatest risk for undergoing tumor transformation and as such represent target cells for such transformation. He suggests that the initial action of a carcinogen is to be selectively toxic for mature differentiated hepatocytes and cause their degeneration. This results in proliferation of "precursor cells" as part of the regeneration process, and simultaneously increases the target cell population for tumor transformation. The actual transformation may be due to the action of an activated virus, a chemical carcinogen, or may be spontaneous, but a critical number of target "precursor cells" is certain to have been created by the action of the initial carcinogen.

After surgical resection of the tumor mass in cases of hepatoma, elevated serum AFP levels have been observed to decrease temporarily,[114] decrease to a steady level,[102] or eventually disappear.[78] In cases of a decreased, but steadily maintained level, it has been suggested that this pattern may be due to incomplete removal of the tumor mass with viable cells continuing to elaborate AFP, but with a lower total production. Complete disappearance of AFP may indicate rejection of remaining tumor cells by a host cellular immune response, but is not generally regarded as confident evidence of cure.[78] Reappearance of AFP or an increase in levels is generally regarded as evidence of recurrence of disease. Therapeutic regimens other than surgical resection

(i.e., chemotherapy, radiotherapy) have generally been regarded as having little if any effect on altering AFP levels.[114,115]

There is no evidence that benign liver tumors (hamartomas, adenomas, hemangiomas) or bile duct carcinomas are associated with elevated serum AFP levels. Hepatic metastases from cancer of the stomach and from pancreatic adenocarcinomas have, however, been reported to have had accompanying elevations in serum AFP.[116]

With regard to the concept of de-differentiation and reversion to fetal growth patterns mentioned earlier, numerous authors have attempted to determine whether the AFP seen in the case of primary hepatocellular carcinoma is the same as that normally elaborated by the fetal liver. On the basis of analyses of physical and chemical properties,[73] and immunologic properties,[67,118,117,74] it has generally been concluded that both normal fetal and cancerous liver cells produce alpha-fetoproteins which are structurally indistinguishable and probably identical.

In addition to hepatocellular carcinoma, elevated AFP levels have also been demonstrated to be consistently associated with teratocarcinomas of the testis and ovary. This observation was independently and simultaneously made for the first time by Masopust et al.[80] and Abelev[77] in 1968. Both experimenters found the protein to be present by gel precipitation in approximately 50% of untreated patients with highly undifferentiated tumors. Proceeding from the original observations of Gitlin and Boesman[118] that the endoderm of the fetal yolk sac was capable of AFP synthesis,

as well as from the fact that structural analogues of the yolk sac are characteristic of teratoblastomas,[119] Abelev[99] suggested the elaboration of AFP by these tumors might be due to the presence in them of functioning yolk sac. Gitlin et al.[120] also suggested that analogues of the endoderm of fetal yolk sac and primary gut present in teratocarcinomas could determine AFP production by them. Work by Mawas et al.[121] and Engelhardt et al.[122] has provided direct confirmation of this hypothesis.

Estimation of AFP levels in patients with teratoblastoma has essentially the same diagnostic and prognostic value as in the case of hepatoma. Detection of AFP by gel diffusion in the appropriate clinical setting can be considered virtually diagnostic of teratoblastoma or hepatic carcinoma. AFP levels can decrease to normal after surgical intervention as in the case of liver tumors, and reappearance of elevated levels carries the same ominous prognosis. Similar to the findings in the case of hepatic tumors, Mawas et al.[123] reported a relationship between age of the patient and the appearance of elevated AFP levels. High levels were found in 56% of patients less than 15 years of age, but in only 15% of older patients. No elevation of AFP levels have been demonstrated in other childhood tumors such as neuroblastoma, nephroblastoma, osteosarcoma, embryonal carcinoma, various brain tumors, liver angioma, and lymphoreticulosarcoma.[80,123]

The presence of elevated AFP levels in certain non-neoplastic liver diseases was first demonstrated by Alpert et al.[97] who

reported such elevations in patients with viral hepatitis. Since that time, transient elevations in AFP levels have been reported by others in cases of viral hepatitis,[125,124] and alcoholic cirrhosis.[127,124,126] Abelev[99] has shown that AFP levels rose between 15 and 30 days after the onset of viral hepatitis and returned to normal between 15 and 26 weeks thereafter. Chandra[100] reported a higher percentage of AFP-positive sera in young hepatitis patients than in adult patients. Karvointzis and Redeker[128] note that the highest AFP levels are found in the most severe cases and that the appearance of AFP in the sera of patients in coma is followed by recovery. This may be due to the association of AFP synthesis with hepatic regeneration.

The association between elevated AFP levels and chronic liver disease was also reported by Belanger et al.[129] These experimenters detected elevated AFP in the sera of eight patients with hereditary tyrosinemia. This inborn error of metabolism is associated with Fanconi's Syndrome, and in addition is marked by progressive degeneration and cirrhosis of the liver. It is characterized by an autosomal recessive pattern of inheritance. In cases of very early onset, hepatic symptoms predominate, and death from hepatic insufficiency may result during the early months of life. The development of malignant hepatoma may occur in those patients who survive long enough, and this may have some bearing on the presence of elevated AFP levels.

As was mentioned earlier in this paper (See "Introduction," p. 1)

Chandra et al.[3] suggested in March 1975 that a strong correlation existed between elevated serum AFP levels and the presence of the CF Gene. Employing techniques of gel diffusion, counterimmunoelectrophoresis, and radioimmunoassay, they measured the levels of AFP in the sera of 22 healthy controls, 18 children with CF, 16 parents of children with CF, 14 siblings of CF children, 7 patients with celiac disease, and 7 patients with bronchiactasis. AFP levels were found to be elevated in all CF patients (Range = 56-8825μg/l; Log mean = 690 ± 1.18), 15 parents (Range = 25-568μg/l; Log mean = 178 ± 0.85), and 7 siblings (Range for all sibs = 6-400μg/l; Log mean = 43 ± 1.60; Range for elevated sibs = 75-400μg/l). All others fell in the normal range of 5-25μg/l. There was no overlap observed between the AFP levels in normal controls and in obligate heterozygotes (parents). No correlation between AFP level and severity of symptoms was found. The experimenters interpreted the bimodal distribution of values among siblings tested as reflecting those heterozygous for the gene (elevated values) and those who were not carriers. They suggest, on the basis of their results, that AFP be considered a marker for the CF Gene, and that serum AFP concentrations should be determined in all first-degree relatives of patients with CF in order to identify the carrier state. Acknowledging that the pathogenesis of liver involvement in CF is unclear, they suggest that the CF Gene may adversely affect normal processes of maturation in the fetus (especially in gut-associated tissues such as the liver)

resulting in the persistence of cell lines capable of synthesizing AFP and simultaneously making the liver more susceptible to damage such as fibrosis, cholestasis, and multilobular cirrhosis.

Since the time that the current study was begun, other investigators have considered the findings of Chandra's group in their own radioimmunoassay studies. Three British investigators[130,131,132] were unable to support the findings while an American experimenter[133] reportedly did. Wallwork et al.[130] examined 37 CF children, 24 of their parents, and 10 of their siblings and found AFP concentrations in all to be well within their normal limits (25 ng/ml) with a median value of 10 ng/ml. Brock et al.[131] reported a similar normal range (3.4-18.0 ng/ml) and found that the seven CF patients tested fell well within it (2.0-9.25 ng/ml). Fitzsimmons et al.[132] examined 46 CF patients, 31 siblings, 80 obligate heterozygotes (parents), and 23 normal controls. This group's findings are interesting in that they found a wider range of AFP values among controls (3-51 ng/ml; median = 9), but they too found no difference between these values and those of the CF heterozygote and sibling groups (CF range = 1-44; median = 11; Heterozygote range = 1-41; Median = 9; Sibling range = 1-55; median = 9). Smith's[133] data, however, does support that of Chandra et al. He examined the sera of seven children with CF and found the mean AFP value to be 1825 ng/ml with a range of 130-2360 ng/ml. Fourteen parents had a mean of 433 (range = 80-1055) ng/ml and three out of eleven siblings showed AFP elevations (840, 1365, and 1980 ng/ml

respectively). The range for normals was not provided.

These contradictory findings suggested the possibility that AFP levels might indeed be used as a marker for the CF Gene and confirmed the need for further work to resolve this important question.

MATERIALS AND METHODS

Subjects

Members of the homozygote group were all outpatients of the YNHH CF Clinic who were having blood drawn for other tests. There were twenty-two subjects in this group, of whom fifteen were male and seven were female. Ages in this group ranged from 6 years to 24 years with a mean age of 13.6 years. Because of the negative results of this study, Swachman scores[134] which were to have been used to correlate CF severity with AFP levels were not determined. None of the subjects were malnourished or cyanotic, and all had normal liver function tests at the time that the specimens were obtained. Diagnosis of CF had been established by pilocarpine iontophoresis with sweat chlorides higher than 60mmol/l.

Members of the heterozygote group included twenty male and thirty-three female parents of children seen in YNHH CF Clinic. All were in good health at the time the specimens were obtained, and all were without any history of chronic disease. Ages in this group ranged from 23 to 56 years with a mean age of 36.7.

There were twenty members of the normal control group. These included five male and seven female children seen at the YNHH pediatric clinic for well child care who were having routine bloods drawn, two female asymptomatic siblings of CF patients, and six

male YNHH medical student and house staff volunteers. Ages in this group ranged from 2 to 34 years with a mean age of 16.1. All were in good health with no history of chronic disease.

All subjects in the three groups were Caucasian.

Collection of Specimens

Specimens from Pediatric and CF Clinics were drawn into a plastic syringe from a clean venopuncture by using a #21 butterfly needle. The blood was then immediately placed in a red-topped glass Vacutainer test tube, refrigerated at 4°C, and allowed to clot. Specimens were then spun at 3000 rpm for 15 minutes. Serum was removed by Pasteur pipet, aliquotted into 2cc bottles, labelled, and frozen at -20°C. Care was taken to thaw each specimen only once, immediately prior to use.

Isolation of AFP

Purification of AFP was accomplished in essentially the manner described by Nishi[73] and employed by Ruoslahti and Seppälä.[135] Known rabbit antiserum to AFP (used by the YNHH Clinical Immunology Lab for AFP quantification) was combined with an AFP source (amniotic fluid and sera from legally aborted fetuses) after determination of the appropriate antigen-antibody ratio by a method of immunoprecipitation in capillary tubes. The antiserum-AFP mixture was

incubated at 37°C for one hour and then stored for fourteen hours at 4°C. The mixture was then spun at 3000rpm for 15 minutes. The supernatant was removed, saving the precipitate of antigen-antibody complexes. The immuno-precipitate was washed several times in normal saline and then dissolved in 0.1 M glycine/HCl (pH = 2) to disassociate the antigen-antibody complexes. Separation of free AFP from free antibody and antigen-antibody complexes was then accomplished by gel filtration on Sephadex G-200. Glycine/HCl (0.05M) in normal saline (pH = 2) was used for elution and the collected fractions were brought to neutral pH (pH = 7.4) with appropriate amounts of 0.2M Phosphate Buffer.

This process was repeated several times as antisera more specific in their binding of AFP became available, resulting in purer isolates of AFP. In order to remove all possible contamination of AFP by components of normal human serum, a pellet was made by polymerizing rabbit anti-whole human serum with glutaraldehyde. The pellet was incubated with the AFP containing eluates for one hour at 37°C and then refrigerated at 4°C for 20 hours. The samples were then centrifuged at 3000rpm for 15 minutes and the AFP-containing supernatant was removed. Immunodiffusion by the Ouchterlony techniques[136] showed lack of immunoprecipitation of purified AFP by rabbit anti-whole human serum, but a strong single line of precipitation by rabbit anti-AFP indicating the purity of the preparation (Figure I). Immunoelectrophoresis also revealed a single line of precipitation.

FIGURE I
Purity of Isolated AFP

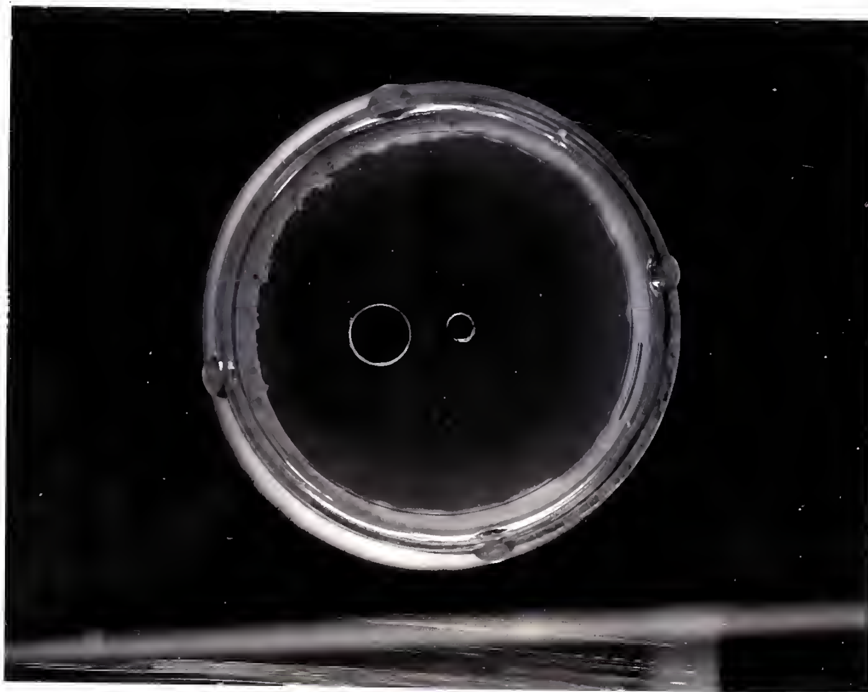


Figure I. Large well contains purified AFP. Small well contains specific anti AFP serum. Single line of immunoprecipitate indicates purity of preparations.

The AFP eluates obtained in this manner was quantitated by standard immunodiffusion techniques employed at the YNHH Clinical Immunology Lab and was found to contain approximately 35 micrograms AFP per milliliter.

Production of Antiserum

Anti-AFP serum used in this study was produced in four laboratory rabbits over a two month period of time. The initial sensitization was accomplished by injection of 1 ml of a suspension of purified AFP in Complete Freunds Adjuvant into the foot pads and dorsal fat pads of the four rabbits. The injections were repeated twice more at four week intervals with 1 ml of a suspension of purified AFP in Incomplete Freunds Adjuvant. The rabbits were bled 10 ml on four separate occasions by intracardiac puncture at approximately three week intervals after the initial sensitization. After each bleeding, the sera obtained were tested for immunoprecipitation of normal human serum and of AFP. The antisera continued to produce specific immunoprecipitation of AFP (Figure II) with no precipitation of normal human serum components, and were utilized in the isolation of additional AFP for injection into the rabbits.

FIGURE II
Specificity of Rabbit Anti-AFP Sera

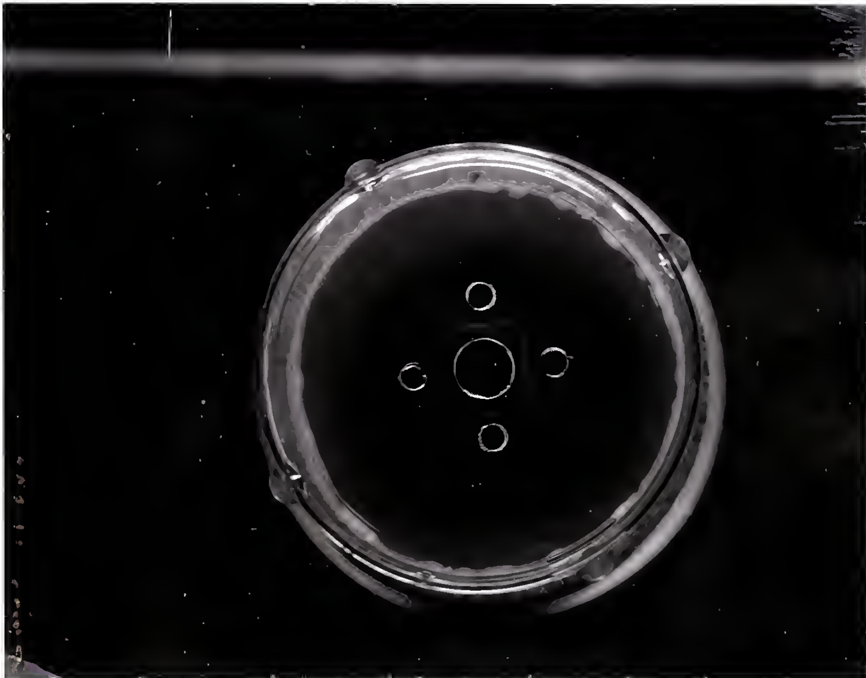


Figure II. Large center well contains purified AFP. Small wells contain one of four different AFP-specific antisera.

Counterimmunoelectrophoresis

All specimens were tested for the presence of detectable AFP by a well established technique of counterimmunoelectrophoresis (CEP) routinely used in the YNHH Clinical Immunology Lab. The

technique involves use of a 3 X 4 inch glass plate coated with 2% Agarose in Barbital Buffer (pH 7.4). Serum with a previously determined quantity of AFP (66 µg/ml) was diluted to an AFP concentration of 300 nanograms per milliliter and was included on each CEP plate. This control was readily detectable as being positive for AFP, thus establishing a sensitivity for the technique of at least 300 nanograms per milliliter. Current re-evaluation of the CEP technique suggests that its sensitivity may actually be in the region of 150 nanograms per milliliter. This re-evaluation, however, is as yet incomplete and so, for the current study, the 300 nanogram figure was employed as the lower limit of sensitivity for the CEP.

Radioiodination of AFP

Iodination of purified AFP was accomplished by a modification of the technique described by Greenwood and Hunter.[137] Twenty microliters of 0.2 M phosphate buffer (pH = 7.4) was added to 200 microliters (7 micrograms) of AFP. To this was added 10 microliters of 5 mCi/ml $Na^{125}I$ in dilute NaOH solution, pH 8-11 (The Radiochemical Centre, Amersham, England). Five microliters of 0.05 M phosphate buffered saline (PBS), pH 7.4 containing 25 micrograms of chloramine-T was immediately added. After approximately 10 seconds' mixing, 10 microliters of PBS containing 150 micrograms of sodium metabisulphite, followed by 20 microliters of PBS containing 332 micrograms of potassium iodide, were added. Incorporation of

^{125}I into AFP was determined by gel filtration on P-30 polyacrylamide gel (1 X 20 cm column), which was used to separate the labeled protein from excess radioiodine. The labeled AFP was collected in six-drop fractions into plastic scintillation tubes (Falcon 16 X 25 mm tubes) containing 0.1 ml of 0.05 M PBS. The degree of radioactivity in the peak AFP-containing fraction was determined by a Nuclear-Chicago automatic gamma sample counter.

In order to remove any rabbit protein (remaining from the AFP isolation process) which might have been randomly iodinated along with the AFP, the iodinated protein was treated with a pellet of glutaraldehyde-polymerized donkey anti-rabbit serum in the manner described for anti-normal human serum in the section on "Isolation of AFP." After removal of the pellet, measurable radioactivity in the labeled protein sample was found to have decreased by approximately 30%, indicating a specific removal of ^{125}I-labeled rabbit protein, and/or a non-specific absorption of protein which may have been damaged in the course of iodination.

The immunoreactivity of the labeled AFP was then determined. In order to do this, the percentage of radioactive AFP precipitated by a 1/2000 dilution of the anti-serum used in the final radio-immunoassay was compared with the precentage precipitated by the same dilution of normal rabbit serum (non-immune). To 20 microliters of protein carrier (1% Bovine serum albumin in PBS, pH 7.4) were added 20 microliters of ^{125}I-labeled AFP (diluted with 1% BSA so as to contain 10,000 counts per minute), and 20

microliters of either 1/2000 rabbit anti-AFP serum or 1/2000 normal rabbit serum. After adequate mixing, this was allowed to stand at 4°C for 20 hours. At this time, the samples were spun in a Sorvall RC2-B refrigerated centrifuge for 20 minutes at 15000 rpm and 5°C. The supernatant and precipitate were then separated, with each being placed in a separate Falcon scintillation tube. The radioactivity present in each tube was then counted for one minute by a Nuclear-Chicago automatic gamma sample counter. The percentage of the total radioactivity for each sample which was present in the immunoprecipitate was then calculated. The presence of 95% or more of the total radioactivity in the supernatant of the normal rabbit serum samples was considered acceptable, with the remaining 5% or less remaining in the original reaction vial due to nonspecific effects. Immunoprecipitation of 25% or more of the labeled AFP by the immune serum was considered as evidence that the radioactive protein was sufficiently immunoreactive for use in radioimmunoassay. ^{125}I-labeled AFP showing less than 25% binding was considered adequate for use in immunoautoradiography.

Immunoautoradiography

Eighty-four (all CF sera and a random selection of 14 normals and 48 heterozygotes) of the ninety-five specimens collected for this study were tested for the presence of detectable AFP by a

technique of immunoautoradiography. A similar technique employed by Abelev et al.[138] was able to detect AFP in the sera of pregnant women and patients with hepatitis. Six standard dilutions of AFP positive fetal serum (previously quantitated in triplicate at 50 micrograms per milliliter) were made so as to contain 150, 100, 70, 50, 30, and 20 nanograms of AFP per milliliter. These dilutions were made by adding appropriate amounts of AFP-negative (by Counterimmunoelectrophoresis) normal human serum and enough ^{125}I-labeled AFP to provide 20,000 cpm in 25 microliters of the final mixture. In order to remove any possible traces of AFP, the normal human serum used for dilution had previously been incubated with a pellet of glutaraldehyde-polymerized specific anti-AFP serum in a manner identical to that described for the purification of AFP with a pellet of polymerized anti-whole human serum (see "Isolation of AFP," p. 31). It was determined by use of a normal rabbit serum control that such a pellet was able to absorb on the order of 100 nanograms of AFP (or 50 nanograms per milliliter for the 2.0 milliliter samples of normal human serum processed for dilution), which should well represent virtually all of the AFP present in a sample of normal human serum (Ruoslahti and Seppälä[135] had found levels of AFP from 1.5-16 nanograms per milliliter in specimens of normal human serum). This determination was made by adding a known amount of AFP (from a fetal serum source) to whole normal rabbit serum, incubating this sample with a pellet of polymerized anti-AFP serum, and then quantitating by

radioimmunoassay techniques the amount of decrease in AFP.

Also prepared were an AFP-positive control quantitated at 94 nanograms AFP per milliliter and a negative control containing only the normal human serum used for dilution with no unlabeled AFP added. To these controls and to each of the sera tested was added enough ^{125}I-labeled AFP to provide 20,000 cpm in 25 microliters. The AFP dilutions (to provide a standard curve for quantification of unknown samples), the two controls, and thirteen unknown samples were set up in duplicate on each immunoautoradiography plate. The plates consisted of 3 X 4 inch glass slides coated with 15.0 milliliters of Special Noble Agar prepared in normal saline and containing 4.0 microliters of anti-AFP serum per milliliter (for immunoprecipitation of any AFP present). Forty-two wells, each holding 25 microliters of sample, had been cut into the agar. The plates were allowed to stand in moist plastic chambers at room temperature for 48 hours. They were then immersed in normal saline and allowed to wash at room temperature for another 48 hours. At that time they were removed from the saline and wrapped in a single layer of Saran Wrap. In a darkroom, 3 X 4 inch pieces of Kodak RP-14 Medical X-ray film were placed in contact with the agar coated side of each plate, and they were appropriately stored so as to avoid exposure to light. After one week, the exposed sheets of film were developed using Kodak Dental X-ray Developer, and then contact printed onto Agfa (Grade 6) High Contrast photographic paper. The diameter of the circle of exposure for

each well was measured with an average of the diameters of duplicate wells being taken. Values to provide standard curves from which the values of unknown specimens could be estimated.

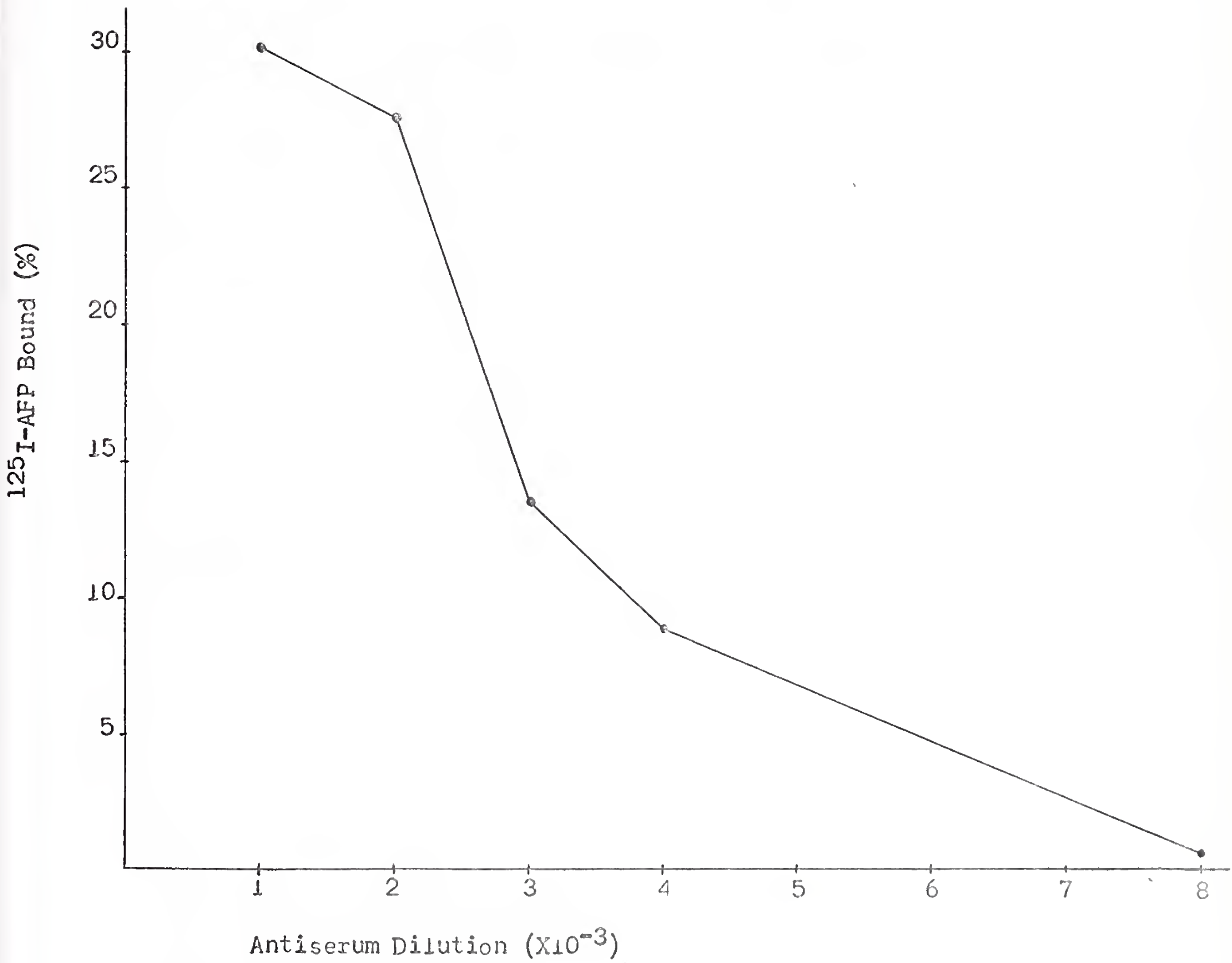
Radioimmunoassay (RIA)

The technique employed for RIA was a non-equilibrium one in which addition of ^{125}I-labeled AFP was made after the test samples had been incubated with the anti-AFP antiserum for 48 hours. The double antibody technique employed by Ruoslahti and Seppälä[135] and by Purves and Purves[140] was considered. This technique, however (attempted with goat anti-rabbit serum), showed no advantage in the ability to precipitate anti-AFP serum bound ^{125}I-labeled AFP over $(NH_4)_2SO_4$ in our hands. We therefore elected to use the ammonium sulfate as it was more readily available. Twenty microliters of a 1% solution of BSA in PBS (pH 7.4) were included in each sample as a protein carrier. Early experiments had exhibited a problem with adherence of labeled AFP to the sides of the reaction vessel. This was eliminated by inclusion of the BSA.

In preparation for the radioimmunoassay, the appropriate dilution of antiserum for use was determined. Anti-AFP serum dilutions of 1/1000, 1/2000, 1/3000, 1/4000, and 1/8000 were prepared with PBS (pH 7.4). Twenty microliters of each dilution were added in duplicate tubes (Fischer polyethylene centrifuge

tubes, 1 ml capacity) containing 20 microliters of a 1% solution of BSA in PBS (pH 7.4). To this was added 20 microliters of ^{125}I-labeled AFP diluted with 1% BSA so as to give 3000 counts per minute. The tubes were mixed with a Vortex mixer and then incubated at 37°C for one hour. They were then refrigerated at 4°C for 20 hours, after which time 0.2 milliliters of a 43.5% solution of ammonium sulfate were added. After mixing, the tubes were again stored at 4°C for 20 hours. They were then spun at 15000 rpm, 5 °C in a Sorvall RC2-B automatic refrigerated centrifuge for 20 minutes. The supernatant was removed by Pasteur pipet and the radioactivity in both supernatant and precipitate were counted. The results are plotted in Figure III.

FIGURE III
Antiserum Dilutions



The antibody concentration binding at least 25% of the precipitable counts, in this case 1/2000, was selected for use in the assay.

In order to obtain a standard inhibition curve from which to determine the level of AFP in unknown samples, standard dilutions of AFP-positive fetal serum (previously quantitated in triplicate) were made using the absorbed normal human serum described earlier as

diluent (See "Immunoautoradiography," p. 38). Seven such dilutions were made so as to contain 15, 25, 50, 75, 100, 150, and 200 nanograms of AFP per milliliter, respectively. Three standard curves were set up in duplicate, one with each of the three batches of unknown sera which were run. In each 1 ml centrifuge tube, 20 microliters of AFP dilution and 20 microliters of a 1/2000 dilution of anti-AFP serum in 1% BSA were added to 20 microliters of 1% BSA in PBS (pH 7.4). This was thoroughly mixed, incubated for one hour at 37°C and then stored for 48 hours at 4°C. At this time 20 microliters of ^{125}I-labeled AFP diluted in 1% BSA so as to contain 3000 counts per minute were added. The samples were again mixed, incubated at 37°C for one hour, and stored at 4°C for 24 hours. A 0.2 milliliter quantity of a 47.5% solution of ammonium sulfate PBS (pH 7.4) was then added. The samples were mixed and stored at 4°C for 24 hours. They were then spun in the Sorvall RC2-B for 20 minutes at 15000 rpm, 5°C. The supernatant was removed by Pasteur pipet. Supernatant and immuno-precipitate were placed in separate Falcon 16 X 25 mm tubes for counting on a Nuclear-Chicago automatic gamma counter. The results were plotted on Semi-Log graph paper (#cpm in precipitate/#cpm in supernatant or BOUND/FREE <u>vs</u> Concentration of AFP in ng./ml). Each series included two control tubes in duplicate. One control contained the same processed normal human serum used for dilution, but had no added "cold" AFP ("Trace Tube"). The second control also contained normal human serum without added AFP, but the anti-AFP

serum was also omitted from this tube. The counts observed in the precipitate of this tube represent non-specific radioactivity, or the Percent of Damage for the assay. This Percent of Damage was subtracted from the precipitated counts for both the standard curve and unknown samples prior to calculating the $^B/_F$ ratio and plotting these values. The $^B/_F$ values appearing in the graphs therefore represent the corrected $^B/_F$ ratios.

The undiluted sera of CF patients, heterozygotes, and normals were tested in duplicate using 20 microliter samples as in the case of the AFP dilutions. The setting up and processing of these specimens was identical to those for the standard inhibition curve save for the substitution of the unknown samples in place of the AFP dilutions. All samples were thawed for the first time immediately before use in the assay.

RESULTS

Counterimmunoelectrophoresis

None of the ninety-five sera tested in this study showed any visible evidence of immunoprecipitation with anti-AFP serum on counterimmunoelectrophoresis. The positive control (containing 300 nanograms AFP per milliliter) included on each plate was clearly distinguishable as positive for AFP, a single line of immunoprecipitation being easily visible.

Immunoautoradiography

The standard curve used to estimate the values of AFP concentration in unknown samples and to establish the lower limits of sensitivity of the technique is presented in Figure IV. It was found that the sensitivity of immunoautoradiography technique was approximately 70 ng/ml in our hands. AFP concentrations above this value were readily distinguishable from each other, while those below 70 ng/ml produced rings of exposure of virtually identical diameter. Ring diameters for all standard curve dilutions and all unknown sera were measured to the nearest 0.5 mm. It was found that none of the 84 sera tested by this technique produced a ring diameter which would indicate an AFP concentration greater than 70 ng/ml. A sample

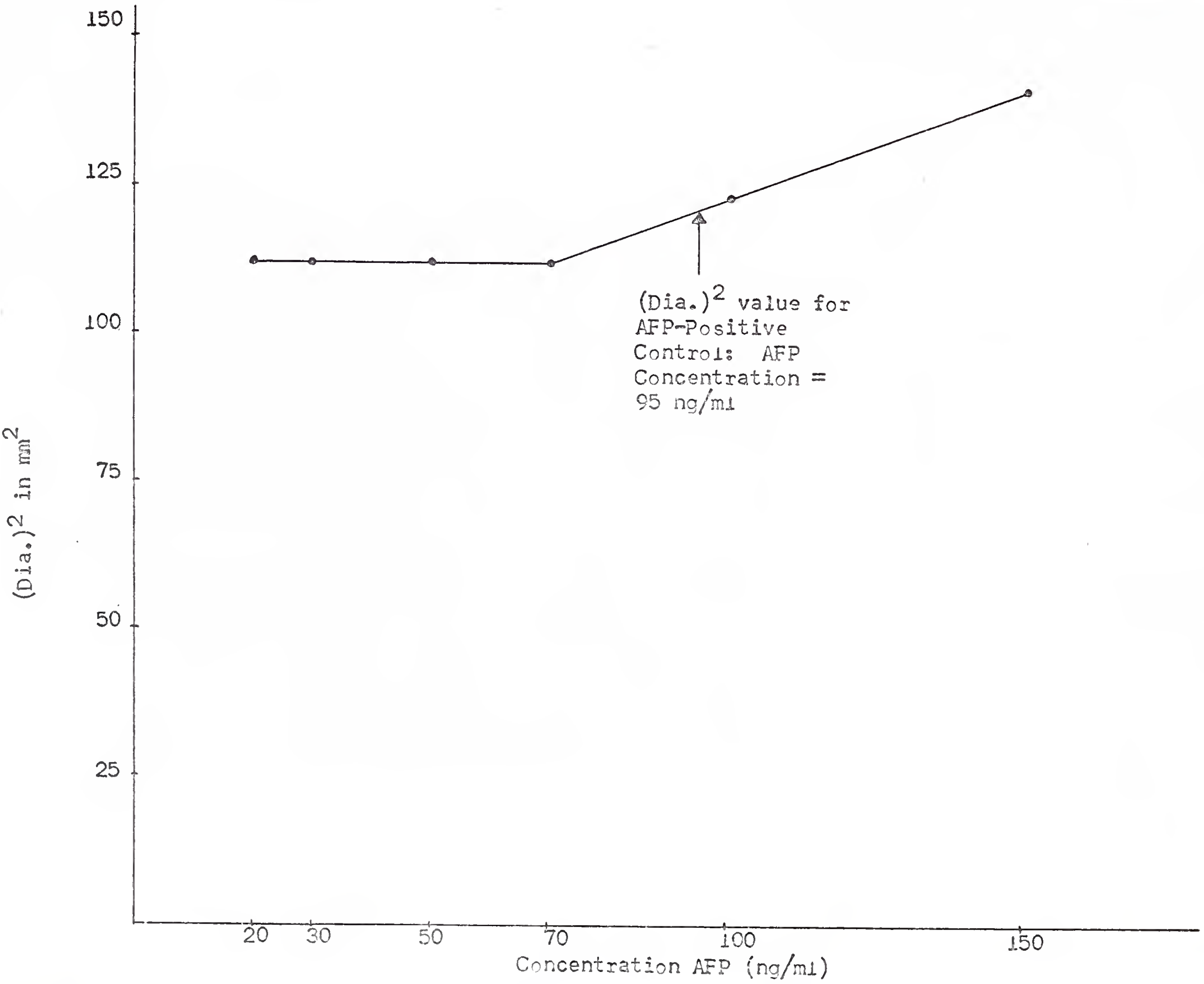


FIGURE IV. Immunoautoradiography Standard Curve.

of the contact prints from which the determinations were made is presented in Figure V. This particular plate contains 13 CF sera in duplicate in the lower four rows. Included on each plate was an AFP-positive control, previously quantitated at 94 ng/ml by gel immunodiffusion techniques. The observed ring diameter for these samples was measured at 11 mm, which, when squared and plotted on the standard curve, showed a value of 95 ng/ml, indicating the accuracy of the immunoautoradiography technique.

FIGURE V. Immunoautoradiography Samples

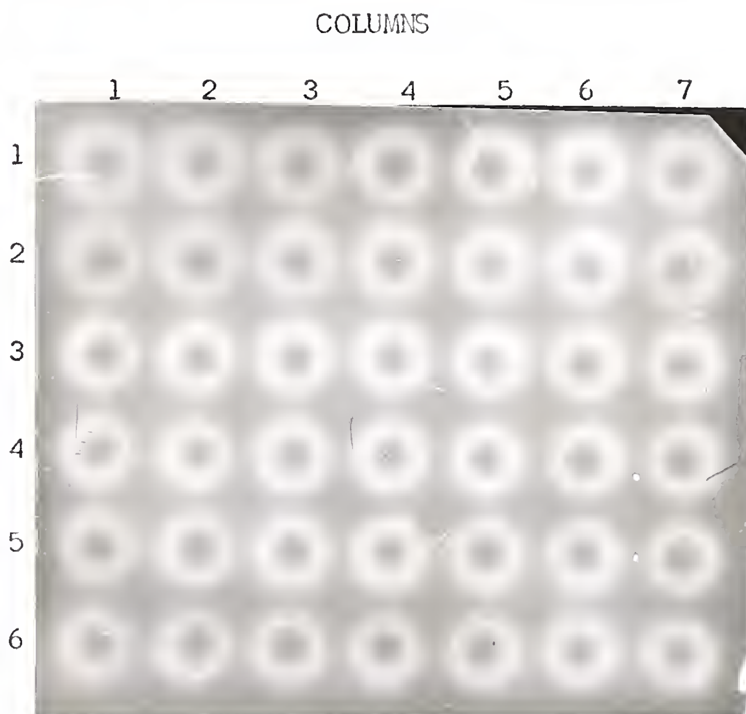


Figure V. All samples are presented in duplicate. Measurements made were of the horizontal diameter of each ring. ROW 1 & 2: AFP dilutions of 150, 100, 70, 50, 30, and 20 ng/ml in COLUMNS 1-7. COLUMN 7 contains AFP-positive control (94 ng/ml). ROW 3 & 4: COLUMN 1 contains AFP-negative normal human serum. COLUMNS 2-7 contain CF sera. ROWS 5 & 6: COLUMNS 1-7 contain CF sera.

Radioimmunoassay

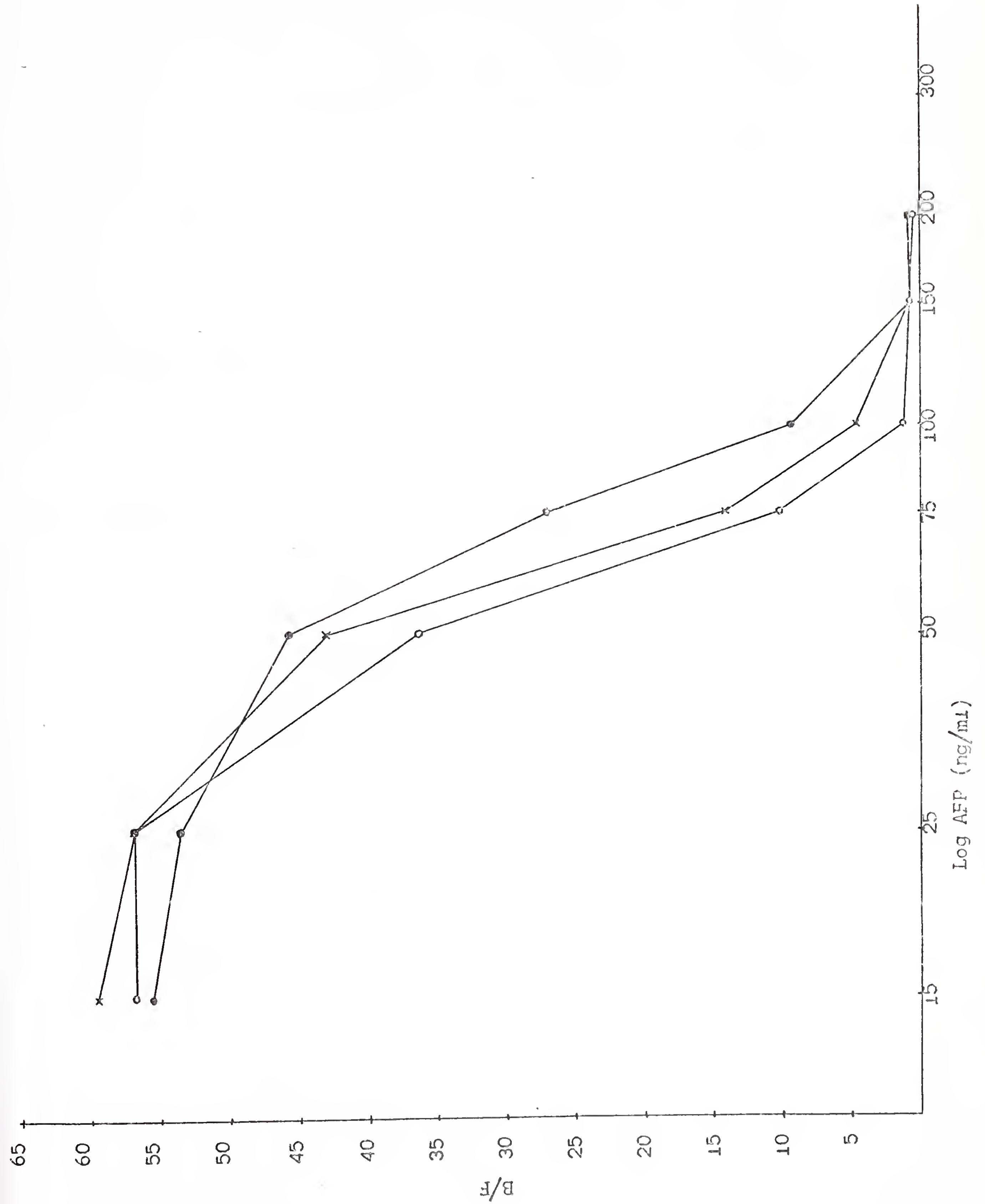
1. Standard Inhibition Curve

The standard inhibition curves from three separate trials are presented in Figure VI, plotted on a semi-logarithmic scale. The relationship between the concentration of AFP, and the inhibition obtained is linear from 25-100 ng/ml. This, then, represents the lower limit of sensitivity (25 ng/ml), and the working range (25-100 ng/ml) of the assay. Values for AFP concentrations less than 25 ng/ml and greater than 100 ng/ml cannot be assigned.

2. Serum Samples

The results of AFP concentration measurements in the unknown sera are depicted in Table II. Log means are presented in addition to the arithmetic means so that the results can be compared directly to those of Chandra et al.[3] Of the 22 CF sera tested, 17 had AFP values in the < 25 ng/ml range; 8 had values in the measurable range (Range = 26-32; $\bar{x} = 28.4$); none had values >100 ng/ml. Thirty-two of 53 parents had AFP values of <25 ng/ml; 20 had measurable values (Range = 25-53; $\bar{x} = 28.8$); and one had a value >100 ng/ml. Both siblings tested had AFP values in the <25 ng/ml range. Of the 18 healthy controls, 10 had AFP values of <25 ng/ml;

FIGURE VI
Radioimmunoassay Standard Curve



8 had values between 25 and 100 ng/ml (Range = 25-28; $\bar{x}$ = 26.6); and none had values >100 ng/ml. The heterozygote serum which was measured at >100 ng/ml was re-evaluated by counterimmunoelectrophoresis and Ouchterlöny gel diffusion and again proved negative, suggesting that if this is a true elevation, the actual value should fall between 100 and 300 ng/ml.

Statistical analysis (Fisher's "Exact Test") evaluating all possible group pairs failed to demonstrate any statistically significant differences ($p > .10$ for all pairs).

TABLE II
Radioimmunoassay Results

NUMBER & SEX	<25 ng/ml NO.	25-100 ng/ml					>100 ng/ml NO.
		No.	$\bar{X}$	L.M.	MED.	RANGE	
PATIENTS 22 M 15 F 7	17	5	28.4	28.3 ±1.1	28	26-32	0
CONTROLS 18 M 11 F 7	10	8	26.6	26.6 ±1.0	26.5	25-28	0
SIBLINGS 2 M 0 F 2	2	0	----	----	----	-----	0
PARENTS 53 M 20 F 33	32	20	28.8	28.3 ±1.1	27.6	25-53	1

$\bar{X}$ = Arithmetic Mean; L.M. = Log Mean ± Standard Error of Mean; MED. = Median.

DISCUSSION

The original report by Chandra et al.[3] of an elevated level of AFP in children with CF seemed based on a reasonable premise. The involvement of the liver in CF has been well documented with localized foci of biliary obstruction and fibrosis commonly found at autopsy, even in infants. In some patients, particularly those in their teens, these changes progress and cause a distinctive type of multilobular biliary cirrhosis with large irregular nodules. The relationship between non-malignant liver disease and elevated AFP levels has been documented,[138,126] and it therefore seemed entirely possible that AFP levels might well be elevated in CF patients with their chronic liver involvement. The CF Gene could well affect normal maturation processes so as to result in the persistence of embryonic cell lines capable of AFP synthesis, and to make the liver more susceptible to damage as Chandra has suggested.[3] The current study has, however, by three separate systems of measurement characterized by differing levels of sensitivity, failed to support the results of Chandra and his co-workers. Counterimmunoelectrophoresis, easily able to demonstrate the presence of AFP concentrations as low as 300 ng/ml (and possibly as low as 150 ng/ml), failed to detect the presence of AFP in any of the samples tested. This already placed the level of AFP concentrations in Cf and heterozygote sera well below

those reported by Chandra et al., and indicated that they were certainly lower than 300 ng/ml. Though clearly lower than the levels reported, the possibility still existed that CF and heterozygote serum AFP concentrations might still fall in an elevated range. This range of possible elevation was further decreased by use of immunoautoradiography. The lower limit of sensitivity of this technique having been established at 70 ng/ml by use of a standard curve, it was found that none of the samples tested fell in the range above 70 ng/ml. An AFP-positive control (previously quantitated at 94 ng/ml) was included on each of the autoradiography plates. Plotting the square of the diameter measured for this sample (diameter ^{2} = 121) yielded a value of 95 ng/ml from the standard curve, indicating the acceptable accuracy of the technique.

Radioimmunoassay with a sensitivity limit of 25 ng/ml established that all but two samples tested fell in the normal range. The sample whose AFP concentration fell in the greater than 100 ng/ml range was retested by counterimmunoelectrophoresis, and was again negative. This suggests that if the radioimmunoassay estimation is correct, the subject's serum AFP concentration lies somewhere between 100 and 300 ng/ml. The subject, an obligate heterozygote, continues to enjoy good health with no evidence of chronic disease. Due to a limited supply of radio-labeled AFP, this subject's serum was unfortunately one of eleven sera which were randomly eliminated from the group evaluated by immunoautoradiography. We are therefore unable to confirm the observation of an elevated

AFP concentration by this technique.

The second elevated serum was quantitated at 53 ng/ml. This value is quite similar to that of 50 ng/ml described by Ruoslahti and Seppälä[141] in a normal (non-carrier of CF) healthy 24 year old male with no detectable liver disease or history of any other chronic disease. The subject in this study, though a heterozygote, is equally healthy. Occasional elevated values in clinically healthy subjects carry an unknown significance at this time. While they may well bespeak the presence of some underlying defect or subclinical disease entity, they must be regarded as normal variants until such a relationship can be demonstrated.

The range of AFP concentrations observed among normals in this study (25 to 28 ng/ml) exhibits a slightly higher upper limit than that of some experimenters[141] (up to 25 ng/ml), but is well within the range observed by others[132] (3-51 ng/ml). It seems clear that there is considerable room for normal variation in the AFP concentrations measured by sensitive techniques such as radioimmunoassay. The mean and median values for the samples which could be accurately quantitated in our study are both higher than those observed in other studies measuring AFP by the same means. The reason for this is that the sensitivity limits of our assay were such that we were unable to accurately quantitate those values which fell in the less than 25 ng/ml range. Thus, our mean and median values represent only those AFP concentrations which

fell in the 25-100 ng/ml range. It is expected, from the number of values which fell below 25 ng/ml, that accurate quantification of these lower values would have resulted in mean and median values more closely resembling those of other experimenters.

We were somewhat disappointed with the narrow working range (25-100 ng/ml) of sensitivity of our assay. Though we were able to compensate for limitations on the upper end of the range by use of the counterimmunoelectrophoresis and immunoautoradiographic techniques, it would have been desirable to have had a lower limit of sensitivity in the region of 1-2 ng/ml, though Ruoslahti and Seppälä have reported a sensitivity threshold of 250 picograms/ml.[135] The limiting factor appears to have been the inability to achieve high enough antibody titers in the two month period of time over which the rabbits were immunized. The specificity of the antibody, despite its low titer, appeared to be high as evidenced by specific immunoprecipitation seen on gel immunodiffusion and immunoelectrophoresis. Evidence of the relatively low antibody titer is provided in Figure III which demonstrates that specific binding by our antibody falls off considerably in going from a 1/2000 to a 1/3000 dilution. Binding is almost non-existent at a 1/8000 dilution. Ruoslahti and Seppälä[135] in contrast, report near maximal binding at an antiserum dilution of 1/25000, with significant binding still occurring with the antiserum diluted 1/3,000,000. This was achieved with a sheep antiserum produced by 5 months' immunization. Because of the low antibody titer, we were also unable to employ dilutions

of our test sera since this would have placed our working range in the region of the sensitivity of the autoradiography technique. Despite these problems, however, our sensitivity threshold is almost the same as the 20 ng/ml level achieved by Purves and Purves[140] who also used undiluted test sera, though their working range was broader (20-1000 ng/ml).

Perhaps our greatest disappointment was in the fact that we were unable to provide evidence in support of the usefulness of serum AFP levels as a marker for the CF heterozygote state. We were somewhat concerned when, after having begun our study, we noted the negative results reported by the three British investigators,[130,131,132] but remained hopeful that we might see positive results in this study until the time that our own data became final.

The reason for the contradictory findings is unclear. In view of the fact that the published data suggesting the correlation between AFP and CF has come from sources on this side of the Atlantic while those denying it come from Europe, it is conceivable that there is a basic difference between CF in the two settings. We know of no such differences, and our data would seem to tip the scales against such a hypothesis. Differences in therapeutic regimes seem unlikely as the cause for elevated AFP levels as elevations were reported in heterozygotes who presumably receive no such treatment.

It is conceivable that Chandra's assay was measuring the

presence of some factor other than AFP. Confirmation that AFP was indeed the factor whose levels were measured could be made by use of immunoabsorbents in the manner described by Ruoslahti and Seppälä.[135] These experimenters absorbed AFP from test samples containing measurable amounts (by radioimmunoassay) of AFP by means of a glutaraldehydepolymerized specific anti-AFP serum in the manner described earlier in this paper. The test sera (with AFP removed) were re-tested by radioimmunoassay for the presence of AFP. Absorbed AFP was freed from the glutaraldehyde pellet by acid dissociation and these samples were then re-tested for AFP content. While the same process could have been employed in our study, the antiserum initially used by us for AFP isolation has been reliably used in the past for AFP detection and quantification, making it quite unlikely that we might have isolated a serum factor other than AFP. In addition, it would seem at this time that the burden of proof is upon Chandra and his group. Should Chandra's antiserum prove to be binding some factor other than AFP, this would still be a highly significant finding as any measurable difference able to reliably differentiate CF patients and heterozygotes from normals would be a noteworthy achievement.

In conclusion, our results lend support to those of other investigators who have failed to find evidence that the measurement of serum AFP concentrations might be used to identify asymptomatic carriers of the Cystic Fibrosis Gene. The need for a reliable method for heterozygote detection in this disease is a critical one in view

of its notably high incidence. Without such a method, efforts to lower the incidence of cystic fibrosis by genetic counseling will remain virtually impossible.

REFERENCES

1. di Sant' Agnese, P.A.: Pathogenesis and Pathophysiology of Cystic Fibrosis of the Pancreas. NEJM, vol. 277, nos. 24, 25, & 26, pp. 1287, 1343, and 1399, 1967.

2. McCombs, M.L.: Research in Cystic Fibrosis: A Review. Texas Rep. Biol. Med., 31(4): 615, 1973.

3. Chandra, R.K., Madhavankutty, K., and Way, R.C.: Serum α-Fetoprotein Levels in Patients with Cystic Fibrosis and their parents and Siblings. British Med. J., 1, 714, 1975.

4. Donnison, A.B., Shwachman, H., and Gross, R.E.: A Review of 164 Children with Meconium Ileus Seen at the Children's Hospital Medical Center, Boston. Pediatrics, 37: 833, 1966.

5. Holsclaw, D.S., Eckstein, H.B., and Nixon, H.H.: Meconium Ileus. Amer. J. Dis. Child., 109:101, 1965.

6. McIntosh, R.: Cystic Fibrosis of the Pancreas in Patients Over 10 years of Age. Acta. Paediatr. Scand., 43:469, 1954.

7. di Sant' Agnese, P.A.: Cystic Fibrosis (Mucoviscidosis). Am. Fam. Phys., 7(3):102, 1973.

8. Lowe, C.U., Bruck, El, and Mosovich, L.L., (eds.): Cystic Fibrosis of the Pancreas, in Barnett, H.L., and Einhorn, A.H., (eds.), Pediatrics, New York: Appleton-Century-Crofts, 1972.

9. Iacocca, V.F., Sibinga, M.S., and Barbero, G.J.: Respiratory Tract Bacteriology in Cystic Fibrosis. Am. J. Dis. Child., 106:315, 1963.

10. Andersen, D.H.: Pathology of Cystic Fibrosis, Ann. N.Y. Acad. Sci., 93:500, 1962.

11. Johansen, P.G., Anderson, C.M., and Hadorn, B.: Cystic Fibrosis of the Pancreas. Lancet, 1:455, 1968.

12. Danks, D.M., Allan, J., and Anderson, C.M.: Genetic Study of Fibrocystic Disease of the Pancreas. Ann. Human Genet., 28:323, 1965.

13. Anderson, C.M., Allan, J., and Johansen, P.G.: Comments on the Possible Existence and Nature of a Heterozygote Advantage in Cystic Fibrosis, in Ross, E., and Stoll, E., (eds.), Modern Problems in Pediatrics: Cystic Fibrosis. Basel, S. Karger Co., 10:381, 1967.

14. Spock, A.: Theories of the Pathogenesis of Cystic Fibrosis. Postgrad. Med., 50:92, 1971.

15. Editorial: Developments in Cystic Fibrosis Research. Lancet, 2:307, 1973.

16. di Sant' Agnese, P.A., Darling, R.C., Perera, A., and Shea, E.: Abnormal Electrolyte Composition of Sweat in Cystic Fibrosis of the Pancreas. Pediat., 12:549, 1953.

17. Munger, B.L., Brusilow, S.W., and Cooke, R.E.: Electron Microscopic Study of Eccrine Sweat Glands in Patients with Cystic Fibrosis of the Pancreas. J. Pediatr., 59:497, 1961.

18. Emrich, H.M., Stoll, E., Friolet, B., Colombo, J.P., Richterich, R., and Rossi, E.: Sweat Composition in Relation to rate of Sweating in Patients with Cystic Fibrosis of the Pancreas. Pediatr. Res., 2:464, 1968.

19. Clarke, J.T., Elian, E., and Shwachman, H.: Components of Sweat. Am. J. Dis. Child., 101:490, 1961.

20. Schulz, I.J.: Micropuncture Studies of Sweat Formation in Cystic Fibrosis Patients. J. Clin. Invest., 48:1470, 1969.

21. Mangos, J.A.: Microperfusion Study of the Sweat Gland Abnormality in Cystic Fibrosis. Tex. Rep. Biol. Med., 31(4):651, 1973.

22. Mangos, J.A., and McSherry, N.R.: Sodium Transport: Inhibitory Factor in Sweat of Patients with Cystic Fibrosis. Science, 158:135, 1967.

23. Kaiser, D., Drack, E., and Rossi, E.: Effect of Cystic Fibrosis Sweat on Sodium Reabsorption by the Normal Sweat Gland. Lancet, 1:1003, 1970.

24. Balfe, J.W., Cole, C., and Welt, L.G.: Red Cell Transport Defect in Patients with Cystic Fibrosis and their Parents. Science, 162:689, 1968.

25. Hadden, J.W., Hansen, L.G., Shapiro, B.L., Warwick, W.J.: Erythrocyte Enigmas in Cystic Fibrosis. Proc. Soc. Biol. Med., 142:573, 1973.

26. Gibson, L.E., Matthews, W.J., Minnihan, P.T., and Patti, J.A.: Relating Mucus, Calcium, and Sweat in a New Concept of Cystic Fibrosis. Pediat., 48:695, 1971.

27. Emrich, H.M.: Excretion of Different Substances in Sweat of Children with Cystic Fibrosis and Controls, in Modern Problems in Pediatrics, vol. 10, Part I, Proceedings of the 4th International Conference of Cystic Fibrosis of the Pancreas (Mucoviscidosis), Rossi, E., And Stoll, E., (eds.), Basel: Karger, p. 58, 1967.

28. Horton, C.R., et al.: Depressed Calcium Transport ATP-ase in Cystic Fibrosis Erythrocytes. Biophys. Res. Com., 40:505, 1970.

29. Mandel, I.D., Kutscher, A., Denning, C.R., Thompson, R.H., Jr., and Zegarelli, E.V.: Salivary Studies in Cystic Fibrosis. Am. J. Dis. Child., 113:431, 1967.

30. Roelfs, R.E., Gibbs, G.E., and Griffin, G.D.: The Composition of Rectal Mucus in Cystic Fibrosis. Am. J. Dis. Child., 113:431, 1967.

31. Chernick, W.S., Barbero, G.J., and Perkins, F.M.: Studies on Submaxillary Saliva in Cystic Fibrosis. J. Pediatr., 59:890, 1961.

32. Gugler, E., Pallavicini, J.C., Swerdlow, H., and di Sant' Agnese, P.A.: Role of Calcium in Submaxillary Saliva of Patients with Cystic Fibrosis. J. Pediatr., 71:585, 1967.

33. di Sant' Agnese, P.A., Grossman, H., Darling, R.C., and Denning, C.R.: Saliva, Tears, and Duodenal Contents in Cystic Fibrosis of the Pancreas. Peds., 22:507, 1958.

34. Roberts, C.B.: Fundamental Defect in Fibrocystic Disease of the Pancreas. Lancet, 2:964, 1959.

35. Chernick, W.S., Barbero, G.J., and Parkins, F.M.: Reversal of Submaxillary Salivary Alterations in Cystic Fibrosis by Guanethidine, Modern Problems in Pediatrics, vol. 10, Part I, Proceedings of the 4th International Conference on Cystic Fibrosis of the Pancreas (Mucoviscidosis), Rossi, E., and Stoll, E., (eds.), Basel: Karger, p. 125, 1967.

36. Bongiovanni, A.M., Yakovac, W.C., and Steiker, D.D.: Study of Adrenal Glands in Childhood: Hormonal Content Correlated with Morphologic Characteristics. Lab. Invest., 10:956, 1961.

37. Garrettson, L.K., Turpin, D.L., Hvidberg, E.F., and Mellinger, T. J.: Para-hydroxy, Phenyl-acetic and Homovanillic Acid Excretion: Variation During Growth and in Cystic Fibrosis and Other Pulmonary Diseases. Am. J. Clin. Path., 55:318, 1971.

38. Rubin, L.S., Barbero, G.J., Chernick, W.S., and Sibings, M.S.: Pupillary Reactivity as a Measure of Autonomic Nervous System Balance in Cystic Fibrosis. J. Pediat., 63:1120, 1963.

39. Dische, Z., Pallavincine, C., Cizek, I.J., Chien, S.: Changes in Control of Secretion of Mucus Glyco-Proteins as Possible Pathogenic Factor in Cystic Fibrosis of the Pancreas. Ann. N.Y. Acad. Sci., 93:526, 1962.

40. Farber, S.: Experimental Production of Achylia Pancreatica. Am. J. Dis. Child., 64:953, 1942.

41. Boyd, E.M., and Jarzylo, S.: Chronic Atropinization and Fibrocystic Disease of the Pancreas. Canad. M.A.J., 82:821, 1960.

42. Ayers, W.B., Stowens, D., and Ochsner, A.: Fibrocystic Disease of the Pancreas: Treatment by Sympathetic Denervation of Pancreas and Presentation of Theory of Neuroeffector Mechanisms: Preliminary Report of Five Cases. JAMA, 142:7, 1950.

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

44. Cherry, J.D., Roden, V.J., Rejent, A.J., and Dorner, R.W.: The Inhibition of Ciliary Activity in Tracheal Organ Cultures by Sera from Children with Cystic Fibrosis. J. Pediat., 79(6):937, 1971.

45. Bowman, B.A., Lockhart, L.H., and McCombs, M.L.: Oyster Ciliary Inhibition by Cystic Fibrosis Factor. Science, 164:325, 1969.

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

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

48. Danes, B.S., Litwin, S.D., Hütteroth, T.H., Cleve, H., And Bearn, A.G.: Characterization of Cystic Fibrosis Factor and Its Interaction with Human Immunoglobulin. J. Exp. Med., 137:1538, 1973.

49. Danes, B.S., and Bearn, A.G.: Oyster Ciliary Inhibition by Cystic Fibrosis Culture Medium. J. Exp. Med., 136:1313, 1972.

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

51. Polley, M.J., and Bearn, A.G.: Cystic Fibrosis: Current Concepts. J. Med. Gen., 11:249, 1974.

52. Wood, R.E., and di Sant'Agnese, P.A.: Bioassays of Cystic Fibrosis Factor. Lancet, 2:1452, 1973.

53. Doggett, R.G., and Harrison, G.M.: Cystic Fibrosis: In Vitro Reversal of the Ciliostatic Character of Serum and Salivary Secretions by Heparin. Nature New Biol., 243:251, 1973.

54. Doggett, R.G., and Harrison, G.M.: Cystic Fibrosis: Reversal of Ciliary Inhibition in Serum and Saliva by Heparin. Tex. Rep. Biol. Med., 31(4):685, 1973.

55. Murakami, A., and Eckert, R.: Cilia Activation Coupled to Mechanical Stimulation by Calcium Influx. Science, 164:325, 1969.

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

57. Doggett, R.G., Harrison, G.M., Patrick, T.A.: Cystic Fibrosis: In Vivo Reversal of the Ciliostatic Character of Serum and Parotid Secretions by Heparin. Nature New Biol., 243:250, 1973.

58. Danes, B.S., and Bearn, A.B.: A Genetic Cell Marker in Cystic Fibrosis of the Pancreas. Lancet, 1:1061, 1968.

59. Atland, K., Schmidt, S.R., Kaiser, G., Knoche, W.: Demonstration of a Factor in the Serum of Homozygotes and Heterozygotes for Cystic Fibrosis by a Non-Biological Technique. Humangenetik, 28:207, 1975.

60. Wilson, G.B., and Fidenberg, H.H.: 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, 1975.

61. Schwachman, H., Araki, H., and Field, M.: Detection of Cystic Fibrosis and Heterozygotes. Lancet, 2:372, 1975.

62. Prehn, R.T.: The Significance of Tumour-Distinctive Histocompatibility Antigens, in Cross-Reacting Antigens and Neoantigens. Trentin, J.J., (ed.), Baltimore: Williams and Wilkins, pp. 105, 1967.

63. Prehn, R.T.: Tumour Antigens, in Immunity, Cancer and Chemotherapy, Mihich, E., (ed.), New York: Academic Press, pp. 265, 1967.

64. Marx, J.L.: Biochemistry of Cancer Cells: Focus on the Cell Surface. Science, 183:1279, 1974.

65. Abelev, G.I., Perova, S.D., Khramkova, N.I., Postnikova, Z.A., and Irlin, I.S.: Production of Embryonal α-Globulin by Transplantable Mouse Hepatomas. Transplantation, 1:174, 1963.

66. Tatarinov, Y.S.: Detection of Embryospecific Alpha-Globulin in the Blood Sera of Patients with Primary Liver Tumour. Vop. Med. Khimil., 10:90, 1964, (English Abstract).

67. Abelev, G.I.: Assecritova, I.V., Kraevsky, N.A.: Embryonal Serum α-Globulin in Cancer Patients: Diagnostic Value. Int. J. Cancer, 2:551, 1967.

68. Alpert, E.: Alpha$_1$-Fetoprotein: Need for Quantitative Assays. NEJM, 290(10):568, 1974.

69. Pedersen, K.: Fetuin, A New Globulin Isolated From Serum. Nature, 154:575, 1944.

70. Bergstrand, C.G., and Czar, B.: Demonstration of a New Protein Fraction in Serum From the Human Fetus. Scan. J. Clin. Lab. Invest., 8:174, 1956.

71. Bergstrand, C.G., and Czar, B.: Paper Electrophoretic Study of Human Fetal Serum Proteins and Demonstration of a New Protein Fraction. Scan. J. Clin. Lab. Invest., 9:277, 1957.

72. Gitlin, D., and Boesman, M.: Serum Alpha-Fetoprotein, Albumin, and γG-Globulin in the Human Conceptus. J. Clin. Invest., 45:1826, 1966.

73. Nishi, S.: Isolation and Characterization of a Human Fetal α-globulin from the Sera of Fetuses and a Hepatoma Patient. Cancer Res., 30:2507, 1970.

74. Ruoslahti, E., and Seppälä, M.: Studies of Carcino-fetal Proteins: Physical and Chemical Properties of Human α-Fetoprotein. Int. J. Cancer, 8:218, 1971.

75. Adinolfi, M., and Gardner, B.: Studies on the Properties of a Human Fetal Serum Protein. Devel. Med. Child. Neurol. 9:609, 1967.

76. Spiro, R.G.: Studies on Fetuin, a Glycoprotein of Fetal Serum. J. Biol. Chem., 235:2860, 1960.

77. Abelev, G.I.: Production of Embryonal Serum Alpha-Globulin by Hepatomas: Review of experimental and Clinical Data. Cancer Res., 28:1344, 1968.

78. Adinolfi, A., Adinolfi, M., and Lessof, M.H.: Alpha-fetoprotein During Development and in Disease. J. Med. Gen., 12:138, 1975.

79. van Furth, R., and Adinolfi, M.: In Vitro Synthesis of the Foetal $\alpha_1$globulin in Man. Nature, 222:1296, 1969.

80. Masopust, J., Kithier, K., and Radl, J.: Occurrence of Fetoprotein in Patients with Neoplasms and Non-neoplastic Diesases. Int. J. Cancer, 3:364, 1968.

81. Waldmann, T.A., and McIntire, K.R.: Serum-Alpha-Fetoprotein Levels in Patients with Ataxia-Telangiectasia. Lancet, 2:1112, 1972.

82. Bergstrand, C.G., Karlsson, B.W., Lindberg, T., and Ekelund, H.: -Foetoprotein, Albumin, and Total Protein in Serum from Preterm and Term Infants and Small for Gestational Age Infants. Acta. Paed. Scan., 61:128, 1972.

83. Seppälä, M., and Ruoslahti, E.: α-Fetoprotein in Normal and Pregnancy Sera. Lancet, 1:375, 1972.

84. Allen, L.D., Ferguson-Smith, M.A., Donald, I., Sweet, E.M., and Gibson, A.A.M.: Amniotic-fluid Alpha-Fetoprotein in the Antenatal Diagnosis of Spina Bifida. Lancet, 2:522, 1973.

85. Harris, R., Jennison, R.F., Barson, A.J., Laurence, K.M., Ruoslahti, E., and Seppälä, M.: Comparison of Amniotic-fluid and Maternal Serum-alpha-fetoprotein Levels in the Early Antenatal Diagnosis of Spina Bifida and Anencephaly. Lancet, 1:429, 1974.

86. Seller, M.J., and Adinolfi, M.: Levels of Albumin, Alpha-fetoprotein and IgG in Human Fetal CSF. Arch. Dis. Child. 50(6):484, 1975.

87. Seppala, M.: Fetal Pathophysiology of Human Alpha Fetoprotein. Ann. N.Y. Acad. Sci., vol. 245, 1975.

88. Seppälä, M., and Ruoslahti, E.: Radioimmunoassay of Maternal Serum Alpha-fetoprotein During Pregnancy and Delivery. Am. J. Obstet. Gynecol., 112(2):208, 1972.

89. Seppälä, M., and Ruoslahti, E.: Alpha-fetoprotein: Physiology and Pathology During Pregnancy and Application to Antenatal Diagnosis. J. Perinatal Med., 1:104, 1973.

90. Seppälä, M., and Ruoslahti, E.: Alpha-Fetoprotein in Maternal Serum: A New Marker for Detection of Fetal Distress and Intrauterine Death. Am. J. Obst. Gynecol., 115:48, 1973.

91. Hirose, A.: Radioimmunological and Autoradiographical Studies on the Alpha-1-fetoprotein in the Obstetrics and Gynecology. Acta. Obst. et. Gynaec. Jap., 20(3):165, 1973.

92. Seppälä, M.: Alpha-fetoprotein Levels in Women Taking Oral Contraceptives. Int. J. Fertil., 18:206, 1973.

93. Wald, N., Barker, S., Peto, R., Brock, D.J.H., Bonnar, J.: Maternal Serum α-fetoprotein Levels in Multiple Pregnancy. Brit. Med. J., 1:651, 1975.

94. Uriel, J., de Nechaud, B., and Dupiers, M.: Estrogen-Binding Properties of Rat, Mouse and Man Fetospecific Serum Proteins. Demonstration by Immuno-auto-radiographic Methods. Biochem. Biophys. Res. Comm., 46:1175, 1972.

95. Swarty, S.K., Soloff, M.S., and Suriano, R.R.: A Comparison of the Estrogen-Binding Activity of Rat and Human AFP, in Alpha-fetoprotein, Masseyeff, R., (ed.), Nice: INSERM, p. 97, 1974.

96. Murgita, R.A., and Tomasi, T.B.: Suppression of the Immune Response by α-fetoprotein. I. The effect of Mouse -fetoprotein on the Primary and Secondary Antibody response. J. Exp. Med., 141:269, 1975.

97. Alpert, E., Uriel, J., and de Nechaud, B.: Alpha$_1$-feto-globulin in the Diagnosis of Human Hepatoma. NEJM, 278:984, 1968.

98. Purves, L.R., Behrson, I., and Geddes, E.W.: Serum Alpha-fetoprotein and Primary Cancer of Liver in Man. Cancer, 25:1261, 1970.

99. Abelev, G.I.: Alpha-fetoprotein in Ontogenesis and Its Association with Malignant Tumors. Adv. Cancer Res., 14:295, 1971.

100. Chandra, R.K.: Hepatitis Antigen and -fetoprotein in Neonatal Hepatitis. Arch. Dis. Child., 48:157, 1973.

101. Editorial: FetoProteins. Lancet, 1:397, 1970.

102. Alpert, E., Hershberg, R., Schurr, P.H., and Isselbacher, K.J.: Alpha-fetoprotein in Human Hepatoma: Improved Detection in Serum and Quantitative Studies Using a New Sensitive Technique. Gastroent, 61:137, 1971.

103. Bagshawe, A., and Parker, A.M.: Age Distribution of Alpha-fetoprotein in Hepatocellular Carcinoma. Lancet, 2:268, 1970.

104. Abelev, G.I.: Alpha-fetoprotein as a Marker. Transplant Rev., 20:3, 1974.

105. Purves, L.R., Branch, W.R., Geddes, E.W., Manso, C., and Portugal, M.: Serum Alpha-fetoprotein. VIII. The Range of Apparent Serum Values in Normal People, Pregnant Women and Primary Liver Cancer High Risk Populations. Cancer, 31:578, 1973.

106. Abelev, G.I., Elgort, D.A., and Perova, S.D.: Quantitative Aspects of Alpha-Fetoprotein Synthesis, in Alpha-Fetoprotein, Masseyeff, R., (ed.), Nice: INSERM, p. 141, 1974.

107. Iwasaki, T., Dempo, K., Kaneko, A., and Onoe, T.: Fluctuation of Various Cell Populations and Their Characteristics During Azo-Dye Carcinogenesis. Gann., 63:21, 1972.

108. Smith, J.B.: Alpha-fetoprotein: Occurrence in Germinal Cell and Liver Malignancies. Amer. J. Med., 51:767, 1971.

109. Ruoslahti, El, and Seppälä, M.: Normal and Increased Alpha-fetoprotein in Neoplastic and Non-neoplastic Liver Disease. Lancet, 2:278, 1972.

110. Alpert, E.: Alpha-1-Fetoprotein: Serologic Marker of Human Hepatoma and Embryonal Carcinoma. Nat. Cancer Inst. Monogr., 35:415, 1972.

111. Watabe, H.: Early Appearance of Embryonic α-Globulin in Rat Serum During Carcinogenesis with 4-dimethylaminobenzene. Cancer Res., 31:1192, 1971.

112. Leduc, E.: Regeneration of the Liver, in The Liver, Vol. II Rouiller, C., (ed.), New York: Academic Press, 1964.

113. Kitagawa, T., Yokochi, T., and Sugano, H.: α-Fetoprotein and Hepatocarcinogenesis in Rats Fed 3-methyl-4-(diethylamino) azobenzene or N-2-fluroenyl-acetamide. Int. J. Cancer, 10:368, 1972.

114. Lin, T.Y.: Primary Cancer of the Liver. Quadrennial Review. Scan. J. Gastroent., 5:223, 1970.

115. Purves, L.R., Bersohn, I., Geddes, E.W., Falkson, G., and Cohen, L.: Serum Alpha-Fetoprotein. IV. Effects of Chemotherapy and Radiotherapy on Serum Alpha-Fetoprotein Levels in Cases of Primary Liver Cancer. So. Afr. Med. J., 44:590, 1970.

116. Masseyeff, R.: Human Alpha-feto-protein. Path. et. Biol., 20:703, 1972.

117. Alpert, E., and Zuckerman, J.: Absence of α-fetoprotein Antigen or Antibody in Maternal Sera. Lancet, 2:465, 1970.

118. Gitlin, D., and Boesman, M.: Sites of Serum α-fetoprotein Synthesis in the Human and in the Rat. J. Clin. Invest., 46:1010, 1967.

119. Teilum, B.: Classification of Endodermal Sinus Tumor (Mesoblastoma Vitellinum) and So-called "Embryonal Carcinoma of the Ovary." Acta. Path. Microb. Scand., 64:407, 1965.

120. Gitlin, D., Perricelli, A., and Gitlin, G.M.: Synthesis of α-fetoprotein by Liver, Yolk Sac, and Gastrointestinal Tract of Human Conceptus. Cancer Res., 32:979, 1972.

121. Mawas, C., Kohen, M., Lemerle, J., Buffe, D., Schweisguth, O., and Burtin, P.: Serum Alpha-1-Fetoprotein in Children with Malignant Ovarian or Testicular Teratomas. Preliminary Results. Int. J. Cancer, 4:76, 1969.

122. Englehardt, N.V., Poltoranina, U.S., and Yazova, A.K.: Localization of Alpha-fetoprotein in Transplantable Murine Teratocarcinomas. Int. J. Cancer, 11:448, 1973.

123. Mawas, C., Buffe, D., Schweisguth, O., and Burtin, P.: Alpha-1-fetoprotein and Children's Cancer. Rev. Eur. Etud. Clin. Biol., 16:430, 1971.

124. Silver, H.K.B., Gold, P., and Shuster, J.: Significance of $Alpha_1$-fetoprotein in Infectious Hepatitis. Clin. Res., 21:525, 1973.

125. Smith, J.B.: Occurrence of Alpha-fetoprotein in Acute Viral Hepatitis. Int. J. Cancer, 8:421, 1971.

126. Silver, H.K.B., Gold, P., Shuster, J., Javitt, N., Freedman, S., Firrlagson, N.: Alpha$_1$-fetoprotein in Chronic Liver Disease. NEJM, 291(10):506, 1974.

127. O'Conor, G.T., Tatarinov, Y.S., and Abelev, G.I.: A Collaborative Study for the Evaluation of a Serologic Test for Primary Liver Cancer. Cancer, 25:1091, 1970.

128. Karvountzis, G.G., and Redeker, A.G.: Relation of Alpha-fetoprotein in Acute Hepatitis to Severity and Prognosis. Ann. Int. Med., 80:156, 1974.

129. Belanger, L., Belanger, M., Prive, L., Larochelle, J., Tremblay, M., Aubin, G.: Tyrosinemie Hereditaire et alpha-1-foetoproteimie, Path. et Biol., 21:449, 1973.

130. Wallwork, J.C., McFarland, H., Hingley, S., Ward, A.M.: Serum α-Fetoprotein in Cystic Fibrosis. Br. Med. J., 2:392, 1975.

131. Brock, D.J.H., Manson, J.C., Raiburn, J.A., Bolton, A.E., and McCrai, W.M.: Serum α-Fetoprotein in Cystic Fibrosis. Br. Med. J., 2:392, 1975.

132. Fitzsimmons, J.S., Smith, N., Hiller, E.J., Wynne, J.: Serum α-Fetoprotein in Cystic Fibrosis. Br. Med. J., 3:344, 1975.

133. Smith, J.A.: Serum α-Fetoprotein in Cystic Fibrosis. Br. Med. J., 3:344, 1975.

134. Swachman, H., and Kulczycki, L.: Long Term Study of One Hundren Five Patients with Cystic Fibrosis. Am. J. Dis. Child., 96:6, 1958.

135. Ruoslahti, E., and Seppälä, M.: Studies of Carcino-Fetal Proteins: III. Development of a Radioimmunoassay for α-fetoprotein. Demonstration of -fetoprotein in Serum of Healthy Human Adults. Int. J. Cancer, 8:374, 1971.

136. Ouchterlony, O.: Antigen-antibody Reaction in Gels. Acta. Path. Microbiol. Scand., 26:507, 1954.

137. Greenwood, F.C., Hunger, W.M. and Glover, J.S.: The Preparation of ^{131}I-labelled Human Growth Hormone of High Specific Radioactivity. Biochem. J., 89:114, 1963.

138. Abelev, G.I., Tsvetkow, V.S., and Biryulina, T.I., et al.: Assessment of the Use of Highly Sensitive Methods of Determining Alpha-fetoprotein for the Diagnosis of Hepatocellular Cancer and Teratoblastoma. Biull. Eksp. Biol. Med., 4:75, 1971. (English Abstract).

139. Holter, H., Hobbins, J., and Baumgarten, A.: Alpha-fetoprotein As An Indicator of Fetal Condition. Lancet, 2:184, 1975.

140. Purves, L.R., and Purves, M.: Serum Alpha-fetoprotein. So. Afr. Med. J., 46:1290, 1972.

141. Ruoslahti, E., and Seppälä, M.: Normal and Increased Alpha-fetoprotein in Neoplastic Liver Disease. Lancet, 2:278, 1972.

142. Seppälä, M., and Ruoslahti, E.: Alpha Fetoprotein in Amniotic Fluid: An Index of Gestational Age. Am. J. Obstet. Gynecol., 114(5):595, 1972.

143. Sizaret, P.: Alpha-Fetoprotein Quantitation by Radioelectrocomplexing. J. Immunol. Meth., 5:283, 1974.

YALE MEDICAL LIBRARY

Manuscript Theses

Unpublished theses submitted for the Master's and Doctor's degrees and deposited in the Yale Medical Library are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but passages must not be copied without permission of the authors, and without proper credit being given in subsequent written or published work.

This thesis by has been used by the following persons, whose signatures attest their acceptance of the above restrictions.

NAME AND ADDRESS DATE