AN EVALUATION OF CYSTIC FIBROSIS SCREENING PROGRAMMES
FOR IMPLEMENTATION IN BRITISH COLUMBIA

by

JANNIE MARTINE SCRIBIN
B.Sc., The University of British Columbia, 1969

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in
THE FACULTY OF GRADUATE STUDIES
(Department of Pathology, Faculty of Medicine)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
August, 1982

© Jannie Martine Scriabin, 1982
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pathology (Medicine)

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date Dec 14/1982
ABSTRACT

Four methods were investigated to determine their suitability for use in a CF screening programme for the province of British Columbia.

A fecal trypsin method which measured trypsin activity by incubating dry stool samples on filter paper cards with the substrate p-tosyl-arginine methyl ester (TAME) and a pH sensitive dye was shown to be non-specific and therefore unsatisfactory.

An attempt to combine a fecal albumin screen with a more specific quantitative immunodiffusion technique for albumin and alpha-1 antitrypsin was unsuccessful.

A meconium albumin assay using the Boehringer-Mannheim Corporation (BMC) test-strip and a more specific fecal trypsin assay which uses the substrate benzoyl-arginine-p-nitroanilide (BAPNA) were incorporated into two pilot projects at Children's Hospital in Vancouver.

The BMC test-strip was simple to use, reliable and inexpensive. Of 8,891 infants tested, 3 positives were diagnosed as suffering from cystic fibrosis and 1 CF patient tested negative. False positives were obtained on 1.3% of infants. The incidence of CF as determined by this screen was 1 in 2000. The meconium albumin screen was satisfactory as a local pilot project but the disadvantages of testing the unstable meconium specimens make the screen unsuitable for a province-wide application.

The BAPNA fecal trypsin method devised by Crossley was used to test 4085 dry stool specimens collected in the hospital and at home. Out of a total number of 190 positive results, none was diagnosed as having CF, giving a false-positive rate of 5.0% for the hospital collected specimens.
and 3.4% for the specimens collected at home. The false positive rate in the hospital collected specimens was due mostly to the large proportion of young infants (under 3 days). The false positive rate of the home collected specimens appeared to be due mostly to the thinner spread of stool sample on the card. Because the quantity of stool sample per test was significantly lower in the home than the hospital collected specimens a new cut-off point for the home collected specimens was considered. Its application, however, did not lower the false positive rate sufficiently. As a result, the high incidence of false positives and the difficulties encountered as a result of this incidence also makes the fecal trypsin screen unsuitable for the province of B.C.

Difficulties encountered during the follow-up of positive results obtained in the two pilot projects are discussed and recommendations are made regarding the efficient and adequate implementation of a follow-up system.
ACKNOWLEDGEMENTS

I would particularly like to thank Dr. Derek A. Applegarth for his expert guidance, for his patience through obstacles and delays and for his faith in the eventual positive conclusion of this research.

Thanks also to Dr. A.G.F. Davidson, for allowing me to get involved in this interesting research, for his guidance and help in establishing the CF screen pilot projects and pursuing the follow-ups.

To Dr. P.E. Reid many thanks for his thorough review of my thesis draft and for his constructive suggestions.

Special thanks to Shirley Turtle for the many hours of meticulous technical assistance and helpful discussions in the laboratory.

Thanks finally to my husband Michael who unselfishly understood my need to devote many hours to this research and gave me his support through difficult times.
CONTENTS

ABSTRACT ................. ii
ACKNOWLEDGEMENTS ........ iv
LIST OF TABLES ........... ix
LIST OF FIGURES .......... x
INTRODUCTION ............ 1

Cystic Fibrosis
Thesis Objective
The Need for a Neonatal Cystic Fibrosis Screen
Criteria for a Successful Screening Programme
Age to be Screened

EXISTING NEONATAL SCREENING PROGRAMMES ................. 10

Sweat Electrolytes
Tests for Detecting Pancreatic Insufficiency
  Meconium Albumin
  Meconium Albumin and Alb:Alpha-1 Antitrypsin Ratio
  Fecal Albumin and Alb:Alpha-1 Antitrypsin Ratio
  Fecal Trypsin

SELECTION AND DEVELOPMENT OF TESTING PROCEDURES ........ 19

BMS TEST-STRIP FOR MECONIUM ABLUMIN ................. 21

Testing Procedure
  Collection of Samples
  BMC Methodology
  Stability Investigation
Results
  Meconium Sample Collection
  Sensitivity
  Specificity
Discussion
  Sample Collection
  Sensitivity
  Specificity
  Incidence of CF in the Population Screened

ROBINSON AND ELLIOTT FECAL TRYPsin SCREEN ............... 32

Testing Procedure
  Collection of Samples
  Trypsin Methodology
  Investigation into Inconsistent Results and Non-Specificity
Results
Sensitivity and Specificity
Investigation into Possible Causes of Inconsistent Results and Non-Specificity

Discussion

Fecal Albumin: Alpha-1 Antitrypsin Ratio

Testing Procedure
Albumin: Alpha-1 Antitrypsin Ratio and Pancreatic Insufficiency
Elution of Stool Samples from Filter Paper Cards
Qualitative Albumin Method

Results
Relationship of Ratio to Pancreatic Insufficiency
Elution of Stool Samples from Filter Paper Cards
Qualitative Albumin Method

Discussion
Relationship of Ratio to Pancreatic Insufficiency
Elution of Stool Samples from Filter Paper Cards
Qualitative Albumin Method

Crosley Fecal Trypsin Method

Testing Procedure
Collection of Fecal Samples
Trypsin Methodology
Investigation to Establish Procedure
Background Interference
Doubling the Substrate Concentration
Validity of Visual Evaluation of Yellow Intensity

Results
Investigation to Establish Procedure
Background Interference
Doubling the Substrate Concentration
Validity of Visual Evaluation of Yellow Intensity
Sensitivity and Specificity
Age of Infant When Hospital Specimen was Collected
Hospital and Home Collected Specimens from the Same Infant
Comparison of Hospital and Home Collected Specimens

Discussion
Investigation to Establish Procedure
Background Interference
Doubling the Substrate Concentration
Validity of Visual Evaluation of Yellow Intensity
Sensitivity and Specificity
Sample Collection
Hospital and Home Collected Specimens
Establishment of New Cut-Off Point
FOLLOW-UP ................................................................. 104

Protocol
Request for Stool Samples for Chymotrypsin
Quantitative Chymotrypsin Analysis
Sweat Electrolytes
Clinical Follow-Up Only
Review of the Files at Medical Records

Results
Meconium Albumin CF Screen
Diagnostic Follow-Up
Medical Records Investigation
Fecal Trypsin CF Screen
Diagnostic Follow-Up
Medical Records Investigation

Discussion
Meconium Albumin CF Screen
Diagnostic Follow-Up
Medical Records Investigation
Fecal Trypsin Screen
Diagnostic Follow-Up
Medical Records Investigation

Difficulties Encountered with the Follow-Up Procedure

SUMMARY AND CONCLUSIONS ........................................... 133

ADDENDUM — AN UPDATE .............................................. 140

APPENDICES ............................................................. 145

A. Screening Card for Collection of Stool Sample for Fecal Trypsin Screen
B. Letter to Parents: Request for Collection of Infant's Stool Sample for CF Screening Programme
C. Calculation of % Error Resulting from Use of Crossley's Fecal Pigment Correction Procedure
D. 1. Letter to Physician: Request for Stool Sample for Quantitative Chymotrypsin Analysis
   2. Instructions for Stool Sample Collection
E. Clinical Questionnaire
F. Letter to Physician: Request for Clinical Follow-up to Physician Who Felt Laboratory Follow-up was not warranted
G. Chi-Square Test for Significance of Difference Between False Positive Rates for Hospital and Home Collected Specimens in Fecal Trypsin CF Screen
H. Chi-Square Test for Significance of Difference Between False Positive Rates for Less than 3-Day Old Infants and At least 3-Day Old Infants
I. Chi-Square Test for Significance of Changes in Results for Hospital and Home Collected Specimens on the Same Infant
J. 1. Chi-Square Test for Significance of Difference Between Distributions of Net Absorbance Readings of Hospital and Home Collected Specimens

vii
2. Test for Significance of Difference Between Means of Hospital and Home Collected Specimens Net Absorbance Values

K. 1. Comparison of Screening Results from Freezer and Room Temperature Stored Specimens
   2. Comparison of Specimen Results Before and After Mailing
   3. Combined Data for Room Temperature Stored Specimens

L. Chi-Square Test for Significance of Differences Between Distributions of Absorbance Values at 460nm of Hospital and Home Collected Specimens

M. Comparison of the Precision of the Results of Hospital and Home Collected Specimens

N. Correlation Between Positive Meconium Screen Results and Presence of Necrotizing Enterocolitis

O. 1. Rank Test for Significance of the Effect of Doubling the BAPNA Substrate Concentration
   2. Test for Correlation Between % Increase in Absorbance Due to Doubling Substrate Concentration and the Original Absorbance Value

P. Conditions Under Which an Equal Percent Reduction in Absorbance at 410 and 460 Nanometers from Hospital to Home Collected Specimens can Occur.

Q. Calculation of Confidence that Child with Positive Fecal Trypsin Screen Result has CF.

BIBLIOGRAPHY ................................................................. 182
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Incidence of CF in North America and Western Europe</td>
<td>2</td>
</tr>
<tr>
<td>II. Meconium Albumin Screening Test for CF</td>
<td>26</td>
</tr>
<tr>
<td>III. Fecal Trypsin Control Specimen Results Robinson and Elliott Method</td>
<td>40</td>
</tr>
<tr>
<td>IV. Albumin and Alpha-1 Antitrypsin Content of Feces Immunodiffusion Method</td>
<td>55</td>
</tr>
<tr>
<td>V. Fecal Pigment Background Interference, Crossley Method</td>
<td>70</td>
</tr>
<tr>
<td>VI. Tests and Their Corresponding Test Blanks in the Crossley Method</td>
<td>72</td>
</tr>
<tr>
<td>VII. Low Trypsin Activity Samples Tested with Crossley Method Using 0.25 and 0.50 mg L-BAPNA per Test</td>
<td>74</td>
</tr>
<tr>
<td>VIII. Comparison of Visual with Spectrophotometric Evaluations of 522 Tests: Crossley Method</td>
<td>77</td>
</tr>
<tr>
<td>IX. Fecal Trypsin Screening Test for CF: Hospital Collected Specimens</td>
<td>78</td>
</tr>
<tr>
<td>X. Fecal Trypsin Screening Test for CF: Home Collected Specimens</td>
<td>78</td>
</tr>
<tr>
<td>XI. Fecal Trypsin Screening Test for CF: Hospital and Home Collected Specimens on the Same Infant</td>
<td>80</td>
</tr>
<tr>
<td>XII. CF Screening Pilot Projects</td>
<td>113</td>
</tr>
<tr>
<td>XIII. Summary of Autopsy Reports Meconium Screening Test Positives</td>
<td>114</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Trypsin Activity with Two Different BAPNA Substrate Concentrations</td>
<td>76</td>
</tr>
<tr>
<td>II. Comparison of Net Absorbance Values from First Assay of Hospital and Home Collected Specimens</td>
<td>81</td>
</tr>
<tr>
<td>III. Comparison of Net Absorbance Values at 460 nm from Hospital and Home Collected Specimens</td>
<td>84</td>
</tr>
<tr>
<td>IV. CF Screening and Follow-Up Protocol Flow Chart</td>
<td>105</td>
</tr>
<tr>
<td>V. Results of the Diagnostic Follow-Up on Positives from the Meconium Screen</td>
<td>116</td>
</tr>
<tr>
<td>VI. Results from an Investigation for Possible Causes of Increased Meconium Albumin in Non-CF Infants</td>
<td>119</td>
</tr>
<tr>
<td>VII. Results of the Diagnostic Follow-Up on Positives from the Fecal Trypsin Screen</td>
<td>120</td>
</tr>
</tbody>
</table>
INTRODUCTION

Cystic Fibrosis

Cystic Fibrosis is the most common lethal genetic disease in Canada. It is an autosomal recessive trait, the disease expressing itself only in the homozygous state. The metabolic defect is unknown. Present statistics indicate that 1 in 20 to 1 in 30 white Canadians carry the gene for cystic fibrosis (CF). The incidence of CF in newborn whites is calculated to be somewhere between 1 in 1,600 and 1 in 3,600. The incidence is very low in the Negro and Oriental population. Table I lists the incidence of CF as determined by various investigators in North America, Europe, Australia and New Zealand.

The genetic disorder results in a general dysfunction of a number of exocrine glands. The mucous glands produce an extremely thick, viscous and sticky mucous which obstructs the ducts of various organs. This affects the pancreas, the lungs, the liver and the gastro-intestinal tract. The CF disease state generally attracts the physicians’ attention with failure of the child to gain weight, steatorrhea and other symptoms of pancreatic insufficiency or chronic respiratory tract infection.
### TABLE I. INCIDENCE OF CF IN NORTH AMERICA AND WESTERN EUROPE

<table>
<thead>
<tr>
<th>Screening Centre</th>
<th>Investigators</th>
<th>Incidence&lt;sup&gt;0&lt;/sup&gt;</th>
<th>No. Screened</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MECONIUM ALBUMIN SCREEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Wales</td>
<td>Prosser et al&lt;sup&gt;63&lt;/sup&gt;</td>
<td>1:1,556</td>
<td>34,228</td>
</tr>
<tr>
<td>Uppsala, Sweden</td>
<td>Hellsing and Kollberg&lt;sup&gt;45&lt;/sup&gt;</td>
<td>1:2,943</td>
<td>8,830</td>
</tr>
<tr>
<td>Milwaukee, USA</td>
<td>Bruns et al&lt;sup&gt;56&lt;/sup&gt;</td>
<td>1:8,114</td>
<td>16,227*</td>
</tr>
<tr>
<td>Philadelphia, USA</td>
<td>Holsclaw, Keith and Palmer&lt;sup&gt;47&lt;/sup&gt;</td>
<td>1:3,361</td>
<td>20,171&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Western Europe, 16 countries</td>
<td>European Working Group for Neonatal Screening&lt;sup&gt;99&lt;/sup&gt;</td>
<td>1:1,936</td>
<td>199,475</td>
</tr>
<tr>
<td>Leeds, England</td>
<td>Evans et al&lt;sup&gt;30&lt;/sup&gt;</td>
<td>1:2,247</td>
<td>15,734</td>
</tr>
<tr>
<td><strong>FECAL TRYPsin SCREEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Zealand</td>
<td>Robinson and Elliott&lt;sup&gt;72&lt;/sup&gt;</td>
<td>1:2,198</td>
<td>6,595</td>
</tr>
<tr>
<td>New Zealand</td>
<td>Crossley, Berryman and Elliott&lt;sup&gt;46&lt;/sup&gt;</td>
<td>1:2,250</td>
<td>4,500</td>
</tr>
<tr>
<td>Australia</td>
<td>Forrest, Wilcken and Turner&lt;sup&gt;32&lt;/sup&gt;</td>
<td>1:4,000</td>
<td>20,000</td>
</tr>
</tbody>
</table>

* racial distribution: 80% white, 19% black, 1% others
+ racial distribution: 75% white, 19.4% black, 2.2% others, mainly Oriental, 2.4% no information recorded
° incidence of cases which tested positive in the screen and were confirmed to be CF
The sweat glands are also affected and exhibit a defect in the tubular reabsorption of electrolytes resulting in an increased concentration of sodium chloride in the sweat.

The disease is found in a wide range of severity, some cases being only mildly affected in either the pancreas or lungs, often not presenting itself until late in childhood. Others are severely affected from birth although as far as it is known the lungs are still healthy at birth.²⁴⁷,⁶⁹

Early Treatment

Early treatment is helpful in all cases and can prevent the often fatal complications of the disease.²⁷,⁴⁴,⁶⁵,⁶⁶ This has been indicated in various ways:

1. The average life span of CF victims has increased considerably over the past 20 years, mostly due to the improvement in treatment but also as a result of the recognition of milder cases and as the result of earlier detection.²⁸,³⁶,⁸²,⁹⁸

2. Studies of siblings indicate that early treatment leads to better prognosis.⁶⁹ In these studies the second child to have CF in a family is diagnosed at an earlier stage because of the family doctor being aware of the genetic trait. The younger siblings received the same treatment but showed significantly better prognosis after 7 years of age than the older sibling who had been diagnosed much later in the development of the disease.
Thesis Objective

The objective of this thesis is to evaluate existing and if necessary to develop new neonatal screening programmes for CF in an attempt to implement one in the province of British Columbia.

The decision to conduct CF screening has been made by others and is beyond the scope of this thesis. Nevertheless, the benefits and potential risks of a CF screening programme are briefly reviewed in the next section for the interested reader.

The Need for a Neonatal Cystic Fibrosis Screen

A. Benefits of Screening

i) The incidence of CF is sufficiently high to justify the application of a neonatal screening programme.

ii) Early presymptomatic diagnosis by screening followed by appropriate treatment will improve the gastrointestinal problems and slow down the progressive bronchopulmonary disease which affect most patients with CF sooner or later.\(^3\)

   a) Affected children can be treated promptly with enzyme supplement and antibiotics.

   b) Prophylactic pulmonary treatment can be initiated prior to the development of irreversible pulmonary complications. The early lung changes in CF often occur within the first three months of life and are felt to be reversible. No pathological abnormality can be shown in the lungs of a CF baby at birth.\(^69\)
iii) The life expectancy of the C.F. patient is increased if diagnosed and treated early. The study by Shwachman indicates that over a 20 year period the life expectancy has improved from approximately 1 to 20 years of age in a group diagnosed early in life. Warwick's statistical study of data obtained from the CF Foundation Registry agrees with this conclusion.

iv) Early diagnosis in a child can result in parents receiving genetic counselling before they consider another pregnancy. Since amniocentesis for identification and subsequent abortion of affected fetuses is not yet available, neonatal screening is the only source of genetic information for prospective parents who are not aware that they carry the CF gene.

v) Cost of the screening programme is minimal considering the potential savings to the community through prevention of complications. The screening cost also compares favourably with other screening programmes. Early treatment results in savings since it leads to a reduction in inpatient care, but against this would need to be set the cost of drugs and outpatient care, which would presumably be administered for a longer time period.

vi) A reliable confirmation test, the sweat electrolyte determination, is available. It does not require sophisticated equipment and facilities.

B. Potential Risks of Screening

i) There is a disagreement over how best to manage the early diagnosed babies, with some physicians advocating non-intervention until
symptoms eventually appear. The objection raised to treating symptom free children is in regards to the possible hazards of prolonged antibiotic therapy which can lead to complications such as the colonisation of the respiratory tract by organisms insensitive to treatment.

ii) The effectiveness of genetic counselling may not warrant screening. Experience in New York has shown that almost half of the families who had produced CF affected children went to further pregnancies after counselling despite the 1:4 risk of a further CF affected child with each conception.

iii) Concern has been expressed that harm could result from parents' knowledge that their child is going to suffer from CF in the time period before the child manifests the disease.

iv) Since CF is not curable, the cost-benefit has been questioned. Early diagnosis does not reduce the cost to the community as drastically as in the case of e.g. PKU where inadequately treated or late diagnosed patients are a 40 to 60 year burden to the community.

v) Psychological stress may be caused to the parents of infants with false positive screening results due to low specificity of the testing method.

The Research Group on Ethical, Social and Legal Issues on Counselling suggests that screening programmes be established only for those genetic disorders that have a fairly high incidence and that the programme be structured on the basis of one or more clearly identified goals. Three distinguishable categories of goals are that the programme:
1) contributes to improving the health of persons who suffer from the genetic disorder,

2) allows carriers of a given variant gene to make informed choices regarding reproduction,

3) move towards alleviating the anxieties of families and communities faced with the prospect of serious genetic disease.

It would appear that a neonatal screen for CF, a genetic disorder whose incidence is relatively high, meets at least the first two of these objectives since early detection will improve and increase the life span of the CF child as a result of earlier treatment and genetic counselling will be available for the parents.

Without screening, approximately 60% of CF patients in Canada are diagnosed during the first year of life and therefore receive treatment at this fairly early stage. Unequivocal proof that earlier treatment yet (presymptomatic) would further improve their condition significantly is not available as yet but the evidence seems to point in that direction.*

The remaining CF patients, the 40% that are diagnosed after 1 year of life, would gain the most from a neonatal screen for the presence of CF may not be suspected clinically for some time and irreversible lung damage may occur. Respiratory and gastrointestinal problems present themselves

* Lubin and Bonner found that the heights and weights of infants with CF diagnosed before 6 months of age fell in the top 25 percent of these measures for those diagnosed after 6 months of age. While Palmer reported that early diagnosis and treatment was associated with a reduced number of hospital admissions, enhanced growth and improved clinical condition.
early in most of these undiagnosed patients. If detected by a CF screen, these patients could be treated accordingly and years of inappropriate treatment could be avoided.

Criteria for a Successful Screening Programme

As recommended by the Committee of the National Academy of Sciences the following points were taken into consideration in evaluating CF screening programmes: i) the validity, reliability and safety of the screening test, ii) costs iii) acceptance of the screening test by the community, including both consumers and practising physicians.

When a testing procedure appeared suitable for a CF screening programme, a pilot project was set up in order to ascertain that the programme's goals were attainable and that acceptable levels of specificity and sensitivity could be reached.

The CF pilot screen was operated from Children's Hospital where the CF Assessment Clinic was located. The clinic had experience with CF and had effective communication with the screening lab and the practising physician who provided primary care to the patient. Presumptive positives were referred to this clinic which was capable of confirming the diagnosis, initiating and monitoring the therapy and counselling the family.

The screening laboratory included in the routine of the testing procedure for C.F. the following recommendations made by the Committee: i) the test was performed under strict quality control procedures with standards and control specimens inserted as unknowns in the daily runs;
ii) a second sample was tested before considering the screening test positive since the possibilities of a test error were great as a result of the nature of the screening test itself and the number of specimens being handled; iii) the results were reviewed periodically to determine if the procedure needed to be altered or the cut-off point needed to be changed; iv) the specimens were saved under conditions that maximized stability in order to enable retesting of specimens should a false negative appear; v) permanent records were kept and tabulated periodically to determine changes in the frequency of both true and false positives and the interval it took for the hospital to send specimens was checked periodically in order to correct delays.

Age to be Screened

Diagnosis, even in the early symptomatic phase, does not exclude the possibility that the lung may be damaged irreversibly. Since evidence to date indicates that the lung is normal at birth, the ideal time to detect the homozygote for the CF gene would be before lung damage has had time to start. Mass screening should therefore be performed on the newborn infant, as soon as possible after delivery.
EXISTING NEONATAL SCREENING PROGRAMMES

Cystic fibrosis is characterized by what is known as the "clinical triad" of chronic pulmonary disease, pancreatic insufficiency and abnormally high concentration of electrolytes in sweat. The detection of the last two are possibilities for a screening test.

I. SWEAT ELECTROLYTES

The principal diagnostic test for CF is the sweat test for the presence of abnormally high levels of sodium or chloride. Between 98 and 99% of the CF patients display an increased level of sodium chloride and this abnormality appears to be present from birth. Although there are reports of isolated cases where an infant has the clinical expression of the disease and a negative sweat test, the abnormal sweat electrolyte results are the most constant symptom.

Attempts have therefore been made to adapt the sweat test to mass CF screening. To date all methods have proved ineffective for the following reasons:

1. The young infant provides serious problems with sweat collection for many newborns produce little or no sweat.

2. The standard sweat sodium and/or chloride determination is too time consuming to apply as a routine screening method especially since
reliable results require stimulation of sweat production to ensure maximal secretory rates. Other methods which are more efficient are too imprecise and unreliable.

3. Since the test is not reliable when performed on the newborn, there is a low degree of patient compliance because the infant must be brought to the hospital for the test at a later date.

As a result screening by measuring sweat electrolytes is limited to high-risk individuals such as siblings and first cousins of CF patients. Several investigators have however attempted to overcome these problems and reports on their successes and failures may be found in the literature.99,97,101

The most common and most reliable method used to analyse sweat is the standard quantitative pilocarpine iontophoresis test of Gibson and Cooke.39 Other methods are conductivity measurements,91,101 osmolality measurements,101 ion-selective electrode,41,42,40 or the indirect method of analysing nail-clippings (salt in nails comes from sweat glands).53

II. TESTS FOR DETECTING PANCREATIC INSUFFICIENCY

Several tests for detecting pancreatic insufficiency are available and have been implemented in mass CF screening programmes. All of them suffer from one main disadvantage, the fairly high false negative rate as a result of the inconsistent presence of pancreatic insufficiency in CF children.
Ten to fifteen percent of CF infants do not display pancreatic insufficiency.\(^{12,23,55,80}\) As a result, this percentage range is quoted by most investigators as the expected false negative rate for CF screening programmes. There are indications lately, however, that these figures may be a little high.\(^{16,75,83}\) It appears that pancreatic functions tests were not always performed promptly on the infants that gave a false negative. As a result there is confusion as to whether the pancreatic deficiency develops in most cases as the child gets older\(^{12}\) or whether most CF children have the deficiency at birth with a few developing normal pancreatic function later in life.\(^{16}\) If the former holds then the % of false negatives could be expected to be higher than 10 to 15%. If the latter holds then the number of false negatives found in a neonatal screening programme as a result of normal pancreatic function should be lower than the 10 to 15% frequently quoted.

The two most frequently used CF screening tests which detect pancreatic insufficiency are the analysis of meconium for albumin and the analysis of feces for trypsin activity. Two other less commonly used screens are also discussed.

A. Meconium Albumin

Meconium is a dark green mucilagenous material present in the intestine of the full term fetus. It is a mixture of the secretions of the intestinal glands and some amniotic fluid.
An increased albumin level is found in the meconium from CF infants. Hellsing and Kollberg who tested the meconium from 1,000 healthy newborns using a single radial immunodiffusion technique report that 59% contained less than 0.3 mg of albumin per g of dried meconium and 99.6% contained less than 4.0 mg per g. A further study in which Kollberg and Hellsing used the same method on a screening series of 8830 healthy infants reports that 99.8% of the meconium specimens had an albumin concentration below 20 mg per g dry weight meconium.

The lowest albumin concentration in 15 cystic fibrosis specimens tested concurrently with their first study was 35 mg per g of dried meconium while most newborn CF infants had a meconium albumin level in the region of 80 mg per g.

The albumin in meconium is probably derived from amniotic fluid swallowed by the fetus in utero. It accumulates in the CF meconium if the pancreatic enzymes are absent or decreased in concentration.

False positives are found in premature infants because many of these infants have low proteolytic activity in the meconium. The presence of blood in the specimen and the presence of a contaminent such as glycerine from a rectal suppository or baby ointments containing protein may also be the cause of false positives.

The sulfosalicylic acid test, Albustix and the BMC test-strip have been used to screen meconium albumin. The last is the most common method used.
BMC Test-Strips

The Boehringer-Mannheim (BMC) test-strip* was designed to detect an albumin content above 20 mg per g dry wt. of meconium.

The BMC test-strips were used in the following studies:

Prosser et al tested 34,228 samples using the sulfosalicylic acid test, Albustix**, immunodiffusion technique and the BMC test-strips for comparative purposes. However, only 2,106 of the samples were tested with the BMC test. One CF patient was detected in this sample and a false positive rate of 0.6% was obtained.


This study reports the results from the testing of 69,000 infants with the BMC test-strip. The results that were compiled for this study were from 16 European centres (plus 2 American centres).

Since some high-risk groups and two American centres had also been included in the study the European incidence could not be determined from the 60 positives detected in the 69,000. However, in a strictly random group of 34,300 neonates from European centres, 19 positive results were obtained giving a frequency of 1 in 1,800. The false negative rate was reported as 0.5%.

In 1976 a report** was published by the European Working Group for Neonatal Cystic Fibrosis Screening based on an extension of the study.

---

* Boehringer-Mannheim Corporation, Mannheim, West Germany
** Ames Company (Division of Miles Laboratory) Elkhart, IN, USA.
by Stephan. A total of 199,475 newborns had been screened. The overall incidence of CF in this group was 1 in 1,936. The false positive rate was 0.5%, and the false negative rate was 15%. As a result of this study, the test-strip became mandatory as a screening test for all neonates in West Germany.


This centre tested 16,224 newborns born in the Milwaukee area hospitals. Two CF infants were diagnosed correctly by the test and two were missed. The false positive rate was 0.9%.


This group screened 20,171 infants. Four positive results were confirmed as CF, 2 CF were missed. A false positive rate of 0.3% was obtained.


Between October 1975 and January 1980, 15,734 babies were screened. Seven of the BMC positives were diagnosed as having CF, with no missed cases. The false positive rate was 0.83%. Eight false positives were associated with the presence of blood in the meconium.

B. Meconium Albumin and Alb:Alpha-1 Antitrypsin Ratio

Ryley*, in an attempt to reduce the number of false positives obtained with the BMC test-strip, combined the meconium albumin screening test with a quantitative immunoelectrophoresis technique for albumin and alpha-1 antitrypsin.
Alpha-1 antitrypsin is normally present in meconium specimens from healthy infants since this protein is resistant to proteolysis by the pancreatic proteases. Its concentration in meconium specimens from CF children is almost the same as the normal range.

Albumin, on the other hand, is found in very low concentrations in meconium from the healthy infants for this protein is liable to proteolysis and is present in increased amounts in specimens from CF children with pancreatic insufficiency. This results in a significantly higher albumin:alpha-1 antitrypsin ratio in meconium specimens from CF children with pancreatic insufficiency as compared to healthy children.

The ratio of albumin to alpha-1 antitrypsin was therefore used by Ryley to exclude pancreatic insufficiency in the healthy infants who gave a positive result with the BMC test. These healthy infants had increased levels in both the albumin and the alpha-1 antitrypsin in the meconium resulting in a normal albumin:alpha-1 antitrypsin ratio.

Meconium specimens from a total of 2,325 babies were examined. Approximately 0.2% of healthy infants gave a positive reaction with the BMC test. It was possible to conclude that these were false positives and that there was very little evidence of pancreatic insufficiency despite the BMC positive result on the basis of the normal albumin:alpha-1 antitrypsin ratio.

C. Fecal Albumin and Albumin:Alpha-1 Antitrypsin Ratio

Although albumin is not present in as high a concentration in feces as in meconium, there is a significant difference in the fecal albumin
concentration from healthy and CF children.

Electroimmunoassay was employed by Ryley\textsuperscript{76,77} to detect albumin and albumin:alpha-l antitrypsin in feces. Feces from infants with CF had an albumin content of more than 2.0 mg per g dry weight and an albumin:alpha-l antitrypsin ratio greater than 3.0.

Ryley reports its usefulness in distinguishing between infants free of CF who gave a positive meconium screening test from infants with CF. Only a limited number of specimens (51 non-CF and 9 CF) were tested in the first report of this study.\textsuperscript{77} The second report\textsuperscript{76} details a 4 year routine screening program in which 15,464 specimens were examined for raised meconium albumin levels by the BMC test-strip method and electro-immuno assay. The incidence of false positive results was 0.5% in either test and this incidence was reduced by 100% by determining the ratio of albumin: alpha-l antitrypsin in subsequent fecal specimens.

D. Fecal Trypsin

A. TAME Method

The first method to be developed for screening stool samples for trypsin was a semi-quantitative test used on specimens collected on swabs. The procedure was used in a small trial screen carried out in Auckland, New Zealand by Robinson and Elliott in 1974.\textsuperscript{77} This method was later modified and used in a mass screening programme in 1975. In the modified method\textsuperscript{72} stool specimens were collected on filter paper cards, allowed to dry and tested with the substrate p-tosyl-L-arginine methyl ester (TAME).
Robinson reports screening 6,595 newborn infants and detecting 3 cases of CF with no known cases of missed CF and a false positive rate of 0.6%.

B. BAPNA Method

A more specific method which uses the substrate benzoyl-arginine-p-nitroanilide (BAPNA) was devised by Crossley in New Zealand in 1977. During overnight incubation, the presence of trypsin enzyme causes the release of yellow p-nitroaniline from the colourless substrate. Results were evaluated by eye and all samples with a weak colour or with a different tint were read spectrophotometrically. Dry stool samples collected on filter paper cards were also used in this method.

In a screen of 4,500 newborn infants, the incidence of false positive results was 0.1%. Two infants with CF were detected in this study with no evidence of false negative results.

Crossley reports that the method was adopted by three centres in New Zealand (Auckland, Wellington and Waikato) testing 700 newborn infants per week.

Forrest adopted Crossley's method for a mass screening study in New South Wales, Australia where 20,000 infants were tested. Three CF were detected and 2 CF were missed, both with normal pancreatic function at the time of diagnosis. The false positive rate was 0.53%.
SELECTION AND DEVELOPMENT OF TESTING PROCEDURES

The testing procedure must meet several criteria to be suitable for a screening programme to detect cystic fibrosis in newborns in the province of British Columbia. Because of the number of tests to be performed on a routine basis, the test must be inexpensive and relatively simple. In addition the screening test must be sufficiently sensitive, specific, precise and accurate to avoid large numbers of false positives and negatives. Since the screening programme will eventually include samples from all the geographically dispersed populations in British Columbia, the testing procedure must meet one other criterion. It must be effective at testing "mailed-in" specimens if a central screening laboratory is incorporated into the programme or alternately it must be easily performed by a variety of personnel in all the various hospitals throughout the province.

According to reviews of all screening methods reported at the time that the initiation of a screening programme was being considered, the most promising in terms of the above mentioned criteria appeared to be analysis of meconium for increased levels of albumin. A pilot project, testing albumin in meconium from the newborns at Vancouver General Hospital (VGH), was therefore initiated in April 1975 and was in operation until June 1979. The author became involved in the meconium
pilot project in June 1977. She was responsible for the follow-up on the majority of the positives obtained with this method from the initiation of the meconium pilot screening programme to its termination.

A neonatal screening method which measured the activity of trypsin in stool samples was the next method to be evaluated by the author (although the collection of fecal specimens had been initiated prior to her investigation). The trypsin method investigated was one developed by Robinson and Elliott, and specifies the use of the synthetic substrate p-tosyl-L-arginine methyl ester (TAME).

This investigation was followed by the author's attempt to adopt the general principal of Ryley's fecal albumin: alpha-1 antitrypsin ratio to a mail in card system.

A second trypsin method, the more specific method of Crossley's which uses the substrate benzoyl-arginine-p-nitroanilide (BAPNA), was the last method to be investigated by the author. Crossley's procedure was incorporated into a pilot project at VGH in November 1977 and was in operation concurrently with the meconium screen for comparative purposes until June 1979. As in the case of the meconium screen, the author was responsible for the follow-up investigation of positive results from the Crossley trypsin screen.

Details on the investigations of these four testing procedures follow in the next four chapters. The protocol, results and discussion of the follow-up performed on infants who tested positive in the two pilot screening projects are presented in the subsequent chapter.
The Boehringer-Mannheim Corporation* (BMC) test-strip for meconium albumin was designed to detect an albumin content above 20 mg per g of dried meconium.** Since this strip was reported to be relatively easy to use, sensitive and reliable,33,88 it appeared to meet British Columbia's specific requirements.

I. TESTING PROCEDURE

A. Collection of Samples

Nurses at the Vancouver General Hospital were instructed to collect the FIRST meconium passed by each infant. If this was not possible, a second meconium was to be collected and labelled as such. The meconium specimen was placed in a styrofoam box, containing a frozen freezer-pack, immediately after collection. The specimens in the styrofoam box were transferred daily to the freezer and the freezer-pack in the box was replaced with a new frozen pack. The frozen meconium specimens were

* Boehringer - Mannheim Corporation, Mannheim, West Germany.
** as detected by a single radial immunodiffusion technique.52
transported once a week to Children's Hospital where they were stored in a freezer until analyzed.

B. BMC Methodology

Principle:

The BMC test is a simple dipstick method which incorporates the principle of ascending chromatography. The test strip is impregnated with tetrabromphenolphthalein ethyl ester as the indicator.

Procedure:

1. The meconium sample was well mixed before testing. Some samples were covered with a thick light brown gelatinous substance, or had a solid mass of the gelatinous substance attached to one end of the meconium sample. This gelatinous substance contained a higher concentration of albumin and was therefore mixed in thoroughly with the rest of the meconium specimen.

2. Using the test-strip as a spatula, a small sample of meconium was taken from the plastic container. The meconium was spread over the entire width of the lower part of the test-strip, approximately 5 to 10 mm along its length.

3. This test-strip was then placed immediately into a small plastic vial containing 3 to 5 drops of deionized water, making sure that part of the meconium layer was above water level.

4. An increased albumin concentration (more than 20 mg albumin per g meconium dry weight) produced, after 15 minutes, an intense blue colour
across the width of the strip and at least half-way along the length of the strip. This colour was not necessarily of uniform intensity but was definitely an intense blue. A positive control was tested concurrently and used for colour comparison. Faint blue colourations or tints of blue on the rims of the test-strip were regarded as negative.

When reading the tests, a check was made to ensure that the water front had advanced up the test-strip well beyond the meconium. If not, it was assumed that proper chromatographic movement had not occurred and the test was repeated.

The characteristic of the specimen was noted*, with special attention to the presence of blood.

The follow-up procedure, discussed later, was initiated on all positive results.

C. Stability Investigation

Early reports indicated that albumin in meconium was relatively stable and that the transport of non-refrigerated specimens through the post did not affect the results. On this basis, the BMC test was initially performed on meconium specimens stored temporarily at room temperature and/or refrigerator temperature prior to their transport to Children's Hospital. However some of these specimens arrived at

* After gaining some experience, it became possible to differentiate visually between meconium, transitional stool and stool specimens.
Children's Hospital with mould growth, some were too dry to test and others gave an atypical reaction with the BMC test-strip.

A stability investigation was therefore performed at Children's Hospital shortly after the program was initiated* testing known positive and negative meconium specimens and meconium specimens to which known quantities of albumin had been added. These specimens were well mixed and divided into several portions. A representative sample was stored at room temperature, 4°C and at -15°C for up to 5, 10 and 14 days.

Results indicated that the albumin was not stable at room temperature, specimens became dry and mould growth which interfered with the reaction appeared in the positive controls. Reconstitution of the dried specimens had also been attempted but this resulted in an atypical reaction. Atypical reactions also occurred occasionally with the 4°C stored specimens. More detailed studies were reported by other investigators verifying that storage at room temperature resulted in a large decrease in albumin concentration (approximately 50%); storage at 4°C resulted in an approximate decrease of 16% and storage at -15°C resulted in a decrease of less than 1%.

The protocol for collection of meconium samples at VGH was therefore changed in May of 1976. All samples were frozen immediately and transferred to Children's Hospital in the frozen state (as outlined under the procedure). This was the collection procedure in use when the author became involved in the project in June 1977.

* Prior to the author's involvement.
II. RESULTS

A. Meconium Sample Collection

As noted in Table XII on page 113, 8891 meconium samples were collected out of a possible total of 10,091. Actually, another 414 specimens were collected by the nurses at VGH but these specimens were either too dry or insufficient in quantity to test for albumin using the BMC test-strip.

Approximately 12% of the specimens tested (1083 out of 8891) lacked the green colour and mucilaginous characteristic that are normally found with the first or even the second meconium specimens. These specimens were thought to be stool specimens.

B. Sensitivity

Three children with CF were detected as having increased albumin in the meconium by the BMC test-strip. One child with CF gave a normal result with this screening test. The sensitivity of the meconium albumin test is therefore 75% (See Table II).

C. Specificity

In the meconium screen, 98.7% of the total number of infants that, to date, appear not to have CF were negative to the test. A false positive rate of 1.31% was therefore obtained, this number representing
the 116 infants with increased meconium albumin that were detected among
the non-CF groups.

<table>
<thead>
<tr>
<th>BMC Test-Strip Result</th>
<th>CF</th>
<th>Non-CF</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>3</td>
<td>116</td>
<td>119</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>8,771</td>
<td>8,772</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8,887</td>
<td>8,891</td>
</tr>
</tbody>
</table>

III. DISCUSSION

The BMC test-strip method was shown to be a reliable and simple
test. No technical difficulties were encountered during the 4 years it
was used to test approximately 9000 specimens. Results were easy to
interpret for the dark blue end-point was distinct.

The method was best performed in a central laboratory. It was
necessary to adhere strictly to the procedure with attention to details
in order to avoid false positives and false negative results. This
agrees with the guidelines set forth by the Committee on Genetics of the
American Academy of Pediatrics¹³ who report that experience with PKU
screening has revealed the necessity of a centralized and carefully
standardized testing programme to maximize both specificity and sensitivity.

Less than 1% of the total number of meconium specimens contained a solid section of lighter coloured gelatinous substance and required an extra thorough mixing as a result of this concentrated protein mass. For the majority of specimens, the testing procedure was a quick two step procedure, applicable to a large series of tests in a screening programme.

Sample Collection

The collection rate was at an acceptable level. The staff at the Vancouver General Hospital collected meconium samples from 92.2% of the newborn infant population under study. The testing rate of 81.1%, although an unfortunate decrease due to dry and insufficient quantity specimens, compares favourably with other screening centres.

Since the concentration of albumin decreases with each meconium passed, in both healthy and CF infants, it is important that the specimen tested be the first or initial meconium specimen. Testing second meconium specimens or transitional specimens (transition from meconium to stool) could lead to false negatives. The questionable meconium* rate of 12% obtained in this meconium screening programme is high. Several attempts were made to lower this percentage by asking the

* Specimens that in colour and consistency resembled stool rather than meconium specimens.
co-operation of the nursing staff but this was not successful. Most of
this communication was however in the form of memos with some telephone
calls placed to the wards. A personal visit in the form of a seminar to
the nurses explaining the importance of collecting the first meconium
specimen might have resulted in a more effective sample collection.
Interest in the seminar could possibly be stimulated by discussing CF as
a disease, the purpose of the screening programme and its effectiveness.

After the initial specimen stability problems were solved (by
storing specimens at 4°C immediately after collection and freezing the
specimen within a couple of hours) no more problems were encountered.
Because of the sample instability unless frozen however, the screen could
not be adapted to a province-wide programme that incorporated testing
facilities at a central laboratory. Previous investigators had
advised against using the BMC test-strip at bed-side. Our own experience
agrees with this. Our lack of technical difficulties with this test was
in part due to the small number of laboratory personnel (2) performing
the test and the very good communication between the two people
involved.

Sensitivity

Since a negative result was obtained with the BMC test-strip for one
of the four infants who had been diagnosed as having CF, the sensitivity
of the meconium albumin test was calculated to be 75%. This rate is
undesirably low and is a definite disadvantage of the meconium screen.
The CF child whose meconium tested negative displayed a normal pancreatic function and as a result a normal concentration of albumin was present in the meconium specimen. The BMC test-strip result was therefore not in error and the problem was a physiological one due to the overlap between the distributions of pancreatic function level of CF patients and healthy infants. This child represented one of the 10 to 15% of infants with CF who have normal or only slightly disturbed pancreatic function as discussed earlier on page 11.

Specificity

Our VGH based, BMC meconium screen specificity of 98.7% is at an acceptable level and in agreement with specificities reported by screening programmes set up in other countries,\(^{10,48,53,90}\) all of which reported a specificity of 99.1% or higher. Our slightly lower specificity may have been due to the large proportion of samples that came from the high risk newborn nursery as is evidenced by the large percentage of premature infants which gave positive meconium results (see Figure VI on page 119).

Incidence of CF in the Population Screened

The incidence of CF in the newborn population screened in Vancouver was 4 in 8,891 or 1 in 2222.
Of the 156,197 live births** in B.C. during the time period that the meconium screen was in effect (April 1975 to June 1979), 36* have been diagnosed as having C.F. as of May 1981.

Four of the 36 CF infants were part of the population screened. The remaining 32 infants lived outside of the area serviced by the Vancouver General Hospital and were therefore not part of this screening programme.

Since CF is more prevalent among Caucasians and rare among Negroes and Orientals, the incidence as determined by the meconium screen for Caucasians could be adjusted to approximately 1 in 2000, if the 9% Oriental and Negro population of Vancouver** is taken into consideration. British Columbia's population as a whole consists of approximately 2.7% Orientals and Negroes**

From literature expectations and our incidence figures, there may be about 39 C.F. patients in B.C. born between April 1975 and June 1979 who are still undiagnosed as of May 1981.

The incidence as determined by this particular screen appears to agree well with incidence figures given in the literature and quoted in Table I on Page 2. However, the statistics used for the VGH based meconium screen include referrals from other hospitals (outborn babies). These referrals alter the percentage of high risk and often premature infants present in the total population. The presence of these infants could influence the incidence of false positives and the incidence of CF.

* BC Cystic Fibrosis Foundation
** Statistics Canada, Vancouver Office
Out of the 4 CF infants in this programme one was in fact a referral patient (lowering the incidence to 1:2964).
ROBINSON AND ELLIOTT FECAL TRYPsin METHOD

An investigation into Robinson and Elliott's\textsuperscript{72} technique of measuring stool trypsin activity on dry specimens of feces from newborn children at 4 to 5 days of age was started in June, 1977 and discontinued in January, 1978.

I. TESTING PROCEDURE

A. Collection of Samples

The nurses at the VGH were instructed to collect a stool sample from each infant on the third day after birth or later.

Three pea size stool samples were placed on filter paper by the nurse as per instructions printed on the screening card (Appendix A).

The card was stamped with the baby's name, date of birth, doctor and date of sampling.

The cards were placed in a styrofoam box containing a freezerpack and transferred daily to the freezer. The stool specimens were transported once a week to Children's Hospital where they were tested.
B. Trypsin Methodology

Principle:

The Robinson and Elliott method detects tryptic activity by measuring the ability of the enzyme to release hydrogen ions from the substrate p-tosyl-arginine methyl ester (TAME) when the stool sample is incubated with this substrate, a buffer and a pH sensitive dye mixture.

Reagents and Material:

1. Sample cards

The sample cards were prepared from filter paper, Whatman #3, flow rate medium. Polyethylene film (35 um) sleeves protected the samples.

2. Buffer, pH 8.2, 0.005 moles per liter.

The buffer contained 0.354 g TRIS-HCl, 0.334 g TRIS, 2.34 g sodium chloride and 2.9 g calcium chloride dihydrate dissolved in a liter of distilled water.

3. Tame Substrate

The substrate consisted of 2.07 g p-tosyl-arginine methyl ester* dissolved in 50 ml of buffer.

* Sigma Chemical Company, St. Louis, Mo., USA.
4. Indicator Solution

The indicator mixture contained 0.2% w/v bromothymol blue in 50% ethanol and 0.2% w/v phenol red in 50% w/v ethanol in the following proportions: 5 ml of bromothymol blue solution, 5 ml of phenol red solution and 50 ml of buffer.

5. Stock Trypsin standard.*

The stock standard was prepared daily by weighing 10 mg of trypsin and dissolving it in 10 ml of buffer.

Procedure:

1. Prior to each run, a fresh mixture of indicator-buffer-substrate was prepared in the proportions of 6:2:1. The pH was adjusted to 8.2 with 0.1 M NaOH.

2. A 6 mm disc containing a representative sample of feces was punched (using a paper hole puncher) from each filter paper card into an appropriately numbered well in a disposable plastic sample tray.**

3. Discs from four control specimens were included in randomly placed positions. The control sample cards were prepared at Children's Hospital from stool specimens containing a known concentration of chymotrypsin and/or trypsin. Both normal and abnormal controls were included in each run.

---

* Sigma Chemical Company, St. Louis, Mo., USA, 16,000 units of activity per mg, 98% protein.

** Clear plastic tray, 96 Wells, Linbro Co., USA
4. Dilutions of the stock trypsin standard were made by pipetting 0.4 ml of stock trypsin standard (1 mg per ml) and of working dilutions of stock standard (1:1000, 1:1500 and 1:2000) into each of 4 wells in the tray.

5. A 0.9 ml portion of indicator-buffer-substrate mixture was pipetted into each well containing the discs and the standard solutions.

6. The tray was mixed gently with a rotating motion and floated in a water bath at 37°C.

7. When the second standard (1:1000 dilution) turned yellow the tray was removed from the water bath. At that point the colour sequence of the set of standards was: Std. #1 (stock std.) - bright yellow, #2 - yellow, #3 - orange and #4 - purple.

8. The colour of each test was recorded.

9. The test was considered to contain a normal trypsin concentration if the indicator colour converted to yellow during the incubation period. If an abnormal result was obtained, the test was repeated in duplicate on discs from the same screening card. If the stool sample smear appeared to be unusually light, the test was repeated in duplicate using 2 discs per well.

C. Investigation Into Inconsistent Results and Non-Specificity

The intentions were to request a stool specimen for quantitative chymotrypsin analyses from infants which gave a presumptive positive result (an abnormally low trypsin activity) indicating a high probability of cystic fibrosis. It was apparent immediately however that the method
was not performing to the same accuracy and precision as reported by
Robinson and Elliott.

Several problems existed:

1. Inconsistent results were obtained when both positive and
   negative controls were repeated from the same sample card. These results
   are reported on pages 39 and 40 and discussed on pages 45 and 46.

2. The reaction rate of the standards varied greatly from one run to
   another (See results on page 41).

3. The number of false positives was very high. (See results on page
   39.)

Because of this variability, the method was examined in detail.
Reagent preparation, sample preparation and storage, environmental
conditions, and non-specificity were investigated.

1. Reagent Preparation

   a) Extra care was taken to prepare the stock standard and working
      standards in order to minimize weighing and pipetting (dilution) errors.
      New trypsin was purchased and two sets of standards, one new and one old,
      were included in the run, one at the beginning and one at the end.

   b) The colour change of the standards obtained during the incubation
      period was different from that described in Robinson and Elliott's
      paper. They mention a distinct colour change from purple to yellow as
      compared with a gradual change from purple to orange, to light yellow, to
bright yellow that was obtained in our laboratory following the procedure outlined above. Several weaker concentrations of the indicator solutions were tested in order to determine whether the precipitate of one of the dyes in the mixture was responsible for the colour variation.

c) A new TRIS buffer with a concentration of 0.050 moles per liter and a pH of 8.0 was prepared. Controls and patients specimens were tested and compared with previous results using the method as outlined incorporating the new buffer.

2. Sample Preparation and Storage

Dry and wet specimens were prepared in the laboratory as follows:

Three sets of cards were prepared from 10 control stool specimens. Each specimen was spread on the filter paper card by pressing on the outside of the plastic film. One of the sets of sample cards was placed in individual envelopes and placed in the freezer immediately. The feces on the other two sets of sample cards were allowed to dry at room temperature for approximately 6 hours. The plastic film was removed in order for the feces to dry properly. Once the samples were dry, the cards were inserted into the plastic film and placed in individual envelopes. One set was stored in the freezer, one set at room temperature. Results from all three sets were compared.

3. Environmental Conditions

Although the tests were routinely carried out in an area exposed to organic solvents (using an uncovered specimen tray), a portion of the tests was repeated under controlled environmental conditions. The water
bath was placed in a fume hood and all efforts were made to avoid environmental contamination of the specimens.

A reagent blank, consisting of a clean disc of filter paper and the reagents in a test well, was included with every set of determinations in order to detect non-specific colour change due to environmental contamination.

4. Investigation Into Non-Specificity

a) Three normal control stool specimens, 3 CF stool specimens and a specimen from a patient with Schwachman Syndrome were autoclaved for 15 minutes at 18 pounds pressure. Both the original specimens and the autoclaved specimens were tested with the Robinson and Elliott method.

b) The method was carried out as outlined but without the addition of the substrate TAME on a selected number of controls: 4 CF specimens, 1 Schwachman Syndrome, and 3 normal controls.

5. Investigation of Interfering Compound

All of the control specimens and the specimens from newborn infants that had been tested with the Robinson and Elliott method, were retested with the method substituting buffer for TAME in order to determine whether the unknown interfering compound was specific for cystic fibrosis.

The fecal specimens that appeared to contain the compound were then exposed to ammonia and retested without TAME.
II. RESULTS

A. Sensitivity and Specificity

The Robinson and Elliott trypsin method was used to test stool specimens from 513 infants and resulted in 42 specimens displaying low trypsin activity. The positive rate was therefore 8.3%.

The sensitivity of the method was evaluated through the use of positive control specimens. A total of 4 specimens known to lack the trypsin enzyme or known to contain an abnormally decreased activity of trypsin were tested (3 CF and 1 Schwachman Syndrome). The number of false negatives obtained for these control specimens varied from day-to-day. Some of these abnormal controls (controls with low trypsin concentration) displayed a more rapid colour change than the normal controls. The results from these control specimens are reported in Table III.
### TABLE III. FECAL TRYPsin CONTROL SPECIMEN RESULTS
**ROBINSON AND ELLIOTT METHOD**

<table>
<thead>
<tr>
<th>Control No.</th>
<th>Diagnosis</th>
<th>Chymotrypsin Activity</th>
<th>No. of Determ.</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>132</td>
<td>CF</td>
<td>negligible</td>
<td>24</td>
<td>3Abn*, 21 N+</td>
</tr>
<tr>
<td>130</td>
<td>CF</td>
<td>below normal</td>
<td>22</td>
<td>22N</td>
</tr>
<tr>
<td>111</td>
<td>CF</td>
<td>negligible</td>
<td>28</td>
<td>16 Abn, 12 N</td>
</tr>
<tr>
<td>129</td>
<td>Schwachman</td>
<td>below normal</td>
<td>22</td>
<td>11 Abn, 11 N</td>
</tr>
<tr>
<td>112</td>
<td>healthy</td>
<td>normal</td>
<td>24</td>
<td>24N</td>
</tr>
<tr>
<td>110</td>
<td>healthy</td>
<td>normal</td>
<td>8</td>
<td>8N</td>
</tr>
</tbody>
</table>

* Pancreatic function assessment based upon quantitative fecal chymotrypsin analysis
* Abn: abnormally low level or lack of trypsin activity.
+ N: normal level of trypsin activity
B. Investigation Into Possible Causes of Inconsistent Results and Non-Specificity

1. Reagent Preparation

   a) The reaction rate of the standards continued to vary greatly from one run to another despite the use of a new bottle of trypsin and the attempts made to minimize weighing and pipetting errors when preparing the stock and working standards. The time period required to reach the stipulated colour change varied from 28 to 43 minutes. The variation in time taken for the tests to reach the end-point (on repeating the same tests in different batches) did not parallel that of the standards.
      The variation was not apparent within the same batch but was a batch-to-batch discrepancy.

   b) When the concentration of both the bromthymol blue and phenol red were reduced to half of the suggested values, the colour change obtained at the end of the incubation period was then a distinct change from purple to yellow. The screening results were not affected by this alteration.

   c) The alteration in buffer concentration and pH produced a less distinct colour change than was obtained with the original buffer concentration of 0.005 moles per liter, pH 8.2. The screening results obtained when several controls, and newborns were tested incorporating the two different buffers were not significantly different.
2. Sample Preparation and Storage

There was no significant difference in results between the 3 sets of controls: one set prepared in a manner identical to that used at the VGH resulting in wet samples; one dried at room temperature and stored in the freezer and one set dried at room temperature and stored at room temperature.

3. Environmental conditions

The results for the control specimens and newborn infants did not improve when the test was performed in a fume hood in another laboratory. The accuracy and precision of the method remained essentially the same.

The reagent blank consistently displayed inactivity.

C. Investigation Into Non-Specificity

a) Autoclaved Control Specimens

The three autoclaved normal control specimens no longer turned the indicator solution yellow within the required time period when tested with the Robinson and Elliott method. In only one of the specimens, however, did the indicator solution remain its original purple colour. The other two specimens did cause some colour change of the indicator solution (to orange) even though the trypsin should have been totally inactivated.
The 3 CF stool specimens, Controls 111, 130 and 132 did not produce a clear bright yellow test solution, within the required time period when the autoclaved specimens were tested. The colour of the indicator solution did change however from purple to orange or to pale yellow during this time period in all 3 CF autoclaved control specimen tests. (These unautoclaved specimens often gave false negative results*, producing a clear bright yellow test solution.)

The stool specimen from the patient with Schwachman syndrome (Control 129) which contained low trypsin activity, displayed total inactivity after autoclaving. The indicator solution remained the original purple colour.

b) Testing with the Robinson and Elliott Method Without TAME

All of the 5 positive controls, stool samples from CF patients known to have abnormally low pancreatic function, turned the indicator solution yellow within the critical time period when tested substituting the buffer for the substrate TAME in the Robinson and Elliott method.

In one of the CF controls (Control 130) the time taken to reach the yellow increased significantly, whereas in the other 4 CF controls the time period was approximately the same when tested with or without the addition of TAME in the test solution.

* Displayed trypsin activity when they were lacking in trypsin enzyme.
The stool samples from the patient with Schwachman syndrome patient and the 2 normal patients did not produce a yellow colour in the test solution that were missing TAME.

5. Investigation of Interfering Compound

When the 513 specimens from newborn infants and the controls were re-tested with the Robinson and Elliott method substituting the buffer for the substrate TAME, 10% of the newborn infant specimens as well as the 5 CF specimens tested earlier turned the indicator solution yellow within the required time period.

When these fecal specimens from newborn infants, that appeared to contain an interfering compound, were exposed to ammonia and re-tested without TAME, a decrease of 8% occurred in the number of positives. However, a decrease of 6% occurred also when the same specimens were repeated without exposure to ammonia.

The results for the CF stool specimens tested without TAME were the same before and after the specimens were exposed to ammonia.

III. DISCUSSION

The initial attempt at setting up a fecal trypsin CF screen was made with the Robinson and Elliott method. As described by the New Zealand authors the method appeared to be suitable for a mail-in screening programme for the province of B.C.

Although 513 stool samples from newborns at the Vancouver General Hospital were tested the project never reached the follow-up stage
because the method proved unsatisfactory from the start. There was a
great day-to-day variability and both false negatives and false positives
occurred regularly with the control samples of known low and normal fecal
trypsin levels.

A cause of the batch-to-batch variation in time taken for the
standards to reach the end-point was not detected. Although the same
type of batch-to-batch variation appeared to be present in the specimens,
the two did not parallel each other. As a result, the reaction rate of
the standards was not a reliable guide for setting the length of the
incubation time period as was suggested by Robinson and Elliott.

The alterations made to the buffer concentration did not improve the
assay. The weaker concentration of indicator solution produced a colour
change that resembled the one described by Robinson and Elliott. It was
thought likely that the precipitation of one of the dyes in the mixture
(probably bromothymol blue) was responsible for the different end-points
obtained (using the concentration specified by the method) as a fine
precipitate formed during the preparation of this solution. Use of the
weaker indicator solution solved this problem. The screening results
however remained imprecise and unreliable using the new indicator
solution. Nor did the wet sample cards received from the VGH or the
storage appear to be responsible for the imprecision. Since the
screening results obtained under strictly controlled environmental
conditions were not significantly different from the results obtained
during routine determinations, environmental contamination with acidic or
alkaline vapours do not appear responsible for the lack of precision.
The consistently inactive reagent blank confirmed this conclusion.
Because the results obtained were not reproducible and because only a small sample of newborns was tested, the specificity, presented as a false positive rate of 8.3%, is inaccurate but it does point to an unacceptably low specificity for a CF screening test.

The sensitivity as evaluated by the CF control specimens was also at an unacceptable level. The false negative results, obtained for the CF control specimens, indicated the presence of a non-specific reaction. This was confirmed by the development of the yellow end-point within the required time period when the CF control specimens were tested without the addition of the substrate TAME. The interfering compound implicated by this non-specific reaction in the absence of TAME and present in the 5 CF specimens appeared to be absent from stool samples from 3 normal controls and 1 Schwachman syndrome patient tested concurrently. The absence of the compound in the normal control specimens was probably only coincidental however for it was present in 10% of the random sample of newborns (n=513) tested later. The interfering compound was therefore not a specific characteristic of CF. Since it did appear, however, in all 5 CF specimens tested, the compound appears to be present in the stool samples of cystics in a much greater percentage than in the normal population. (The probability that it would show up in all 5 CF specimens due to chance alone when it is present in 10% of the population is $1.0 \times 10^{-5}$; essentially a negligible chance.) There is the possibility that the interfering compound is present in the stool samples from all cystics and from that standpoint it would be of interest to test other CF specimens and to identify the compound or compounds in question.
The compound (or compounds) was heat sensitive as indicated by the greatly decreased reaction rate obtained upon testing the autoclaved CF stool specimens with the R & E method. This heat sensitive compound was not present in significant amounts in the 3 normal control specimens that were autoclaved and retested and it appeared to be totally absent from the stool specimen obtained from the patient with Schwachman syndrome. Since the absence of the interfering compound (capable of producing a colour change in the absence of TAME) in the 3 normal control specimens appeared to be coincidental it is doubtful that there is any significance to its absence in the specimen from the Schwachman syndrome patient.

It was theorized that the interfering compound might be an organic acid and that the poor reproducibility might be related to the slow and varied dissolution rate of the acid(s) from the various stool samples. This theory was not substantiated. The results obtained from the testing of specimens before and after ammonia exposure were not significantly different. The decrease in number of positives that occurred when positive specimens were retested probably reflected the poor precision of the method rather than any action of ammonia on the interfering compound. Also the indication that the compound or at least one of the compounds is heat sensitive casts doubt on it being an organic acid. As a result no definite conclusions could be drawn as to the characteristic of this interfering compound.

Since the method failed to distinguish between CF patients and normals there seemed to be no purpose in continuing the investigation.
Fecal Albumin:Alpha-1 Antitrypsin Ratio

Since stool samples from CF children with pancreatic insufficiency were reported to contain a significantly higher albumin:alpha-1 antitrypsin ratio as compared with healthy children, an attempt was made to set up a screening programme that incorporated this ratio. The plan was to analyse stool samples qualitatively for albumin with a follow-up albumin:alpha-1 antitrypsin ratio determination on those specimens that displayed an increased albumin concentration.

Theoretically this ratio could be considered an index for pancreatic insufficiency and should result in a lower false positive rate than obtained with the fecal albumin test alone. To adopt the screening programme in B.C, however, methods have to be designed that would be sufficiently sensitive and specific to measure the albumin and albumin:alpha-1 antitrypsin ratio in small samples of dried feces spread on filter paper cards.

I. Testing Procedure

A. Albumin:Alpha-1 Antitrypsin Ratio and Pancreatic Insufficiency

First it was necessary to verify the relationship between albumin:alpha-1 antitrypsin ratios and pancreatic insufficiency. Albumin
and albumin:alpha-1 antitrypsin ratio were determined using an immunodiffusion plate method on stool specimens from 8 CF children with pancreatic insufficiency, 4 CF children on enzyme therapy, a child with Schwachman syndrome, 7 healthy infants with known normal pancreatic function (normal quantitative chymotrypsin concentration) and 7 random samples from healthy children assumed to have normal pancreatic function. The specimens were numbered randomly and treated as "blind controls".

The stool specimens were frozen as soon as possible after collection. None was visibly contaminated with blood. Prior to analysis a portion of the specimen was thawed and dried for 48 hours in a dessicator under vacuum.

A 10% w/v stool solution was prepared in 0.05 moles per litre TRIS acetate buffer, pH 7.3, containing 0.3 moles per liter NaCl and 0.1 moles per liter EDTA.* The mixture was mixed for 30 minutes with a Vortex mixer, and centrifuged at 3000 x g for 30 minutes to remove the insoluble material. The supernatant was then applied to each of the specific immunodiffusion plates.

a) Albumin

1. A 20 microliter portion of supernatant and 10 microlitre portion

---

* EDTA is necessary for detection of albumin by the immunodiffusion technique. A faint precipitin ring was obtained in the absence of EDTA, a distinct ring when buffer containing EDTA was used. EDTA removes possible interfering cations.
of buffer were applied in two stages to the albumin plate*, which has an assay range of 2.8 to 44.4 mg per dl.

2. The diameters of the precipitin rings were measured and the concentrations were read off a calibration curve prepared at the same time.

b) Alpha-1 Antitrypsin

1. A 20 microliter portion of supernatant was applied in two stages to an alpha-1 antitrypsin plate*, which has an assay range of 0.8 to 12.5 mg per dl.

2. The intensity of the precipitin rings was increased by treatment with 3, 4-dihydroxyphenylalanine (DOPA) solution as outlined by Madhosingh and Wood.

B. Elution of Stool Samples From Filter Paper Cards.

Dried stool samples were prepared using the 23 stool samples from CF and non-CF children (listed above) as follows:

1. A pea sized sample of stool specimen was placed on the filter paper card in the center of a marked 2.5 mm circle. Three samples from one specimen were smeared on one card.

2. The card was covered with plastic film.

* LC Partigen Immunodiffusion plates, Behring Institute, Canadian Hoechst Ltd., Montreal
3. The outside of the plastic film was pressed to spread the specimen evenly within the circle. The plastic film was removed immediately.

4. The specimen was left at room temperature for approximately 4 hours to dry and then placed in a paper envelope and stored in the freezer.

A method was devised to elute the stool samples from the filter paper cards resulting in a solution that contained approximately 10 g of feces per dl in TRIS acetate buffer containing sodium chloride and EDTA. This concentration of fecal solution, applied to the plates as outlined earlier should have resulted in precipitin rings for the albumin present in stools from CF children and for the alpha-1 antitrypsin present in all 27 stool specimens. However, neither the albumin nor the alpha-1 antitrypsin was detected on any of the immunodiffusion plates.

The extraction procedure was therefore modified to provide a more concentrated fecal solution and employed on 8 CF stool specimens known to contain an increased concentration of albumin as follows:

1. Two discs of filter paper, each containing a representative sample of specimen, were clipped into a small test tube using a standard paper hole puncher.

2. A 0.5 ml sample of buffer was added and the solution was mixed on a vortex mixer periodically for 30 minutes.

3. The fecal solution was centrifuged for 15 to 20 minutes at 3000 x g for 20 minutes.
4. The fecal solution was then placed in a dessicator under vacuum for 48 hours until completely dry.

5. The residue was dissolved in 50 microliters of distilled water resulting in a 20% w/v feces solution. (Ten discs of filter paper containing dried samples of feces were weighed individually. The average weight of feces on a filter paper disc was 5 mg. The feces from two discs was extracted into 50 microlitres of solution.)

These extracts were applied to the albumin immunodiffusion plate as specified earlier.

Visible precipitation rings were obtained for the 8 CF stool specimens and as a result the procedure was repeated on all of the 27 stool specimen cards and applied to both albumin and alpha-1 antitrypsin plates.

The resultant precipitin rings on the albumin plates were smaller than expected and no rings were detected for any of the specimens on the alpha-1 antitrypsin plates. Since it appeared that the extraction procedure was inadequate, various technical modifications were made such as altering the extraction time and mixing vigour, increasing the centrifugation speed and time. The eluants were changed substituting water for the buffer used initially and buffer for the water used in the final extraction. These various modifications were tested for their effectiveness on stool specimens to which a known amount of albumin had been added.

An attempt was also made to concentrate the final fecal solution applied to the immunodiffusion plate. The volume of distilled water used to dissolve the feces, however, could only be reduced within certain
limits. These limits were set by the necessity to have sufficient volume to wash the dried feces off the side of the test tube and to dissolve the feces so that a clear suspension and homogenous solution was obtained for application to the immunodiffusion plate.

C. Qualitative Albumin Method

Concurrently with the above investigations an attempt was made to find a suitable qualitative albumin method to be used to screen the stool samples.

Ideally, the albumin method should give a positive result for stools containing albumin above 2.0 mg per g of dry weight and a negative result for normal stool specimens which contain less than 0.1 mg per g.

Several methods were investigated testing a select number of stool specimens, controls and random specimens from healthy newborns. The methods investigated were: Lowry, Brom-cresol green dye binding method and Albustix.*

II. RESULTS

A. Relationship of Ratio to Pancreatic Insufficiency.

All 8 of the stool specimens from CF children with pancreatic

* Ames Company (Division of Miles Laboratories) Elkhart, In, USA.
deficiency produced a measurable ring on the albumin immunodiffusion plate, indicating an abnormally high concentration of albumin.

Seventeen of the remaining stool specimens (12 normal, 1 Schwachman syndrome and 4 from CF children on enzyme therapy) contained a normal concentration of albumin. These samples contained albumin levels below the sensitivity of the immunodiffusion plate indicating a concentration of less than 0.28 mg of albumin per g dry weight of feces. Two of the stool specimens from healthy newborns contained an abnormally high concentration of albumin.

Measurable precipitin rings were obtained on the alpha-1 antitrypsin plates for 13 out of the total 27 stool specimens tested. The remaining 14 specimens contained alpha-1 antitrypsin in concentrations beyond the range of the calibration curve indicating a concentration of more than 1.5 mg of alpha-1 antitrypsin per g dry weight of feces. These were re-tested using 10 microliters of supernatant or less instead of the 20 microliters specified.

The results of the albumin, alpha-1 antitrypsin, and albumin:alpha-1 antitrypsin ratio for the 27 stool specimens investigated are summarized in Table IV.

B. Elution of Stool Samples from Filter Paper Cards

Precipitin rings appeared on the albumin immunodiffusion plates for the 8 CF stool sample extracts prepared from the sample cards. The concentrations obtained for these samples were approximately 65% less than when the determination was performed on the original stool sample.
TABLE IV.  
ALBUMIN AND ANTITRYPSIN CONTENT OF FECES  
IMMUNODIFFUSION METHOD

<table>
<thead>
<tr>
<th>Spec (n)</th>
<th>No. of Spec (n)</th>
<th>Albumin (mg/g dry wt.)</th>
<th>Alpha-1 Antitrypsin (mg/g dry wt.)</th>
<th>Albumin: alpha-1 antitrypsin ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mean ± S.D. limits of values</td>
<td>mean ± S.D. limits of values</td>
<td>mean ± S.D. limits of values</td>
</tr>
<tr>
<td>CF with pancreatic insufficiency</td>
<td>8</td>
<td>3.6 ± 2.9 1.0-9.2</td>
<td>0.56 ± .26 0.30-1.02</td>
<td>5.7 ± 2.1 3.3-10.2</td>
</tr>
<tr>
<td>CF on enzyme therapy</td>
<td>4</td>
<td>0.28*</td>
<td>1.6 ± .34 1.20-2.03</td>
<td>0.23</td>
</tr>
<tr>
<td>Schwachman</td>
<td>1</td>
<td>0.28</td>
<td>0.94</td>
<td>0.29</td>
</tr>
<tr>
<td>Known normal pancreatic function</td>
<td>6</td>
<td>0.28</td>
<td>0.95 ± .62 0.31-1.82</td>
<td>0.90</td>
</tr>
<tr>
<td>Healthy children</td>
<td>6</td>
<td>0.28</td>
<td>2.2 ± 1.6 0.73-2.86</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1.6</td>
<td>3.42</td>
<td>0.49</td>
</tr>
</tbody>
</table>

* with the method outlined, the lowest concentration detectable is 0.28 mg. albumin/g. dry wt.
This poor extraction of stool constituents resulted in an inability to detect the alpha-1 antitrypsin. None of the specimen extractions resulted in visible precipitin rings indicating that the concentration of alpha-1 antitrypsin in the extracts was below 0.8 mg per dl of extract, the lowest concentration detectable on the LC Partigen immunodiffusion plates.

The percentage recovery did not increase with any of the modified extraction procedures as indicated by the lack of a significant increase in the albumin concentration detected. Although it was possible to increase the concentration of fecal extract slightly by reducing the volume of buffer used to dissolve the feces in the final step of the extraction procedure, it was not possible to concentrate the extract sufficiently to detect the alpha-1 antitrypsin on the immunodiffusion plate.

C. Qualitative Albumin Method

1. Lowry Method

The Lowry method detected protein in all of the 27 control stool specimens. A quantitative value was not obtained for these specimens for the blue colour produced was much darker in colour than the highest standard, beyond the range of photometric accuracy of the spectrophotometer and beyond the concentration range of adherence to Beer's law for the method.

It was however possible to assess the specimens qualitatively. The protein concentrations of all of the stool specimens were greater than 25
mg per g of the dry weight of feces as compared with previous result of 1.0 to 9.2 mg per g of dry wt. obtained for the CF specimens using the immunodiffusion method. The specimens that contained the highest concentration of protein as detected by the Lowry method were the stool specimens from normal patients which were previously determined to contain the lowest concentration of albumin, less than 0.28 mg per g dry weight of feces, using the immunodiffusion method.

It was not worthwhile to repeat the determinations on the stool samples using a smaller sample since the Lowry method obviously was measuring the total protein present as well as other interfering substances and not the albumin of interest.

2. Dye Binding Method

The bromcresol green dye binding method produced an unusual colour change when used to test the control stool samples. The fecal pigments appeared to mask the effect of albumin on the bromcresol green solution. The resultant colour could not be related to albumin concentration.

3. Albustix

It was also not possible to detect the increased albumin concentrations in the 8 CF stool specimens with Albustix. A 10% w/v fecal solution was required to satisfy the sensitivity of the Albustix reagent strip of 20 mg albumin per dl of solution. The protein and fecal pigments present in a fecal extract of this concentration interfered with the detection of albumin by the Albustix method.
III. DISCUSSION

Relationship of Ratio to Pancreatic Insufficiency

The albumin concentration range of 1.0 to 9.2 mg per g dry weight of feces from infants with CF agrees fairly well with that reported by Ryley (reported in the next paragraph). The alpha-1 concentrations of both CF and non-CF stool specimens appear to be slightly lower than Ryley's. The difference is however insignificant when the small sample size is taken into consideration. As a result of these lower alpha-1 antitrypsin concentrations however, the albumin:alpha-1 antitrypsin ratios for infants with CF were also slightly lower than those reported by Ryley.

Ryley reported a mean of 4.7 (+1.6) mg of albumin and a mean of 0.9 (+0.4) mg of alpha-1 antitrypsin per g of dry weight of feces from CF infants. For feces from non-CF infants Ryley reported a mean of 2.0 (+1.0) mg of alpha-1 antitrypsin per g dry weight of feces. The albumin:alpha-1 antitrypsin ratio reported by him for feces from CF infants was 6.7 (+3.6).

Most of the remaining stool specimens (17 out of 19) contained a normal concentration of albumin which according to Ryley is less than 0.1 mg of albumin per g dry weight of feces. The method as outlined did not result in precipitin rings for these specimens because the albumin concentrations of these samples were below the sensitivity of the plate. The data obtained, however, seemed sufficient for the purpose of this investigation.
The relationship between an abnormally high albumin:alpha-1 antitrypsin ratio and pancreatic insufficiency was supported by the data obtained. An abnormally high albumin:alpha-1 antitrypsin ratio, greater than 3.3, was obtained for all of the 8 CF stool specimens from children known to have pancreatic insufficiency. The remaining 19 specimens (16 non-CF children and 4 CF children on enzyme therapy) had albumin:alpha-1 antitrypsin ratios less than 1.0.

A normal albumin:alpha-1 antitrypsin ratio was obtained for stool specimens from 2 healthy children despite the increased fecal albumin concentration in both specimens. The specimens were probably more concentrated than normal causing an increase in both the albumin and the alpha-1 antitrypsin. As a result a normal albumin:alpha-1 antitrypsin ratio was obtained for these 2 specimens. It appears therefore that the albumin:alpha-1 antitrypsin ratio is a more specific measurement for pancreatic insufficiency than the measurement of albumin alone.

Elution of Stool Specimen from Filter Paper

The extraction procedure devised to detect the abnormally high concentrations of albumin in dry stool samples on discs of filter paper appeared to be adequate and suitable for use with the immunodiffusion plate. These same extracts did not contain sufficient alpha-1 antitrypsin however to be detected with the alpha-1 antitrypsin immunodiffusion plate method. The % recovery of alpha-1 antitrypsin was too low resulting in alpha-1 antitrypsin levels in the extract being below the sensitivity of the IC Partigen plates.
The analysis of dry stool specimens on filter paper cards for albumin presented another problem. Spurious results were obtained in the quantitative albumin determinations. Since feces are not sterile when passed these results were probably due to the action of bacterial proteases.

Qualitative Albumin Method

None of the methods investigated was suitable for screening the dry stool samples on filter paper cards for an abnormally high albumin concentration.

The Lowry method presented specificity problems. The falsely high results obtained for stool samples from both normal and CF patients were probably due to the measurement of all of the protein present in the sample rather than a specific albumin measurement as well as other interfering substances.

Interference from the fecal pigments appeared to be the major problem with the dye binding method, the bromcresol green method, and the Albustix reagent strips. The colour change that was to be related, in each method, to the albumin concentration was masked in these methods.

A literature search did not uncover a qualitative albumin method with the necessary specificity and sensitivity.
CROSSLEY FECAL TRYPsin METHOD

Since the fecal trypsin method of Crossley, Berryman and Elliott was also designed to test dried feces collected on filter paper cards, it seemed an obvious alternative to the unsuccessful TAME method discussed above.

I. TESTING PROCEDURE

A. Collection of Fecal Samples

   a) The collection procedure previously established at the VGH for the Robinson and Elliott TAME method was continued. The same screening cards (Appendix A) were used for collecting dried fecal samples for the Crossley method which was initiated in November, 1977.

   b) Home collections were started in September 1978. The mothers were given the filter paper card, plastic sleeve, envelope and a letter (Appendix B) at VGH on the day of discharge from the hospital asking them to collect the stool specimen at home within the first two weeks of life of the child. The specimen was to be mailed to Children's Hospital on the day of collection.
B. Trypsin Methodology

Principle:

A colourless substrate, benzoyl-arginine-p-nitroanilide (BAPNA) is employed in this method for the determination of trypsin in feces. When this substrate is hydrolyzed by trypsin, yellow p-nitroaniline is released. Samples from infants with CF, who lack trypsin, give negligible colour.

Reagents:

1. Buffer

   The buffer contained 0.1M TRIS-HCl, 0.04 moles per liter CaCl₂ and 0.08 moles per liter NaCl and had a pH of 8.2

2. BAPNA Substrate

   The substrate was prepared from 0.65 mg of pure L-isomer BAPNA* per ml of distilled water. The solution was protected from light while it was heated in hot water for a few minutes to dissolve the BAPNA. It was stable for several weeks if stored at room temperature in the dark.

* Merck, Sharpe and Dohm, Montreal, Canada.
3. Stock Trypsin Standard

The stock standard was prepared every two weeks by dissolving 1 mg of porcine trypsin*, in 1.00 ml of 0.001 M HCl. This solution was stored at 4°C. The stock standard contained 15,680 units of trypsin activity per ml of solution.

4. Working Trypsin Standards

The stock standard was diluted 1:10, 1:100 and 1:10,00 with the buffer.

Procedure:

1. A 6 mm disc containing a representative sample of feces was punched with a standard paper punch from each screening card into numbered 12 x 75 mm test tubes. Care was taken to obtain consistent sample thickness by visually selecting an appropriate area on the card.

2. A 10 microlitre portion of each working standard was pipetted into properly labelled test tubes resulting in 1.0, 0.1 and 0.01 microgram trypsin per test tube representing 15.68, 1.568 and 0.1568 units of trypsin activity* respectively.

3. Discs from control specimens, 2 normal and 2 untreated CF, were included in the set of determinations, interspersed throughout the tests at random.

* Sigma Chemical Company, St. Louis, Mo, USA 16000 Units of activity per mg., 98% protein in that particular sample. Since each bottle of purchased porcine trypsin varies slightly in trypsin activity, reference will be made to the trypsin standard in terms of weight rather than activity.
4. A blank filter paper disc was placed in a test tube for the reagent blank.

5. An equal volume of BAPNA solution was mixed with an equal volume of buffer and 0.8 ml was added to each test tube using a pipette dispenser.

6. The test tubes were placed in a dark cupboard for 15 to 20 minutes. Each tube was then vortexed briefly to loosen the plastic film from the specimen and the tubes were returned to the dark cupboard to stand overnight.

7. In the morning, between 17 to 18 hours later, the test tubes were removed from the cupboard and examined visually as follows:

   a) Pairs of test tubes were examined against a white background for a clear fluorescent yellow colour that compares in intensity to the 1.0 ug working standard (approximately 15 units of activity, depending on the particular lot of porcine trypsin). Samples that were equal to or greater than the yellow colour of the standard were considered to contain a normal concentration of trypsin.

   b) Solutions that were paler in colour than the 1.0 microgram standard or solutions with a different tint due to fecal background colour were placed aside for spectrophotometric examination along with the reagent blank and standards.

8. Distilled water, 2.2 ml, was added to the reagent blank, standards and each of the abnormally coloured samples. The tubes were placed in the dark for 15 to 20 minutes.

9. Samples that appeared turbid or cloudy were centrifuged for 5 minutes to remove the fecal debris.
10. Spectrophotometric absorbance readings at two wavelengths were taken of the reagent blank, standards and samples:
   
a) The reagent blank was read against distilled water at a wavelength of 410 nm. The instrument was then zeroed on the reagent blank and the standards and tests were read.

   b) The standards and tests were read against distilled water at 460 nm.

11. The two absorbance readings for each solution were used to calculate a net absorbance according to the following formula suggested by Crossley (see also explanation next page):

\[
\text{Abs}_{410} - 2 \times \text{Abs}_{460} = \text{net absorbance}
\]

If a net absorbance value of 0.300 or less was obtained, the assay was repeated, in triplicate, on the same specimen. If the fecal-smear on the test card appeared to be unusually light, the test was repeated in triplicate using 2 discs per test tube.

A specimen was considered to have an abnormally low trypsin concentration if the average of the triplicate net absorbance values was less than 0.300. The follow-up procedure, discussed later, was then initiated.

C. Investigation to Establish Procedure

Background Interference

1. Fecal Pigments

Soluble pigments present in stool samples add to the colour of the
test solutions. These pigments, especially bile pigments, could contribute significantly to the absorbance reading at 410 nm and may lead to false negative results. Crossley's method attempts to correct these falsely elevated $\text{Abs}_{410}$ readings by reading the solution at 460 nm as well as 410 nm. According to Crossley, the absorbance reading obtained at 460 nm is due mostly to fecal pigments with p-nitroaniline contributing a small amount. To compensate for these pigments, the reading at 460 nm is multiplied by 2 and subtracted from the 410 nm reading.

Since the average mean ratio of $\frac{\text{Abs}_{410}}{\text{Abs}_{460}}$ obtained by Crossley was 1.38 (S.D. of 0.20) the background interference would appear to be overcompensated for in some tests.

Because of a concern that this overcompensation may lead to false positives an investigation was made into this background interference correction procedure. Various fecal samples, representing a wide range of background colours and a wide range of trypsin concentrations were studied. These samples, 48 patient samples and 17 control samples, were tested with the Crossley method with and without the addition of the substrate, BAPNA.

2. Test Banks

Since fecal pigments and turbidity appear to interfere with obtaining a reliable absorbance reading for the produced p-nitroaniline, a correction blank for each test, or a TEST BLANK, was considered. These blanks, each containing a sample disc and 0.8 ml of buffer, were
incubated overnight with their respective test solutions; diluted with 2.2 ml of distilled water and read spectrophotometrically.

Both the tests and the test blanks were read only at 410 nm. At 410 nm the absorbance of the test represents the yellow p-nitroaniline produced by the trypsin enzyme and also any fecal pigment and turbidity that may be present. The absorbance of the test blank would be due to only the fecal pigment and turbidity related to that particular fecal sample. The subtraction of the Test Blank Abs\textsuperscript{410} from the Test Abs\textsuperscript{410} would therefore correct for the presence of any fecal pigment and turbidity. The 460 nm reading was therefore no longer required.

Several test blanks (2 or 3) were prepared for each test in order to check into the reproducibility of the absorbance readings of the blanks.

3. Turbidity

After the 17 to 18 hr. incubation period, the tests were evaluated by eye and 2.2 ml of distilled water was added to those that needed to be read spectrophotometrically. The force of the water stream mixed the solution so that an even distribution of colour resulted. The stool particles themselves however were also floating in the mixture and these interfered with the spectrophotometric reading. The tubes were therefore allowed to stand for 15 to 20 minutes, during which time most of the debris settled to the bottom of the tube leaving a clear solution.

A few tests, approximately 1%, remained turbid and these were centrifuged to clear the solution.
Doubling the Substrate Concentration

Crossley performed the first assay using 0.25 mg L-BAPNA per test. Re-assaying was done in duplicate using 0.25 mg and 0.50 mg L-BAPNA per test. She reports that by re-analyzing the positive samples with the more concentrated solution of BAPNA the frequency of positive results was reduced from 0.4 to 0.1%.

This concept was evaluated:

a) Thirty samples that gave net absorbance values of 0.300 or less on all four assays (positives had been re-assayed in triplicate) were re-tested using 0.25 mg BAPNA and 0.50 mg BAPNA. The results of the 0.50 mg BAPNA were compared to the 0.25 mg BAPNA substrate results.

b) Working standards of various concentrations of trypsin were tested with the Crossley method using 0.25 and 0.50 mg L-BAPNA per test in order to investigate the kinetics of the enzyme reaction.

Validity of Visual Evaluation of Yellow Intensity

In the method, as outlined earlier on page 64 under Procedure, spectrophotometric readings are to be taken only on those tests that appear less intense in yellow colour than the 1.0 microgram standard (representing approximately 15 units of trypsin activity) or that appear to have an unusual colour tint when examined by eye. This visual evaluation of the yellow intensity of the tests was recommended by Crossley.
In order to establish the validity of this visual evaluation, all of the tests were evaluated both visually and spectrophotometrically during this pilot project. Detailed comparisons were made of the visual and spectrophotometric readings on two sets of samples (approximately 500 samples per set) at different times, to establish correlation between the visual evaluation, the 410 nm absorbance reading and the final result. The time periods chosen were: one at the beginning of the project (May, 1978) and one when a new technologist was performing the test (August, 1978).

II. RESULTS

A. Investigation to Establish Procedure

Background Interference

1. Fecal Pigments

Results from the investigation into a representative sample of fecal specimens with a wide variety of background pigments and a wide range of trypsin concentrations are listed in Table V. The absorbance readings at wavelengths 410 and 460 nm are given for the samples tested with and without the substrate BAPNA.

The absorbance readings at 410 nm for the tests performed without BAPNA (Column b) represent the readings for the fecal background interference at that wavelength. The absorbance readings at 410 nm for the tests performed with BAPNA represent the absorbance due to the fecal
TABLE V. FECAL PIGMENT BACKGROUND INTERFERENCE, CROSSLEY METHOD

| SAMPLE DESCRIPTION* | WITH BAPNA absorbance | WITHOUT BAPNA absorbance | | |
| | Colour of Test Solution | 410nm | 460nm | Net a | Colour of Test Solution | 410nm | 460nm | Ratio b/c | % Error |
| Standard: | | | | | | | | |
| 1.0 microgram | YELLOW | 1.55 | .072 | 1.487° | colourless | 0 | 0 | - | - |

| Stool Samples: | | | | | | | | |
| brown, normal | YELLOW | 1.675 | 0.164 | 1.347+ | pale yellow | 0.106 | 0.066 | 1.61 | -3 |
| dark brown | YELLOW | 1.756 | 0.132 | 1.492 | pale yellow | 0.141 | 0.081 | 1.74 | -1 |
| greyish brown | hint of yellow | 0.032 | 0.007 | 0.018 | hint of yellow | 0.014 | 0.008 | 1.75 | -8 |
| shiny, dark brown | brownish yellow | 1.400 | 0.450 | 0.500 | brownish yellow | 0.540 | 0.320 | 1.69 | -21 |
| light brown | pale | 0.150 | 0.012 | 0.126 | colourless | 0.005 | 0.003 | 1.67 | -2 |
| greenish brown | yellow | 1.621 | 0.116 | 1.389 | hint of yellow | 0.037 | 0.015 | 2.47 | +2 |
| green | greenish yellow | 1.636 | 0.108 | 1.420 | light green | 0.050 | 0.029 | 1.72 | -1 |
| green | YELLOW | 1.630 | 0.097 | 1.436 | hint of yellow | 0.027 | 0.020 | 1.35 | -1 |
| shiny, greenish brown | hint of yellow | 0.052 | 0.012 | 0.028 | hint of yellow | 0.020 | 0.011 | 1.82 | -6 |
| greenish yellow | greenish yellow | 2.078 | 0.395 | 1.288 | marked green | 0.465 | 0.302 | 1.54 | -10 |
| greenish yellow | YELLOW | 1.662 | 0.094 | 1.474 | hint of yellow | 0.042 | 0.020 | 2.10 | 0 |
| yellow | YELLOW | 1.595 | 0.087 | 1.421 | hint of yellow | 0.024 | 0.020 | 1.20 | -1 |
| yellowish brown | YELLOW | 1.676 | 0.138 | 1.400 | light yellow | 0.109 | 0.079 | 1.38 | -3 |

In the Crossley trypsin method, p-nitroaniline is measured at 410nm and 2Abs_460 is subtracted from the Abs_410 to compensate for fecal background interference on the assumption that the pigments absorb twice as much at 410nm as at 460 nm. The % error caused by this assumption, instead of using the actual ratio (b/c), is reported in the last column.

* visual appearance of stool on filter paper screening cards.
° average readings of 5 determinations for the standard
+ average readings of a minimum of 3 determinations of the stool samples.
background interference and the p-nitroaniline produced as a result of
the enzyme degradation of BAPNA.

Crossley claims that the 460 nm readings in the tests performed with
BAPNA (Column a), when multiplied by 2, provides an estimate of the fecal
background interference at 410 nm present under normal conditions of the
test. As a result, Table V includes a column that reports the % error
caused by using this factor of 2 in the fecal pigment correction for all
stool samples even though there is considerable variation in pigment
content among the samples. (See Appendix C for the calculation of the %
error).

In order to calculate the % error it was necessary to compare the
absorbance readings of the fecal pigments at 410 nm (Column b) to the
absorbance of the fecal pigments at a wavelength of 460 nm (Column C),
both in the absence of p-nitroanaline. The ratio $\frac{Abs_{410}}{Abs_{460}}$ for a
selection of fecal pigments is included in Table V, Column b/c. This
table includes only a few results of the 65 specimens tested. The ratio
varied from 0.92 to 2.47 with an average of 1.48 and an S.D. of 0.385 for
the 65 specimens tested with and without BAPNA. Four of the specimens
had an $\frac{Abs_{410}}{Abs_{460}}$ ratio over 2.0, ranging from 2.09 to 2.47.

2. Test Blanks

Absorbance readings from various pigmented fecal samples, which
produced a relatively high fecal background interference, and their
respective test blanks are represented in Table VI (Samples no. 1 to 4).
The table also includes the absorbance readings for both test and test
blanks for several control specimens (Samples No. 5 to 8).
TABLE VI.

TESTS AND THEIR CORRESPONDING TEST BLANKS IN THE CROSSLEY METHOD

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Colour of Test</th>
<th>Solution</th>
<th>Absorbance at 410 nm</th>
<th>Test Blanks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Test</td>
<td>i</td>
<td>ii</td>
</tr>
<tr>
<td>1</td>
<td>clearly YELLOW</td>
<td>2.131</td>
<td>.290</td>
<td>.303</td>
</tr>
<tr>
<td>2</td>
<td>YELLOW</td>
<td>2.138</td>
<td>.170</td>
<td>.185</td>
</tr>
<tr>
<td>3</td>
<td>YELLOW</td>
<td>2.238</td>
<td>.356</td>
<td>.365</td>
</tr>
<tr>
<td>4</td>
<td>orange-yellow</td>
<td>0.087</td>
<td>.072</td>
<td>.043</td>
</tr>
<tr>
<td>5</td>
<td>greenish YELLOW</td>
<td>2.243</td>
<td>.022</td>
<td>.013</td>
</tr>
<tr>
<td>6</td>
<td>clearly YELLOW</td>
<td>1.640</td>
<td>.059</td>
<td>.041</td>
</tr>
<tr>
<td>7</td>
<td>yellow</td>
<td>0.358</td>
<td>.016</td>
<td>.017</td>
</tr>
<tr>
<td>8</td>
<td>hint of yellow</td>
<td>0.053</td>
<td>.022</td>
<td>.015</td>
</tr>
</tbody>
</table>
3. Turbidity

Centrifugation cleared the test solutions in most situations. In those few tests where the test solution was still turbid after centrifugation, re-assay was necessary. If the turbidity was due to a heavy fecal smear on the screening card, the sample was re-assayed using a smaller stool sample.

Temperature affected the turbidity of the test solutions. This was noticed after incubating tests at room temperature overnight for the usual 17 to 18 hours in the middle of the summer. The nights were very warm and this increase in temperature caused an increase in the number of turbid tests that did not clear when centrifuged. This problem may have been one of bacterial growth and was avoided by incubating the tests in an environment where the temperature remained fairly constant and close to normal room temperature (21°C).

Doubling the Substrate Concentration

The results from a random sample of 10, out of the 30 samples tested in duplicate with 0.25 mg and 0.50 mg L-BAPNA per test are listed in Table VII.

One of the samples in Table VII, no. 8, shows a decreased net absorbance value for the 0.50 mg L-BAPNA test result as compared with the 0.25 mg L-BAPNA test result. There were a total of 4 samples in the 30 retested that gave a similar decreased net absorbance value when the analysis was repeated using the more concentrated substrate solution. The decrease ranged from 5.9% to 55% with a mean of 30%.
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Net Absorbance Values*</th>
<th>% Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.25 mg</td>
<td>0.50 mg</td>
</tr>
<tr>
<td>4</td>
<td>.248</td>
<td>.689</td>
</tr>
<tr>
<td>8</td>
<td>.213</td>
<td>.201</td>
</tr>
<tr>
<td>15</td>
<td>.107</td>
<td>.129</td>
</tr>
<tr>
<td>16</td>
<td>.250</td>
<td>.785</td>
</tr>
<tr>
<td>18</td>
<td>.244</td>
<td>.491</td>
</tr>
<tr>
<td>20</td>
<td>.136</td>
<td>.167</td>
</tr>
<tr>
<td>23</td>
<td>.285</td>
<td>.464</td>
</tr>
<tr>
<td>24</td>
<td>.118</td>
<td>.331</td>
</tr>
<tr>
<td>26</td>
<td>.212</td>
<td>.317</td>
</tr>
<tr>
<td>28</td>
<td>.183</td>
<td>.192</td>
</tr>
</tbody>
</table>

* Samples that resulted in net absorbance values less than 0.300 when initially tested with the Crossley method as outlined.
+ Results given represent the mean value of duplicate testing.
Results obtained when working standards of various concentrations of trypsin were tested with the Crossley method using a 0.25 mg and 0.50 mg L-BAPNA per test are plotted in Figure I.

Validity of Visual Evaluation of Yellow Intensity

The data from the study in which two sets of spectrophotometric readings were compared to visual evaluations were essentially the same. As a result only one set of data is presented. The second detailed study (August, 1978) is presented in Table VIII.

In the detailed study the visual evaluation of the tests involved differentiating between a clear bright yellow as produced by the 1.0 µg standard, representing approximately 15 units of trypsin activity (recorded as "YELLOW" in Table VIII, indicating that the test colour was considered to be equal to or greater than the 1.0 µg standard), "LIGHT" yellow when the intensity was less than that of the standard but still an obvious yellow, "PALE" yellow when only a hint of the colour was present, "COLORLESS" and "TINTED". The latter referred to the presence of either a brownish or greenish fecal pigment or other abnormal tint.

B. Sensitivity and Specificity

The fecal trypsin screen using Crossley's method did not detect any CF children (Tables IX and X). The sensitivity of this method has therefore not been established to date.
FIGURE I.

TRYPsin ACTIVITY WITH TWO DIFFERENT BAPNA SUBSTRATE CONCENTRATIONS

The broken line represents the relationship between absorbance and trypsin concentration using the Crossley method with 0.25 mg of L-BAPNA per test. The solid line was determined using 0.50 mg of L-BAPNA per test. Various concentrations of standards were used following the method as outlined under the procedure.
TABLE VIII.

COMPARISON OF VISUAL WITH SPECTROPHOTOMETRIC EVALUATIONS
OF 522 TESTS : CROSSLEY METHOD

<table>
<thead>
<tr>
<th>COLOUR</th>
<th>Visual Evaluation</th>
<th>Spectrophotometric Readings</th>
<th>SPECTROPHOTOMETRIC READINGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>Abs. at 410nm</td>
</tr>
<tr>
<td>TESTS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Std. 1.0 ug</td>
<td>9</td>
<td>100</td>
<td>1.390-1.605</td>
</tr>
<tr>
<td>Yellow</td>
<td>375</td>
<td>71.8</td>
<td>1.390-2.078</td>
</tr>
<tr>
<td>Light yellow</td>
<td>59</td>
<td>11.3</td>
<td>0.365-1.099</td>
</tr>
<tr>
<td>Pale yellow</td>
<td>22</td>
<td>4.2</td>
<td>0.354</td>
</tr>
<tr>
<td>Colourless</td>
<td>3</td>
<td>0.6</td>
<td>0.84-0.87</td>
</tr>
<tr>
<td>Tinted</td>
<td>68</td>
<td>13.0</td>
<td>0.315-1.894</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.183-0.637</td>
</tr>
</tbody>
</table>

The Crossley method for fecal trypsin is designed so that only those tests which appear to be a lighter yellow than the standard or have a different tint are read spectrophotometrically. If the net absorbance is less than 0.300, the specimen is considered to have an abnormally low trypsin concentration (35/522=6.7% positive on first assay in this small sample).
### TABLE IX. FECAL TRypsIN SCREENING TEST FOR CF: HOSPITAL COLLECTED SPECIMENS

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Crossley BAPNA Test Result</th>
<th>CF</th>
<th>Non-CF</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>0</td>
<td>160</td>
<td></td>
<td>160</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>3,050</td>
<td></td>
<td>3,050</td>
</tr>
</tbody>
</table>

### TABLE X. FECAL TRYPsin SCREENING TEST FOR CF: HOME COLLECTED SPECIMENS

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Crossley BAPNA Test Result</th>
<th>CF</th>
<th>Non-CF</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>0</td>
<td>30</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>845</td>
<td></td>
<td>845</td>
</tr>
</tbody>
</table>

The specificity of the fecal trypsin screen was calculated to be 95.0% for the hospital (VGH) collected specimens (Table IX) and 96.6% for the specimens mailed in from home (Table X). The false positive rates were 5.0 and 3.4% respectively and the chi-square test indicates no significant difference between these two rates (calculation in Appendix G).
C. Age of Infant When Hospital Specimen Was Collected

A large proportion of mothers and their infants were discharged from the hospital on the 4th day after birth and as a consequence of this the nurses ran into difficulties with obtaining a stool specimen from the baby on day 4.

Approximately 49% of the specimens received were from infants that were less than 3 days old when the specimen was collected. Of the CF screen "positive" population 78% were less than 3 days old. There were however only 39% under 3 days of age in the group that tested negative with the CF screen. This is a difference of 39%; a highly significant difference indicating that there is a relationship between the positive results and the high proportion of infants under 3 days of age (Appendix H).

D. Hospital and Home Collected Specimens From the Same Infant

During a 10 month time period, 692 infants were tested by the fecal trypsin screening method on both VGH and home collected specimens. One infant displayed low fecal trypsin activity in both specimens. Further follow-up however indicated that these results were both false positives for the child did not appear to have CF.

The false positive rates for these results from tests performed on the same infants were 5.1% for the VGH collected specimens and 2.9% for the specimens mailed in from home (Table XI).
TABLE XI. FECAL TRYPsin SCREENING TEST FOR CF: HOSPITAL AND HOME COLLECTED SPECIMENS ON THE SAME INFANT

<table>
<thead>
<tr>
<th></th>
<th>VGH</th>
<th>HOME</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>1</td>
<td>34</td>
</tr>
<tr>
<td>-</td>
<td>21</td>
<td>636</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>670</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>657</td>
</tr>
<tr>
<td></td>
<td></td>
<td>692</td>
</tr>
</tbody>
</table>

Results of a McNemar test for the significance of changes (from positive to negative and vice versa between VGH and home collected specimens) indicates no significant difference between the two collection methods as far as specificity is concerned (calculation in Appendix I).

E. Comparison of Hospital and Home Collected Specimens

The net absorbance values of a representative sample of VGH collected specimens were compared with representative sample of home collected specimens covering the same time period. The distributions of the net absorbance readings are displayed in Figure II. An application of the Chi-square test indicated that there was a significant difference between the two populations of absorbance values and the means were also shown to be significantly different (Appendix J).
FIGURE II.

COMPARISON OF NET ABSORBANCE VALUES
FROM FIRST ASSAY OF HOSPITAL AND
HOME COLLECTED SPECIMENS

Net absorbance: Hospital collection

Net absorbance: Home collection

- 485 -

n 485
\bar{x} 1.068
S.D. 0.385
St. Err. 0.0175

- 410 -

n 410
\bar{x} 0.941
S.D. 0.377
St. Err. 0.0174
The VGH group represents 215 positive results obtained on the first assay which decreases to 160 positive results when the sample is retested in triplicate. This is a decrease of 25.6%. The home group decreases 47.4% from 57 positives in the first assay to 30 positives after re-testing.

Four possible reasons for the lower net absorbance values in the home collected specimens are: loss of enzyme activity in the stool sample due to the mailing process, increase in fecal background interference in stool sample collected from older child at home, uneven spread of specimen and/or thinner spread of specimen on home screening card. These four possible reasons for the significantly lower net absorbance values in the home collected specimens were investigated.

1. Comparison of Freezer to Room Temperature Stored and Mailed-in Specimens

   a) Control specimens (6) and newborn specimens (14) were stored at room temperature and in the freezer for varying time periods from 3 to 7 days and analyzed by the Crossley method.

   Of the 20 specimens, 12 displayed a decrease in enzyme activity after the storage at room temperature. The Sign test was applied and indicated that there was a 25.1% probability that the decrease in results on the room temperature stored specimens was due to random error alone (Section 1, Appendix K).

   b) As a check on the stability of the trypsin activity in the mail, 25 specimen cards were prepared (controls and healthy newborn infants specimens) and mailed to Children's Hospital from various areas in
Greater Vancouver. Trypsin determinations using Crossley's screening method were performed before and after mailing.

A decrease in trypsin activity was displayed by 14 out of the 25 "mailed-in" cards. The sign test was applied to the results and indicated that there was a 27.0% probability that the decrease could be due to random error alone (Section 2, Appendix K).

c) Since a decrease in trypsin activity was obtained in both the room temperature stored specimens and the mailed-in specimens (also at room temperature), the data of these two groups were combined and the Sign test was applied. There was a 14.6% probability that the decrease in room temperature stored specimens was due to random error alone (Section 3, Appendix K).

2. Comparison of Fecal Pigment Interference in Hospital and Home Collected Specimens

It was postulated that the lowered net absorbance values from the home collected specimens could be partially due to the presence of a larger concentration of fecal pigment compared to the VGH collected specimens. This increased amount could lead to lower net absorbance values when 2 Abs\textsubscript{460} is subtracted from the Abs\textsubscript{410} (overcompensation). To investigate this possibility the absorbance values at 460 nm from a representative sample of VGH collected specimens and home collected specimens were compared. The results from each of these samples are presented in Figure III. These bar graphs visually indicate that the home Abs\textsubscript{460} values appear to be smaller than the VGH Abs\textsubscript{460} values. Statistically it was shown that the home Abs\textsubscript{460}
FIGURE III.

COMPARISON OF ABSORBANCE VALUES AT 460 NM FROM HOSPITAL AND HOME COLLECTED SPECIMENS

Absorbance at 460 nm: Hospital collection

Absorbance at 460 nm: Home collection

n 682
\bar{x} 0.992
S.D. 0.0635
Std. Err. 0.0024

n 633
\bar{x} 0.0877
S.D. 0.0699
Std. Err. 0.0024
values had a significantly lower mean and that the distribution had
shifted to the left. The difference between the means, 0.0115, is more
than 3 standard deviations greater than 0.* That this shift is highly
significant was verified by a Chi-Square test (Appendix L).

3. Precision of Repeated Analysis on the Same Screening Card

Poor preparation of screening cards at home with uneven
thickness of the stool sample could possibly lead to wider dispersion of
results compared to the VGH collected results. This would also result in
poor precision of the four determinations performed on each of the first
assay positive specimens (i.e. poor precision when re-testing).

The standard deviations of these four determinations on the
first assay positives of the home collected specimens were compared with
the VGH collected specimens. Because there were indications that the
S.D. varies with the absorbance range, only those specimens that gave a
net absorbance value between 0.250 and 0.300 were used for the
comparison. Results from 37 VGH samples (4 determinations for each
sample) were compared with 10 sets of results from home collected samples
and no significant difference is S.D.'s was detected (Appendix M) between
the two groups.

Visual examination of the screening cards prepared at home also
indicated that the cards prepared at home by the mothers appeared to be
equal in consistency of spread to those prepared by the nurses at VGH.

* By the central limit theorem, the S.D. of the difference between
the means is 0.00297^2 + 0.00245^2 = 0.00371
4. Thinner Spread on Home Collected Cards

There are two possible reasons to suspect that the quantity of fecal sample on the screening cards from home may be less than from VGH.

a) A large proportion of the total number of stool samples from VGH were transitional stool samples (transition from meconium to stool) which are fairly dense samples. The stool samples passed by the slightly older infant at home tend to be more liquid, and would therefore coat the screening card in a thinner layer.

b) Mothers might be a little more reluctant than the nurse to spread a pea size sample on the screening card.

The different distribution of home results could therefore be due to less sample on a majority of the screening cards prepared at home. Visual examination of the home collected screening cards indicated that although the thickness of the stool sample did not warrant the use of 2 discs per test, the majority of them did appear to be slightly decreased in thickness compared to the VGH cards.

III. DISCUSSION

The second method to be investigated for incorporation into a fecal trypsin CF screen, the Crossley et al method, proved to be reliable and became part of a pilot project at VGH after a thorough investigation of some of the problem areas and questionable steps in the procedure.
Investigation to Establish Procedure

1. Background Interference Correction Procedure

a) Fecal Pigments

Crossley's background correction procedure, where $2\text{Abs}_{460}$ is subtracted from $\text{Abs}_{410}$ is based on two of her findings:

i) the endogenous absorbance at 410 nm was never more than twice that at 460 nm.

ii) p-nitroaniline had an absorbance at 460 nm about 10% of that at 410 nm.

Our results varied slightly from hers. The absorbance readings of p-nitroaniline are dependent upon the instrument and the chemical purity. The p-nitroaniline used in our laboratory gave an average absorbance reading at 460 nm of approximately 4.4% of that at 410 nm. This means that when the correction procedure is used that in addition to subtracting the fecal pigment absorbance some of the absorbance due to the p-nitroaniline is also being subtracted (i.e. $2 \times 4.4\% \text{Abs}_{410}$) biasing the net absorbance. This additional subtraction alters the net absorbance by a constant percentage however and is therefore acceptable since it does not affect the ranking of the results. All it means is that approximately 91% of the yellow colour due to the p-nitroaniline is estimated in all of the tests.

Most, but not all, of the ratios of $\text{Abs}_{410}/\text{Abs}_{460}$ of the various fecal pigments from 65 newborn stool samples studied in our laboratory were lower than the factor of 2 employed in the correction procedure. Presumably using a factor of 2 should therefore correct for a very high
percentage of pigments but not all. If false negatives are to be avoided altogether however it would appear that a larger factor (e.g. 2.5) should be used. This larger factor would however increase the probability of false positives in samples with a lower $\frac{\text{Abs}_{410}}{\text{Abs}_{460}}$ ratio due to its particular fecal pigment if this is combined with a trypsin activity level at a low normal level.

In the representative sample given in Table V on page 70, 11 of the 13 samples had a ratio of $\frac{\text{Abs}_{410}}{\text{Abs}_{460}}$ less than 2. The percentage error reported for these 11 specimens indicate that the use of the factor 2.0, instead of the lower factor determined for those particular pigments, resulted in overcompensation. In all 11 samples the % error is on the negative side; i.e. the calculated net absorbances after the fecal pigment correction were lower than they should have been.

The degree of error of the net absorbance value obtained using Crossley's correction procedure is dependent on several factors:

i. the larger the deviation between 2.0 and the actual ratio, the larger the % error.

ii. the greater the concentration of fecal pigment the larger the error because the

$$\% \text{ error} = 1 - \frac{2.0}{\text{true ratio}} \times \text{concentration of fecal pigment.}$$

iii. the % error is greater in those stool specimens with low trypsin concentration because the proportion of fecal pigment to concentration of $p$-nitroaniline is increased. (i.e. the fecal pigment absorbance is a larger fraction of the total absorbance at 410 nm)
The effects of the above mentioned factors are evident in the % error results reported in Table V.

All but two of the errors obtained in that representative sample of 13 tests were within a reasonable level, especially since the method is not a quantitative one. The concern is that the % error does not cause a low normal to drop below the cut-off point and become a false positive. A larger error such as the -21% error obtained in the worst case is however possible and could potentially lead to false positive results in a specimen with a borderline trypsin activity level.

In the total number of pigmented samples tested with and without BAPNA (65), an error of 10% or more due to overcompensation occurred in 5 samples (7.7% of the samples). Since only those test solutions which have an unusual tint or appear to be less intense in yellow colour than the 1.0 ug standard (approximately 15 units of trypsin activity) when examined by eye are read spectrophotometrically, the correction factor will be used on only approximately 30% of the total number of specimens tested. Overcompensation is however only a possibility in those test solutions which contain a considerable amount of pigment and these are present in approximately 15% of the total number of tests (Table VIII, page 77). A rough estimate of the potential number of false positives resulting from the use of this correction procedure is, therefore, 7.7% of 15% = 1.2% of the total number tested. Only those stool specimens which display low normal levels of trypsin are in danger of becoming false positives as a result of this overcompensation error.

Approximately 50% of the specimens evaluated visually as "tinted" had low absorbance readings at 410 nm. A conservative estimate, therefore, of
the percentage of tests that could potentially result in a false positive as a consequence of the error in the pigment correction procedure is 0.6%. This is an acceptable level for a CF screen.

The investigation into the fecal pigment correction procedure was performed on the hospital collected specimens. The home collected specimens appear to contain a smaller number of strongly pigmented stool specimens. As a result, less than a 0.6% false positive rate, due to the correction procedure error, is expected for the home collected specimens.

b. Test Blanks and Turbidity

The use of a Test Blank for each test could theoretically eliminate the error due to endogenous absorbances, which contribute to the test absorbance at 410 nm. The fecal pigment and turbidity may contribute significantly. The concept of incorporating a Test Blank into the procedure was however discarded for two reasons:

i. The Test Blank absorbance readings reported in Table VI on page 72, appear to be fairly imprecise as a result of the semi-qualitative sampling technique used. This method of correcting can only be accurate if the Test Blank disc contains the same amount of feces as the Test disc. Two Blanks prepared concurrently gave imprecise results due to the variability of the thickness of feces on the screening card discs. This method was therefore not an improvement with respect to % error to Crossley's correction procedure.

ii. The introduction of a test blank contradicts one of the earlier requirements for a screening test procedure: that the procedure should be simple and require very little time to set up.
Although a test blank is necessary only for those tests that need to be read spectrophotometrically (30% of the total number tested), the blank would need to be prepared for all of the tests since there is no way of predicting which tests would or would not have to be read on the spectrophotometer ahead of time.

Alternatively, to set up a test blank later would mean the test would have to be repeated alongside of it for the two need to be incubated under the same conditions and for the same length of time.

Since it is possible to eliminate the turbidity in most test solutions by temperature control during the incubation period and by allowing the test solutions to stand for 15 to 20 minutes prior to the spectrophotometric reading a Test Blank is no longer necessary to solve the turbidity problem. For the few solutions that remained turbid despite adherance to the above mentioned precautions centrifugation cleared the solution. Since very few test solutions needed centrifugation (less than 1%) this increased the workload a negligible amount.

It is important to clear the solutions prior to the spectrophotometric reading because turbidity could theoretically result in false positives (again only on specimens with low normal trypsin levels) using the correction procedure outlined. An error occurs with turbid solutions when twice the absorbance at 460 nm is subtracted from the absorbance at 410 nm because the scattering of light at 410 nm and at 460 nm due to insoluble particles is essentially equal.
2. Doubling the Substrate Concentration

The investigation into the procedure of using a substrate with twice the concentration for re-assaying positive tests indicated that it was not a valid procedure to employ even though Crossley reports that it reduced her positive rate by 3 percentage points.

The results from the 30 samples tested with both 0.25 mg and 0.50 mg L-BAPNA displayed a general increase in net-absorbance values with the more concentrated substrate. Technical errors appear to be the only explanation for the 5 samples that gave a decreased net absorbance value when re-assayed. Because of this discrepancy a rank test was applied to the absorbance values obtained for both assays and this statistical test indicated that a significant increase in values was obtained when the tests were re-assayed with the 0.50 mg BAPNA (see Appendix 0, Section 1).

The % difference between the mean of the 0.25 mg BAPNA results and the 0.50 mg BAPNA results was calculated for each sample and a linear regression analysis performed. No significant correlation was found between % increase and the absorbance reading (see Appendix 0, Section 2).

The results obtained can be explained in terms of the enzyme kinetics of the reaction involved. Since higher absorbance values were obtained when the tests were re-assayed with the more concentrated substrate, the enzyme reaction could not have been in zero order kinetics. This is not surprising since the reaction is allowed to proceed for 17 to 18 hours. The substrate concentration will most likely not remain in excess for most of the samples during this time period. However, it appears as if the substrate may have been in excess for the
lower trypsin concentrations since a linear relationship between absorbance and trypsin concentration was obtained for the low concentrations of standards (Figure I, page 76). This relationship appears to hold up to the 0.7 unit trypsin standard using 0.25 mg BAPNA and the 1.4 unit trypsin standard using 0.50 mg BAPNA.

With the 0.25 mg BAPNA, the substrate therefore appears to be in excess for tests with net absorbance values under 0.300. This absorbance value varies considerably from run to run as a result of wavelength error* and variation in incubation time and temperature. (e.g. ± 0.100 for the 1.0 g standard).

A safe estimate would be that the linear relationship between trypsin concentration and absorbance holds for those specimens that gave a net absorbance reading up to at least 0.200. This infers zero order kinetics and as a consequence of this, the reaction rate is independent of substrate concentration. Doubling the substrate concentration would therefore not significantly increase the amount of p-nitroaniline formed for these specimens. This would also explain why Crossley found that the net absorbances of 7 C.F. samples were not significantly increased. Presumably the trypsin concentrations of these C.F. specimens were sufficiently low that the enzyme reaction was essentially in zero order.

The test solutions with higher concentrations of trypsin, however, would be affected by the increase in BAPNA. These specimens would have the reaction in first order for a large portion of the incubation period

* Wavelength error occurs because the wavelength chosen to read the p-nitroaniline, 410 nm, falls on a steep slope of the spectral absorbance curve of p-nitroaniline with its wavelength maximum at 385 nm.
and the increased substrate would therefore cause an increased production of p-nitroaniline. This would result in a reduction in the number of positives since tests with net absorbance values close to 0.300 would now read considerably higher.

This significantly increased production of p-nitroaniline will occur however with all specimens that give a net absorbance value of approximately 0.200 or higher when re-assayed with the double substrate concentration. C.F. specimens with low residual trypsin may also convert from a positive to a negative. It is therefore not advisable to repeat the assay using double substrate. It essentially amounts to the same thing as lowering the cut-off point for the re-assay results. This cut-off point was established at 0.300 on the basis of 0.25 mg BAPNA per test to avoid false negatives and cannot be used with a 0.50 mg BAPNA per test.

A more logical procedure to eliminate a screening error as a result of poor precision is to repeat the positive samples in triplicate and base a final decision on the average of the 4 net absorbance values although this would increase the cost and time of screening.

3. Validity of Visual Evaluation of Yellow Intensity

Since it is difficult to judge colour intensity by eye and since this judgement is dependent upon background colours and individual colour perception an investigation into the consistency of the eye evaluation procedure suggested by Crossley seemed necessary.
A comparison of the visual evaluation and the spectrophotometric readings of 522 random samples indicates that the eye evaluation is reliable and can be incorporated into the screening procedure as a time and labour saving measure.

Approximately 70% of the samples tested appeared visually to be equal to or greater in intensity than the 1.0 μg standard (representing approximately 15 units of trypsin activity). These tests would therefore be considered normal and would not be carried through the procedure any further. Almost 1/3rd of those thought to be equal to the standard in colour visually were actually less intense when measured spectrophotometrically. Individual differences would alter this proportion. One technologist, for instance, became quite expert at evaluating the yellow intensity. All the tests evaluated by her as YELLOW fell within a fairly narrow absorbance range of 1.0 to 2.0 at 410 nm while the absorbance range obtained for YELLOW by another technologist reached as low as 0.561. The technique of the first technologist was therefore adopted and all tests were evaluated comparing a maximum of 4 tests with the 1.0 μg standard against a white background.

The error that was made on 30% of the visual evaluations by falsely rating them YELLOW would not have resulted in false negative screening results if they had only been examined visually because a fairly large safety margin exists. Not until the 410 nm absorbance drops to 0.331 in the LIGHT yellow group did the net absorbance drop to a value below 0.300 giving a positive result. Out of the 59 LIGHT yellow tests only 2 fell in this category.
If the initial eye evaluation had been relied on for the 522 tests in the study presented in Table VIII and only those tests that were not evaluated as YELLOW had been read, then the 35 tests that gave net absorbance below 0.300 would all have been read spectrophotometrically. The eye evaluation screening procedure would therefore have been successful in detecting all of the positives in that particular random sample. This was also the situation in the other random sample of approximately 500 tests studied in detail.

Further evidence to verify the validity of the initial eye evaluation selection procedure was made by comparing the eye evaluation to the absorbance readings of all the positive tests obtained in this fecal CF screen pilot project. On the first assay, 272 tests out of the total 4081 specimen cards tested had a net absorbance of less than 0.3 and were therefore presumptive positives. Three of these tests were considered to be YELLOW when evaluated visually. These 3 would therefore not have been read on the spectrophotometer and would have been considered normal leading to false negatives results.

The three discrepancies were due to errors that were detected when the specimens were re-assayed. In one of the tests the visual evaluation did not agree with the absorbance readings simply because the absorbance readings were in error. When the test was repeated in duplicate the visual and spectrophotometric readings agreed.

The other two discrepancies were due to incorrect visual evaluations. In both, the moderately high concentration of yellow p-nitroaniline noted did not agree with net absorbance values of around 0.2. When the tests were repeated, it was noticed that both test
solutions had an unusual tint and that the latter had not been noticed on the first assay. These discrepancies occurred within two weeks after the procedure was initiated. It drew attention to the importance of visually examining the test solutions for background pigments. These pigments interfere with the visual evaluation of the yellow intensity and if present the test must be read spectrophotometrically.

Sensitivity and Specificity

Since none of the infants screened by the fecal trypsin screen have been diagnosed as having CF, the sensitivity of the method has not been established to date. The method did however consistently detect the abnormal controls prepared with stool specimens from known CF infants that displayed pancreatic insufficiency.

The specificities of the hospital and home collected specimens were essentially the same with 95.0 and 96.6% respectively. The two screening populations were therefore evaluated as one unit.

The false positive rate on the first assay for trypsin on the dry stool specimens was 6.7% which was lowered to 4.7% when the test was repeated on the same sample. The specificity of the Crossley method on both VGH and home collected specimens combined was 95.3%.

A false positive rate of 4.7% is too high from both a financial and ethical standpoint. Follow-up problems resulting from such a high false positive rate are discussed on page 128.
Sample Collection

The collection rate of fecal specimens at the Vancouver General Hospital was 68.7% which was much lower than that of the mecononium CF screen. The stool specimen collection rate is also low compared with the sample collection of the PKU screen which is operating in the province of B.C. and has a collection rate of approximately 95%.

If the CF screen is to be effective, the 68.7% rate must be increased. A possible reason for the low collection rate could have been the concurrently running meconium screen. It may have drawn attention away and de-emphasized the importance of the fecal screen since both were screening for CF.

There is no doubt that the high percentage (49%) of under 3 day old infants present in the VGH population tested increased the false positive rate. Trypsin activity increases significantly within the first few days of life, as indicated by the values in ug per g reported by Mullinger: day 1 - 73, day 2 - 135, day 3 - 332 and day 4 - 291 μg per g.

After day 3 the trypsin activity reaches a fairly stable level. The CF screen should therefore be performed on stool specimens from infants that are at least 4 days old. Since many infants are discharged from the hospital by the 4th day, it is necessary to collect the stool specimen to be screened at home.

The home collection rate was lower, at an unacceptable level of 45.6%. No data are available to determine the cause of this poor compliance rate. It is assumed that a high percentage (if not all) of mothers received the request letter and the collection card when
discharged. However the mothers may have been influenced slightly by the attitude of the VGH personnel towards yet another CF screen (the third on the same child!). There was some indication that a few physicians, when asked by the parents about the screen, responded with a lack of enthusiasm and discouraged the stool collection. As the high false positive rate became apparent to the physicians with time the credibility of the screen became questionable.

Hospital and Home Collected Specimens

The high false positive rate of the hospital collected specimens (5.0%) was due mostly to the young age of the infant at time of collection and to a small degree to the error produced by the fecal pigment correction procedure.

A significant decrease in the false positive rate was expected with the home collected specimens since the latter would be from children over 4 days old. The decrease was however not obtained. Although all but 1 of the VGH collected positive results became negative when tested on the same patients' home collected specimens, "new" positive results appeared. All but 1 of these positives obtained were from infants who had previously tested negative.

The one patient that tested positive on both the VGH and home collected specimens was not diagnosed as CF but was a normal healthy child. It is probably coincidental that the two positive results were obtained on the same patient.
The false positive rate obtained for the home collected specimens (3.4%) does not compare favourably to Crossley's rate of 0.1%* obtained on 2500 babies tested in New Zealand and Forrest's rate of 0.02% obtained on 20,000 babies tested in Australia.

The false positive results obtained in this study on home collected specimens were due to factors other than age.\(^{31}\)

One difference apparent between the two populations, of hospital and home collected specimens was the shift to the left of the distribution of net absorbance values from the home collected specimens. These values were significantly lower than those of the VGH collected specimens even though they were collected during the same time period and analyzed at the same time with the same set of reagents. The decrease in net absorbance values appears to be due to a thinner spread of stool sample on the screening card prepared at home as compared to the VGH prepared card. Visual examination indicated that the home cards did have a lighter spread of stool. Stool samples from the slightly older infants at home tend to be more liquid than the hospital samples, especially if the child is breast fed.

Other evidence that the low net absorbance values were caused by a thinner spread of stool is that the mean of the absorbances at 410 nm and the mean of the absorbances at 460 nm decreased by the same amount between hospital and home specimens (see Figures II and III). The only ways in which the equal reduction could occur are:

* Crossley's false positive rate is 0.4% after the initial assay, reducing to 0.1% after re-assay of the same specimen using double the substrate concentration.
i) that the home collected stool smears are thinner

ii) that there is a loss of trypsin activity as well as a reduction in the concentration of fecal pigment in the home collected specimens and that the two reductions are approximately equal. (See Appendix P)

Since the probability of the latter is extremely small it is concluded that the stool smears are thinner.

The decrease in net absorbance values did not appear to be caused by a loss of enzyme activity as a result of mailing in the stool samples. There was no significant difference between the results obtained on 25 samples before and after mailing. Nor was a significant difference obtained when 20 samples were stored at room temperature for 3 to 7 days and compared to the same sample stored in the freezer.

Two other contributing factors were considered: that the lowered net absorbance values in the home collected specimens were due to a larger concentration of fecal pigment leading to overcompensation or that the home prepared stool specimen was spread unevenly in thickness on the cards leading to wider dispersion of results. Neither factor was found to have any significant effect on the difference between the net absorbance values of the hospital and home collected specimens.

Establishment of a New Cut-Off Point

Since there were indications that on the average less p-nitroaniline was measured in the test solutions of the home collected specimens, a lower cut-off point could be applied to the CF screen which makes use of this collection procedure.
Crossley's cut-off point which was initially adopted by us for the hospital collected specimens and by Forrest in Australia was an absorbance reading for the p-nitroaniline produced of 0.300 (i.e. a net absorbance lower than 0.300 was considered positive). The mean net absorbance values for the population of VGH collected specimens was 1.068 with an S.D. of 0.385. The cut-off point was therefore approximately 2 standard deviations below the mean \([1.068 - 2(0.385)=0.298]\). If the same criterion is used to establish the cut-off point for the home collected specimens, the new cut-off point will be \(0.94-2(0.377)=0.187\) which could be rounded off to 0.200 for convenience.

Using the new cut-off point, the positive rate for the home collected specimens is lowered from 3.7 to 1.9%. This is a significantly lower rate than the false positive rate obtained for the VGH collected specimens but is still not ideal and much higher than the rates obtained in Australia and New Zealand.

The concern with lowering the cut-off point is that while it increases the specificity to 98.1% it will likely also decrease the sensitivity. Since CF patients were not detected with this pilot project it is not possible to determine the effects on the latter to see if the sensitivity remains at an acceptable level.

It is possible however to determine the effect the new cut-off point would have on the positive control specimens. All of the CF control specimens would have tested positive with the new cut-off point of 0.200 including those that were mailed to the laboratory as part of the investigation into the effects of mailing. The control cards would have to be prepared spreading the stools of known trypsin concentration the
same thickness as the home collected cards. However, this would only serve to lower the net absorbance values further and would not have affected the final outcome of the results.

A false positive rate of 1.9% and a predicted incidence of 1:2000 of CF implies by Bayes theorem a less than 2.6% probability that a child with a positive result does in fact have CF (Appendix Q). A lower false positive rate is therefore desired in order to increase the physicians' confidence in the test and to reduce unnecessary follow-up costs - both financial and emotional.
FOLLOW-UP

I. PROTOCOL

A follow-up was initiated on all presumptive positives for cystic fibrosis from two testing procedures: either an increased meconium albumin concentration as detected by the BMC test on specimens collected at the Vancouver General Hospital from April 1975 until June 1979, or a decreased fecal trypsin concentration as detected by Crossley's BAPNA procedure which ran concurrently from November 1977 until June 1979.

The two tests were used in parallel. An infant was considered to be positive for CF if the child tested positive in either one of the tests and negative if he tested negative in both tests.

The follow-up protocol is illustrated in Figure IV.

The letters to the physicians and the clinical questionnaire used in the follow-up procedure were prepared by the CF screening programme committee consisting of Dr. A.G.F. Davidson, Dr. D.A. Applegarath and Dr. L.T.K. Wong. These letters were initially prepared for the meconium screen and modified later to include the stool trypsin screen when both were in operation at the VGH simultaneously.
FIGURE IV. CF SCREENING AND FOLLOW-UP PROTOCOL FLOW-CHART

Newborns at VGH

- Meconium Samples
  - BMC Strip-test for ALBUMIN
    - Normal Results (negative for CF)
    - Abnormal Results (positive for CF)
      - Result recorded
      - No further action taken
  - Stool Sample Cards
    - Crossley BAPNA test for TRYPsin
      - Abnormal Results
        - Test repeated in triplicate on the same sample
        - Normal
        - Abnormal

Infants at Home

- Stool Sample Cards
  - Crossley BAPNA test for TRYPsin
    - Abnormal Results
      - Test repeated in triplicate on the same sample
      - Normal
      - Abnormal

Letter mailed to physician:
- request for stool sample
  - Quantitative Stool assay for Chymotrypsin
    - Normal
    - Abnormal
      - Physician informed
      - Physician contacted for clinical evaluation
        - Well, no signs of CF
        - Questionable Symptoms
          - Well, no signs of CF
            - Request for a 2nd stool sample
              - Chymotrypsin
                - abn.
                - normal
              - Normal
              - Abnormal
                - CF likely
                - CF not detected
                  - Child referred to CLINIC
            - no further action taken
    - Abnormal
      - CF likely
      - CF not detected
        - Child referred to CLINIC
      - no further action taken

SCREENING

DIAGNOSTIC FOLLOW-UP
A. Request for Stool Sample for Chymotrypsin

The process started with a letter mailed to the pediatrician or family physician informing him of the abnormal result and asking for a stool specimen for quantitative chymotrypsin analysis. Instructions for sample collection were enclosed. The physician was also asked in this letter to contact the CF Assessment Clinic at Children's Hospital immediately if the child had respiratory or gastrointestinal symptoms suggestive of CF.

Response to this initial letter was very poor. An additional paragraph was therefore inserted offering to contact the parents directly to arrange the stool collection. This increased the response rate considerably. (The letter and stool collection instructions are enclosed in Appendix D.)

1. Lack of Response

If a stool specimen was not received from the child within two weeks after the letter was mailed, the doctor was contacted by phone to determine whether his office had received the letter and whether the child in question was his patient. A verbal offer to contact the parents directly was made and this offer was usually accepted. An inquiry was made at the same time as to the child's clinical picture.

In several cases the doctor's office neglected to notify the CF assessment clinic laboratory at Children's Hospital that the family had
moved or that we had the incorrect physician's name. This information was not obtained until the follow-up telephone call.

If the name of the family physician was not known, the parent's telephone number was obtained from Medical Records at VGH and the mother was contacted by telephone for this information. This was accomplished without mentioning the CF screening programme or the positive result. In a few cases where the family lived outside the Greater Vancouver area or the family did not have a telephone, a letter was written to obtain the doctor's name.

2. Direct Contact with Parents:

The majority of physicians preferred that we contacted the parents directly. The sample collection and delivery instructions were given by telephone to one of the parents and the written instructions were mailed to their home on the same day in order to remove any doubts or confusion in the parent's mind.

If the stool specimen was not received within two weeks the parents were contacted by telephone again and a friendly reminder was given. This was repeated as many times as necessary until the specimen was received.

B. Quantitative Chymotrypsin Analysis

The follow-up stool specimens were analyzed for chymotrypsin by the method of Smith et al. Chymotrypsin was chosen because it was reported
to show a clear correlation with pancreatic exocrine insufficiency and its secretion was shown to be affected earlier and more severely than trypsin secretion. 

1. Abnormally Low Chymotrypsin Activity

Since a spot stool specimen rather than a 24 or 72 hour stool specimen was analyzed, there was a low probability that the abnormally low result was due to normal day to day variation. This possibility had to be kept in mind, even though the correlation between spot stool specimen results and 24 hour specimen results had been reported as very good. As a result, a re-assay on a new sample was requested by telephone if an abnormally low chymotrypsin value was obtained. The physician was also asked for a clinical evaluation of the child at this point in time. If it was not possible to contact the physician directly, the request for a second stool specimen was placed with the office and the physician was asked to fill out a clinical questionnaire (Appendix E) which was promptly mailed out to him on the same day.

If the clinical picture was suggestive of CF, the second stool chymotrypsin analysis was omitted and a sweat electrolyte determination was ordered immediately.

If the second stool specimen also displayed low chymotrypsin activity, sweat electrolytes were determined.
2. Normal Chymotrypsin Activity

If the quantitative chymotrypsin analysis performed on the follow-up stool specimen was normal the result was mailed to the physician, along with the clinical questionnaire. If the child's clinical picture was not suggestive of CF no further action was taken.

In one instance, the clinical picture was questionable and the physician was encouraged to order a repeat chymotrypsin analysis on a second stool specimen and/or to order a sweat electrolyte determination despite the initial normal chymotrypsin.

C. Sweat Electrolytes

The detection of abnormally high levels of sodium and chloride in the sweat was used as the principal diagnostic criterion of CF. Since sweat collection can be a problem in the first few weeks of life, this test was usually performed when the infant was at least two weeks of age. The pilocarpine iontophoresis sweat test was performed at various laboratories and the results were mailed to the CF Screening Programme at Children's Hospital.

1. Abnormal Sweat Electrolyte Concentrations

If the concentration of the sweat electrolytes was abnormally high the analysis was repeated for confirmation. If the results from the
repeat analysis were also abnormally high then CF was likely and the child was referred to the CF clinic for further assessment and confirmation.

2. Normal Sweat Electrolyte Concentrations

If the sweat electrolyte concentrations were normal CF was considered unlikely. It would be up to the physician to decide on the basis of the sweat electrolyte result, the clinical picture of the child and the family history whether or not CF was still a possibility and whether a repeat sweat analysis was warranted.

D. Clinical Follow-Up Only

In some cases follow-up specimens for further laboratory analysis were not obtained. With a few infants, this was because the physician felt that the request for the stool specimen or sweat test would result in severe anxiety in the mother and that it was more beneficial to the child and the family to just monitor the child carefully from a clinical standpoint.

In these situations, the physician was contacted by telephone when the child was one year old and again at two years of age in order to determine if the child was "well with no clinical symptoms of CF". Letters were mailed out to these physicians (Appendix F) along with the clinical questionnaire. Stool specimens were sometimes obtained at this later date when the physician felt that the mother was more able to cope
with the situation or the clinical picture warranted a laboratory follow-up.

In a few cases, the child and family moved out of the greater Vancouver area. In these situations the family was contacted by mail to obtain the name of the new family physician. The new physician was notified of the CF screening result and asked to fill out a clinical questionnaire. For a few infants we received a stool sample for chymotrypsin analysis through the mail or the physician mailed us a sweat electrolyte result but for most of these infants a clinical follow-up was all that was obtained.

When possible the physician was re-contacted by telephone for a second clinical evaluation when the child was 2 years of age.

E. Review of the Files at Medical Records

Another type of follow-up that was performed for the purpose of evaluating these two pilot projects was a comprehensive review of each baby's hospital records filed in the Medical Records Department at VGH. The baby's condition at birth was noted and a search was made for possible reasons for false positive results.

In the initial follow-up procedure the secretarial staff mailed a letter informing the physician of a positive result, and requesting a follow-up. From March to June, 1979 funds were available to hire a part-time person specifically for the task of notifying the physicians of positive results and retrieving follow-up specimens by mail and telephone. The author became involved with the follow-up procedure in
May 1979 and assumed full responsibility in July 1979. She personally completed the follow-up on 57% of the meconium screen positives and 70% of the fecal trypsin screen positives.

The review of the files at the Medical Records Department at the VGH was performed by the author on all positives received in both screening programmes.

II. RESULTS

The number of babies tested and the number of presumptive positives for CF in each of the two pilot projects, the meconium albumin and the Crossley fecal trypsin method, are listed in Table XII.

A. Meconium Albumin CF Screen

1. Diagnostic Follow-Up

As indicated in Table XII, during a 4 year period 119 positives (increased albumin concentration) were detected out of a total of 8891 infants tested. From this number of positives, 12 infants died shortly after birth before further follow-up could be performed. The autopsy reports summarized in Table XIII, implied that these infants did not have cystic fibrosis. It was therefore necessary to follow up 107 infants for diagnostic studies and this follow-up was completed on 103 of these infants (96%). Follow-up data were not obtained on 4 of the infants: 3 of the children were adopted and one family moved shortly after the birth
<table>
<thead>
<tr>
<th>Specimen</th>
<th>Time Period</th>
<th>Population*</th>
<th>Number Tested</th>
<th>Abnormal Finding</th>
<th>Testing Procedure</th>
<th>Number &quot;Positive&quot;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meconium</td>
<td>April/75 - June/79</td>
<td>10,091</td>
<td>8,891</td>
<td>Albumin Increase</td>
<td>BMC Strip-Test</td>
<td>119</td>
</tr>
<tr>
<td>Stool: VGH</td>
<td>August/77 - June/79</td>
<td>4,675</td>
<td>3,210</td>
<td>Trypsin Decrease</td>
<td>Crossley BAPNA</td>
<td>160</td>
</tr>
<tr>
<td>Home+</td>
<td>September/78 - June/79</td>
<td>1,920</td>
<td>875</td>
<td>Trypsin Decrease</td>
<td>Crossley BAPNA</td>
<td>30</td>
</tr>
</tbody>
</table>

* Number of babies born at Vancouver General Hospital (VGH) plus number of babies transferred to VGH, shortly after birth, from other hospitals.

+ The mothers were given a CF screening card when discharged from VGH and were asked to mail in a stool specimen from home.
<table>
<thead>
<tr>
<th>NEWBORN NO.</th>
<th>FINAL PATHOLOGICAL DIAGNOSIS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pulmonary interstitial emphysema, bilateral pneumothoraces subependymal plate hemorrhage with intraventricular hemorrhage</td>
</tr>
<tr>
<td>2</td>
<td>Meningitis, bleeding diathesis with intraventricular and pulmonary hemorrhage, kernicterus</td>
</tr>
<tr>
<td>3</td>
<td>Sudden unexpected death syndrome, cyanosis of digits and mucous membranes, cerebral edema with congested cortical veins, petechiae on pleura and capsule of thymus</td>
</tr>
<tr>
<td>4</td>
<td>Respiratory distress (cause not determined), perforation of small intestine</td>
</tr>
<tr>
<td>5</td>
<td>Aspiration pneumonia, cotton fibre embolus in lung, post natal growth retardation, suggestion of necrotizing enterocolitis</td>
</tr>
<tr>
<td>6</td>
<td>Postmeningitic hydrocephalus, postoperative right ventriculoperitoneal shunt, subdural hematoma; secondary to shunt</td>
</tr>
<tr>
<td>7</td>
<td>Intrauterine growth retardation, necrotizing enteropathy, pulmonary edema</td>
</tr>
<tr>
<td>8</td>
<td>Severe perinatal asphyxia, necrotizing enterocolitis with pneumatosis cystoides intestinalis and perforation, pulmonary congestion with intra-alveolar hemorrhage</td>
</tr>
<tr>
<td>9</td>
<td>Perinatal asphyxia, bronchopulmonary dysplasia, necrotizing enteropathy, stomach, ileum and proximal colon, acute myocardial necrosis, hepatic steatosis and cholestasis</td>
</tr>
<tr>
<td>10</td>
<td>Perinatal asphyxia, necrotizing enterocolitis, sepsis, subependymal plate hemorrhage with intraventricular extension</td>
</tr>
<tr>
<td>11</td>
<td>Down's syndrome, exaggerated atelectasis in both lungs, massive bilateral subependymal cell plate hemorrhages</td>
</tr>
<tr>
<td>12</td>
<td>No autopsy performed. Cause of death given as respiratory failure.</td>
</tr>
</tbody>
</table>

* Common final diagnosis for all infants except No. 3 is preterm delivery and neonatal death. No. 3, also a premature birth, died at 6 months of age.
of the child leaving no forwarding address and no other leads as to their new location.

Of the 103 remaining children, a stool was obtained for chymotrypsin for 67 infants. Stool chymotrypsin was normal in 45 of these. There were no clinical suspicions of CF on the part of the attending physician and no further action was taken. Twelve infants had abnormal chymotrypsin results. These were repeated in 4, with normal results. In 2 there was no clinical problem, and no further action was taken. While another 2 had sweat assays performed, with normal results. The remaining 8 infants who had a repeat chymotrypsin analysis, again had abnormally low results and sweat electrolyte determinations were performed on all 8. Normal sweat results were obtained on 5 of these infants with no clinical suspicion of CF on the part of the attending physician. Abnormal results were obtained on the remaining 3 infants and these infants were diagnosed as having cystic fibrosis.

Fourteen of the infants who had an initial abnormal meconium screen had sweat analysis performed without first assaying a stool specimen for chymotrypsin. All 14 had normal results.

It was not possible to obtain a biochemical follow-up on 32 infants. In these cases, either the physician did not feel that any biochemical follow-up was warranted or the parents refused to co-operate. Follow-up in the form of contacting the physician for a clinical report has continued for at least 2 years on these infants and none of them have shown any need for further study.

The results of the 103 follow-ups, categorized according to the initial test or evaluation that was performed, are presented in Figure V.
FIGURE V. RESULTS OF THE DIAGNOSTIC FOLLOW-UP ON POSITIVES FROM THE MÉCONIUM SCREEN

I. INITIAL CHYMOTRYPSIN

Normal 45 → no further action taken
Abnormal 12

repeat chymotrypsin

Normal 4 → no further action taken
Abnormal 8

confirmation, sweat analysis

Sweat analysis

Normal 2
Abnormal 3 → CF Assessment Clinic

CF 3

II. INITIAL SWEAT ANALYSIS

Normal 14 → no further action taken

III. CLINICAL EVALUATION

Child "well, with no symptoms of CF, over 2 years old" 32

TOTAL 103
2. Medical Records Investigation

Since 3 out of the 119 positives were true positives, 116 false positives were investigated by reviewing the hospital files on these infants for possible causes of an increased albumin in the meconium.

a) Prematurity

A high incidence of prematurity was found among the infants that gave false positive results (81%). As noted from the autopsy reports in Table XIII, all of the 12 infants that died shortly after birth were born prematurely. Of the remaining 104 infants that survived, 82 had also been born prematurely.

b) G.I. Disturbances and Necrotizing Enterocolitis

Other similarities noted were that 26 infants out of the 104 had G.I. disturbances, 18 of these being necrotizing enterocolitis. Among the autopsy reports another 5 listed necrotizing enterocolitis as part of the final pathological diagnosis, making a total of 23 necrotizing enterocolitis cases out of the 116 false positives (20%).

Other G.I. disturbances among the 82 premature live infants were 3 abdominal distention, 2 ileal atresia, 1 intra-abdominal abscess, 1 meconium gastritis and 1 Hirschsprung's disease.

c) Diseases Related to Prematurity

The other diseases that occurred in several of the live infants were ones that were related to their prematurity such as hyaline membrane
disease (22), and respiratory distress syndrome (9). These same diseases were also listed in the autopsy reports.

The results from the Medical Records investigation on the 104 children that gave false positives are presented in Figure VI.

B. Fecal Trypsin CF Screen

1. Diagnostic Follow-Up

Testing the VGH and home collected stool screening cards with Crossley's trypsin method, 190* positives were detected out of a total number of 4085 samples tested (Table XII). These represented 3393 infants since 692 infants were tested by both methods.

Diagnostic follow-up was completed on 175 infants (92%). The follow-up results, a discussion of which follows, are summarized in the accompanying flow chart (Figure VII) where they are categorized according to the initial biochemical test or evaluation that was performed. Of the 175 children followed up, stool samples for chymotrypsin analysis were obtained from 152 of which 149 gave a completely normal result and no further action was taken. Three of the 152 chymotrypsin analysis had borderline low chymotrypsin levels. Repeat chymotrypsin determinations were performed on 2 of these infants both of which were normal and on the third infant a sweat test was done which was also normal. Of the

* 190 positives were detected but these represented 189 infants since one child tested positive on both hospital and home collected specimens.
FIGURE VI. RESULTS FROM AN INVESTIGATION FOR POSSIBLE CAUSES OF INCREASED MECONIUM ALBUMIN IN NON-CF INFANTS

I. PREMATURE INFANTS

82

G.I. Disturbances 24
- necrotizing enterocolitis 16
  - abdominal distention 3
  - ileal atresia 2
  - intra-abdominal abscess 1
  - meconium gastritis 1
  - Hirschsprung's disease 1

- Ingestion of maternal blood 2
- Intrauterine growth retardation 1
- Hemolytic disease of the newborn 3
- Hyaline membrane disease 22
- Respiratory distress syndrome 9
- Down's syndrome 1
- Meconium aspiration 1
- No special problems listed 19

II. FULL TERM INFANTS

22

- Necrotizing Enterocolitis 2
- Meconium Aspiration 2
- Healthy 18
FIGURE VII. RESULTS OF THE DIAGNOSTIC FOLLOW-UP ON
POSITIVES FROM THE FECAL
TRYPSIN SCREEN

I. INITIAL CHYMOTRYPSIN

- Normal 149 → no further action taken
- Abnormal 3 → Sweat Analysis
  - repeat chymotrypsin
    - Normal 1
    - Normal 2

II. INITIAL SWEAT ANALYSIS

- Normal 6

III. CLINICAL EVALUATION

- Child "well with no symptoms of CF, over 2 years of age" 14
- Child "well with no symptoms of CF, under 2 years of age" 3

TOTAL 175
remaining 23 who were followed up 6 had normal sweat tests and 17 were only evaluated clinically. The latter 17 were felt by the family physician or pediatrician to have no clinical symptoms which would suggest cystic fibrosis and the physicians concerned felt that they did not warrant further follow-up.

Twelve patients were lost to biochemical follow-up despite the request of the physician that a chymotrypsin analysis be performed. The lack of further laboratory testing on these 12 infants was due to various reasons: 3 mothers were unwilling to co-operate, 6 mothers promised to deliver stool specimens for chymotrypsin analysis but had not done so at the time this thesis was being prepared and 1 pediatrician would not give his permission for direct communication with the parents of three infants, insisting on arranging for the stool specimen collection himself. These specimens have not been received to date. These infants are however completely healthy and will be re-evaluated periodically.

Three infants are lost to follow-up of any sort, for their families had moved shortly after the birth of these infants and left no forwarding address and we were unsuccessful in our attempts to locate them.

2. Medical Records Investigation

All of the positives from the VGH and home collected specimens tested for trypsin appear to be false positives. A search of the Medical Records files on these infants did not provide explanations for the false positives. Most of the infants were full term healthy babies at birth
(91.5%). Exceptions were: 7 premature birth, 1 Down's syndrome, 4 intrauterine growth retardation, 2 meconium aspiration, 2 hemolytic disease of the newborn and 1 infant that was small for gestational age.

Serum bilirubin determinations had been performed on many of the infants involved in the CF screening programme during their hospital stay. Thirty-nine percent of the VGH collected CF screening positives and 40% of the home collected CF screening positives had a serum bilirubin above the normal level for their age at birth.

A regression test indicated no linear relationship between the absorbance at 460 nm obtained with the Crossley method and the serum bilirubin concentration.

III. Discussion

A. Meconium Albumin CF Screen

Diagnostic Follow-Ups

The follow-up which was completed on 96% of the infants who obtained a positive result with the meconium screen appears on the surface to have been thorough.

In actual practice however the follow-up procedure was inadequate. In most cases too much time elapsed between the positive result, locating the patient and obtaining a sample for the initiation of the follow-up investigation. Although 93% of the positives were detected before May 1979, only 43% had been followed up by that date. The delay was
Initially due mostly to inadequate manpower but a further delay resulted when all efforts were directed at setting up the new fecal trypsin screen. This delay in follow-up resulted in several problems:

1. Theoretically either true (CF diagnosed) or false positive results from the meconium screen could have been of benefit to the physician because false positive results were associated with other problems such as G.I. disturbances (discussed later). Further biochemical testing would have benefited the patient under both of these circumstances if it had resulted in early diagnosis which then enabled the infant to obtain early medical treatment. The value of further diagnostic testing was questionable however when it was delayed to the point where treatment for the symptoms was well underway or completed when the physician received the results.

2. The initial contact with a report of the positive result was usually prompt but further follow-up was often delayed. The importance of the test was greatly diminished in the eyes of the physician when he was recontacted after some time had passed since the birth of the child. Co-operation for further testing was understandably more difficult to obtain.

3. The longer the delay the more difficult it became to trace the family. This was due to various reasons: e.g. the infant was no longer seeing the pediatrician, the family doctor had been changed, the family had moved or in situations where the infant had been referred to VGH from another hospital the infant had returned to his home outside of this area. This hampered further biochemical testing and in some cases prevented it.
4. Some parents were difficult to deal with when contacted at a later date. The delay seemed to increase their anxiety. The parents seemed to assume there was another serious problem with their child (most had been high risk premature). Unfortunately the manpower to deal with exceptionally anxious parents was also not available. In a few cases, the parent was not satisfied with the physicians explanation of the screen and the infant's results, and placed a call to Children's Hospital but was unable to obtain peace of mind because a proper system had not been set up to deal with such calls.

5. Other parents reasoned that if a request for a stool specimen was coming this late it couldn't be very important and therefore they could not be bothered to collect a stool specimen.

The difficulties in obtaining stool specimens for chymotrypsin analysis is reflected in the high percentage (31%) of follow-ups that consisted only of a clinical evaluation by the physician.

Medical Records Investigation

The investigation of the patients, detailed records on file in the Medical Records department at the Vancouver General Hospital proved to be worthwhile. Out of a total of 104 infants, 87 infants records contained a possible explanation for the presence of the increased albumin concentration in the meconium specimen.

In the remaining 17, a clinical reason for detecting an increased albumin concentration was not apparent. These infants had all been classified as "healthy newborn infants" with no complications.
1. Healthy Infants

One infant out of the 18 had been given a glycerine suppository prior to the meconium collection. Glycerine has been reported to produce the typical blue colour with the BMC test-strip.

On re-examination of the laboratory records no biochemical explanations were present for the false positive results on the remaining 17 tests. None of the specimens had been reported as appearing bloody. Two of the specimens appeared to visually resemble stool specimens rather than meconium but this could lead to false negative results not false positives.

Whether these 17 positives were due to the failure of the BMC test-strip or whether these specimens actually contained an increased concentration of albumin due to some unknown cause is not known.

2. Premature Infants

Premature infants often have lower than normal pancreatic activity due to the slow development of necessary enzyme systems. Albumin accumulates in the meconium as a result of a decrease or absence of these pancreatic enzymes and results in a false positive in the CF meconium screen.

3. G.I. Disturbances

In the meconium specimens from infants with G.I. disturbances, the specimens most likely did contain an increased concentration of protein that was detected by the BMC test-strip. This increased protein could
have been due to inflammatory protein discharge, occult blood or a general concentration of the meconium specimen due to malabsorption.

4. Necrotizing Enterocolitis

There were reports of 50* cases of necrotizing enterocolitis (NEC) in the total population of 8,891 infants tested with the meconium albumin assay using the BMC test-strip. This is an incidence of 5.62 NEC per 1000 infants at VGH, both inborn and outborn. This incidence is high compared with the incidence of NEC reported in the literature: e.g. 2.02 cases per 1,000 births during a two year period (1975 to 1977) with 13,860 births and 3.61 per 1,000 births in 1978 as compiled by Finter and Moriartey in Alberta and an incidence of 3.95 per 1,000 births during a 20 month period (July 1977 to February 1979) with 8841 births, inborn and outborn, in Atlanta, Georgia as reported by Stoll et al.

The higher VGH incidence rate for NEC is partially as a result of VGH being a centre for high risk newborns but is probably also due to the manner in which the statistics were collected. In both of the studies by Finer and the one by Stoll, the babies with clinical symptoms which were suspect NEC but without radiographic evidence for NEC were excluded from the analysis. This procedure was not followed to compile the statistics for this meconium screen. All cases diagnosed by the pediatrician or family physician as NEC were included in the data even though positive confirmation with x-ray analysis had not been obtained for all of the cases.

* According to an analysis of the hospital files on patients whose files were retrieved by the Medical Records department through a diagnosis classification process.
According to the above calculated incidence of NEC for VGH, 5.62 in 1,000 births, one would expect to find 0.67 NEC cases among the 119 false positives, whereas 23 were present. This is a significant increase. (See Appendix N, section 1)

However, 82 of the 119 infants, on which positive screening results were obtained were premature infants. The incidence of NEC among premature babies increases drastically, the rate increasing with decreasing birth weight. A rough estimate of the incidence of NEC for premature babies, calculated using the date presented in Stoll's paper, would be 18 per 1,000 births. According to this rate, one would expect to find 1.5 NEC cases among the 82 premature infants that gave a false positive result with the BMC test-strip. Again the actual number present was significantly higher, at 21 (See Appendix N, section 2). There was therefore a definite correlation between the positive results obtained in the meconium screen and the presence of NEC.

These statistics point to the possible usefulness of the BMC test-strip (or other meconium albumin assay) for the early detection of NEC. The meconium albumin results were available within 2 to 7 days of the birth of the child. Since the specimen is collected within a day, the collection procedure could be re-organized so that the results are available within 2 to 3 days. The age at diagnosis of NEC of the infants at VGH ranged from 1 to 90 days, with most (62%) between 3 to 15 days. Most infants who develop NEC do not develop the clinical symptoms of NEC for 2 to 5 days. At VGH, during the time period under study only 3 infants were diagnosed under 3 days. As a result the BMC test-strip appears to have potential as an indicator of the possible development of NEC.
NEC and give the physician an early warning of this high mortality rate disease.

There is however one major drawback. The test detected 23 NEC out of a total of 50 infants diagnosed as having NEC. Twenty-seven infants tested negative with the BMC test-strip. The negative results could not be correlated with a suspicious and therefore possibly incorrect diagnosis of NEC.

If the BMC test-strip were used as a diagnostic test for NEC the physicians would have to be made aware that although a positive result could be an early warning sign for NEC, a negative result does not exclude the possibility of NEC developing.

B. Fecal Trypsin CF Screen

Diagnostic Follow-Up

Out of a total of 190 positives, 175 children were followed up. All of the 175 children were felt not to have cystic fibrosis and to-date information of a CF diagnosis has not been received.

The completion of the diagnostic follow-up on the positives obtained with the fecal trypsin CF screen was at a slightly lower percentage (92%) than the meconium screen.

Follow-up was not delayed to the same extent on these positives as with the meconium screen, because some additional manpower had been made available. As a result stool specimens for chymotrypsin analysis were obtained for a large proportion (81%) of the completed follow-ups.
Despite this, there were some difficulties in attempting to complete 8% of the follow-ups. The resistance encountered in obtaining the follow-up stool specimens appeared to be due mainly to the lack of confidence in the reliability of the Crossley method and in the questionable significance of the low trypsin result. This view was presented by the physicians themselves. Many expressed the opinion that further testing was not warranted although a few were kind enough to request a specimen "for the sake of the project" from mothers who they felt would not get anxious as a result of the request.

This view was also forwarded by the mothers who were reluctant to comply to the request of a stool sample for chymotrypsin. According to these mothers they had been informed by their physicians that the stool collection was not necessary and that it was not important*.

Because most of the positives were from healthy children who were a few weeks old at the time of the follow-up request, the physicians relied more on us to contact the parents directly. This did result in a more efficient retrieval system. This agreed with studies in Quebec in 1972 which revealed that compliance rates for obtaining a follow-up sample improved greatly when the onus for collecting and sending a test was placed on the parents rather than on the physician. This system appeared to arouse anxiety in some of the parents (or at least made us aware of the anxiety that does result). Lack of a system for handling anxious parents caused similar problems in the fecal trypsin screen as

* These instructions may however have been given to reduce the anxiety that appeared in some of the parents.
were present in the meconium screen. Several telephone calls, that we
know of, were placed to the Children's Hospital by anxious parents and
these calls were not channeled to a professional person capable of
dealing with such matters.

Medical Records Investigation

All of the positives detected in the fecal trypsin screen using the
Crossley method appear to be false positives to date. The few problem
areas or disease states that were present among the false positive
population such as premature births, Downs syndrome and hemolytic disease
of the newborn, were present in percentages that would be expected in the
normal infant population.

A large proportion (40%) of the infants that gave a positive result
had increased serum bilirubin levels at birth. Neonatal jaundice among
the normal newborn population is however found in approximately 60% of
newborns. 96

C. Difficulties Encountered with the Follow-Up Procedure

The form letter was not very successful in obtaining a follow-up
specimen in either screen. Many were filed away and forgotten*. It was
therefore necessary to place personal phone calls to the physicians in

* Probably, the intentions were to review the letter with the mother
when the infant and mother had the next appointment.
order to complete the follow-up. It was also important to follow-up the
original request with a second phone call (to the physician or the
parents) within a reasonable time period. This stressed the importance
of the request (especially to the parents) and usually resulted in
compliance.

An additional problem, not anticipated in advance,* was the lack of
resourcefulness of some parents for locating a container for the
collection of the stool specimen. There was also hesitation in mailing
the specimen in their containers and therefore the request for collection
was conveniently ignored. Containers and mailing instructions were made
available for these parents when we were made aware of their problem.

The follow-up procedure aroused anxiety in some parents. This
appeared to be slightly worse in the meconium screen and thought to be
due mostly to the large delay in forwarding the request.

The anxiety associated with a screen should be reduced through
delicate treatment of the parents. Information should be provided about
CF, the objective of the test and the follow-up procedure. Once the
parents are told of the screening result, prompt completion of the
follow-up test is necessary to minimize their anxieties. Again because
of minimum manpower the latter was not always accomplished.

Anxiety could possibly also be reduced by not making the parents
aware of the exact disease at such an early stage in the follow-up
procedure. The anxious state in the parents seemed to be triggered by
the term "Cystic Fibrosis" and all of its ramifications. Towards the end

* The assumption had been made that the doctor's office would be
looking after all the stool collections.
of the pilot project the use of the words Cystic Fibrosis was avoided with the fecal trypsin screen. Parents were informed that their child had been screened by a neonatal screening programme and could possibly have a digestive enzyme deficiency. The physicians of these infants were completely in favor of this approach. Although the sample was small, there was a strong indication that this approach reduced the amount of anxiety. This type of approach also has a negative side to it, however, for if the importance of the screen is de-emphasized in order to reduce anxiety, the importance of the follow-up is also de-emphasized and the parent is more likely to respond with less urgency or not at all. This holding back of information is probably not advisable for an established screening programme, but may be justifiable for a pilot project that has not-yet been proven reliable.
SUMMARY AND CONCLUSIONS

Cystic fibrosis, which occurs most frequently among Caucasians, is a condition whose early diagnosis and the resultant opportunity for effective treatment could be missed. The incidence of CF as determined by a meconium albumin pilot screen in operation in Vancouver from 1976 to 1979 was 1 in 2000. This incidence is sufficiently high to warrant a CF screening programme for the province of British Columbia.

Four methods were investigated to determine their suitability for use in a cystic fibrosis screening programme for the province of British Columbia. The essential objectives of a satisfactory screening procedure for use in the geographically dispersed areas of B.C. are that it should provide the highest possible specificity and sensitivity and be adaptable to a mail-in programme.

Two of the procedures, the Robinson and Elliott fecal trypsin method and the determination of the fecal albumin:alpha-1 antitrypsin ratio, were shown to be unsatisfactory.

The Robinson and Elliott trypsin method initially appeared to have potential because it was performed on dry stool samples on filter paper cards which would be mailed to a central laboratory from the geographically dispersed areas of B.C. In our hands, however, the method failed to detect known CF specimens and to separate non-CF from control specimens. The method was shown to be non-specific because the colour
change of the assay was due not only to trypic activity but also to
other unknown compound or compounds. Since this compound was not
identified, the significance of its presence in stool specimens from
cysts is not known. The interference of this compound and the
resultant lack of specificity makes the Robinson and Elliott method
unusable for the determination of fecal trypsin activity.

The albumin:alpha-1 antitrypsin ratio has been used by Ryley to
differentiate between what appears to be pancreatic insufficiency in
healthy infants from an actual pancreatic insufficiency. This concept
was appealing because its use, in combination with a fecal albumin
screen, could potentially lower the number of false positives that would
theoretically be obtained with that type of a screen. The investigation
did confirm that the ratio could be used to differentiate between fecal
specimens with an abnormally high albumin concentration from cystics
(with pancreatic insufficiency) and from non-cystics. However the
efforts made to find a qualitative albumin method for screening the
stool samples and to adopt the quantitative albumin and alpha-1
antitrypsin immunoelectrophoresis to the analysis of dry stool specimen
on filter paper cards failed.

Of the other two procedures investigated, the meconium albumin assay
(BMC test strip) and Crossley's fecal trypsin assay, the former had
already been incorporated into a pilot project at the Vancouver General
Hospital. Since both methods seemed promising, the latter was
incorporated into a second pilot project, and fairly large scale
investigations were conducted.
The Boehringer-Mannheim Corporation test-strip, a strip that detects an albumin content over 20 mg per g of meconium, proved to be a simple, reliable and inexpensive test. It was used to assess 8,891 infants yielding 119 positive results. Of these 12 were from premature babies who had died shortly after birth but who showed no signs of CF. Follow-up was completed on 96% of the remaining infants. Of these, 3 infants were diagnosed as having cystic fibrosis. One false negative occurred, totalling 4 CF and giving an incidence of 1:2223 for the population tested. The negative result was from a CF child who had low normal pancreatic function at birth. The false positive rate was 1.3% which is at an acceptable level. The false positive results were related to premature birth and gastro-intestinal disturbances. A significant correlation was shown to be present with the positive BMC test-strip result and necrotizing enterocolitis (NEC) indicating that the further investigation into using the BMC test-strip as a diagnostic test for NEC would be worthwhile.

The meconium screen did give satisfactory results. The fact that the test is not specific for CF is a disadvantage to a screening programme for CF but it is not a drawback in itself since it is also important to recognize early the other clinical conditions associated with a high concentration of albumin in the meconium. The screen was reasonably sensitive given its limitation of identifying only patients with CF who have intrauterine pancreatic insufficiency. The main disadvantages of the method are related to sample collection. Sample collection is time constrained leading to a low collection rate. Good precision with the BMC strip is only obtained if all of the testing is
done in a central laboratory but this leads to difficulties in the organization of the sample collection away from larger cities since the meconium is unstable and needs to be transported frozen. These disadvantages make the method unsuitable for adoption as a CF screen for the whole province of B.C.

A fecal trypsin method published by Crossley et al provided a satisfactory solution to the sample collection for B.C. The trypsin activity in fecal samples spread on filter paper cards and mailed to a central laboratory was shown to be stable.

The method was used to test dry stool specimens from 4085 infants. None of them to-date have cystic fibrosis. The false positive rate was 4.7% which is an unacceptably high level financially and ethically and leads to difficulties in adequately following-up the patients.

Two questionable areas of Crossley's method, the correction procedure for interfering fecal pigments and the visual evaluation of the test solutions were investigated and verified. Although the correction procedure for fecal pigments will lead to a few false positive results due to overcompensation, the data indicate that the number will be less than 0.6% as a conservative estimate. The visual evaluation was shown to be reliable, eliminating approximately 70% of the spectrophotometric work load.

The use of a substrate solution twice the original concentration for re-analysing positive samples was shown to be unnecessary. This step in Crossley's procedure was therefore not adopted in this trial screening programme.
The majority of the stool specimens (3210) for the trypsin screen were collected in the hospital. The false positive rate for this population was 5.0% and due mostly to the young age (under 3 days) of the infants tested whose trypsin enzymes are normally not present to the same level as 4 days or older infants.

The remainder of the specimens (875) were collected by parents at home and were therefore from infants over 4 days old. These specimens were mailed in to the laboratory. The false positive rate for this population was 3.4%, a rate that was not significantly different from the hospital collected rate.

The average concentration of p-nitroaniline in the test solutions of the home collected specimens was however significantly less than that present in the test solutions of hospital collected specimens. This was shown to be due to the thinner spread and therefore lower trypsin activity of feces on the cards prepared at home. Since the original cut-off point chosen by Crossley was based on their data, our cut-off point for the home collected specimens could theoretically be lowered even further based on our data if its effect on specificity and sensitivity were carefully monitored. This could only be done however on a large scale evaluation of home collected specimens. This was not feasible economically and would cause further undue anxiety.

For this reason, and because of the additional concern that the false positive rate could not be lowered sufficiently to enable the efficient completion of adequate follow-up, the trypsin CF screen was also considered unsuitable for the province of B.C.
Many difficulties were encountered in obtaining a follow-up on the presumptive positives obtained in each CF screening pilot project. The difficulties in the meconium screen were caused by a delay in initiating the follow-up. As a result of this delay some anxiety was caused in parents that could possibly have been avoided, considerable more time and effort was required to carry out the follow-up, and the test results were of less potential value to the physician. The difficulties in the fecal screen were due to the high incidence of false positives which discredited the programme. As a result less co-operation was obtained from both the physicians and the mothers in collecting the follow-up stool sample for chymotrypsin analysis.

Unfortunately, both screening programmes were affected by budget constraints. At peak periods of laboratory work, and holiday time, lack of manpower resulted in an excessive delay time in analysing and reporting the biochemical follow-up, unnecessarily lengthening the anxiety periods of some parents. Several phone calls were placed to the family doctor or pediatrician or to Childrens Hospital by parents who were anxiously waiting for the results of the follow-up stool chymotrypsin tests.

The experiences gained in following up the positive results from the meconium albumin and fecal trypsin screen led to the following recommendations:

1) Adequate information should be presented to parents before their infant is screened. This may delay anxiety at the time of the test and result in greater willingness to bring in the sample (or infant) for the follow-up test.
ii) Prompt initiation of the follow-up and prompt completion of the test is mandatory in order to obtain a sufficiently high compliance rate, provide the physician and infant with the full benefit of the programme and minimize the parents' anxieties.

iii) The persons responsible for retrieving the follow-up specimen, requested to confirm a presumptive positive in the screening programme, should be able to explain both the screen and the follow-up test if called upon to do so. They should be experienced in dealing with anxious parents and should be readily available to the parents.

iv) If the follow-up requires the retrieval of a stool or urine sample from the infant, convenience official mailers should be provided to make the collection as easy as possible for the parent.
ADDENDUM — AN UPDATE

In 1979, shortly after a decision had been reached at Children's Hospital to terminate the two existing CF screening pilot projects of meconium albumin and fecal trypsin, Crossley reported an exciting new advance in CF screening. The New Zealand group had measured immunoreactive trypsin (IRT) in CF children and found that in the first few months of life all CF children had a raised serum IRT (including CF children in whom fecal trypsin activity was not significantly decreased). IRT measurement appeared therefore to be capable of eliminating one of the strongest objections to both the meconium albumin and fecal trypsin screens, the high false negative rate that results because they test for pancreatic insufficiency. It also appeared as if the IRT method would eliminate or at least decrease in number the false positive results obtain in the fecal trypsin screen because all 11 CF children tested with the IRT method were clearly distinguishable not only from corresponding controls but also from non-CF babies with persistently low stool trypsin. The IRT method appears to be capable of detecting CF children who are essentially free of symptoms and these are the patients who are likely to benefit the most from the screen. 

Crossley had performed the IRT analysis on dried blood spots. This is a great advantage because dried blood spots are collected for several neonatal screening tests in many countries. It is advantageous from both
a practical and economical standpoint to screen for several diseases from one sample. The cost of each additional new test usually adds little to a screening programme cost. In British Columbia dried blood spots on filter paper are routinely collected throughout the province and used to screen for phenylketonuria (PKU) and congenital hypothyroidism (T4). The quantity of blood collected from each infant is sufficient to perform three analyses, PKU, T4 and IRT and test for all three genetic disorders.

The IRT assay is a relatively simple radioimmunoassay suitable for screening large numbers of specimens. Commercial kits are available which use the protein binding principle. The method requires fairly expensive gamma counting equipment but since Children's Hospital had this equipment on hand already for the T4 screen the cost of the IRT analysis is reasonable and approximately equal to that of the T4 screen.

When reports from other retrospective studies confirmed Crossley's finding that increased blood IRT was a characteristic of all newborn CF infants whether or not they have residual exocrine pancreatic function, Children's Hospital initiated a pilot IRT screen. Only samples from newborn infants are tested since IRT value decreases in CF children, approaching the normal range or lower in the older CF patient. The present theory is that IRT is raised in the newborn as a result of spillage from the pancreas into the circulatory system because of blocked pancreatic ducts. In the older child the IRT level is thought to fall as a result of the deterioration of pancreatic function.

In 1980, Crossley's second publication on the IRT method included a prospective study testing over 5,040 newborns. The false positive rate was 0.67% which is at an acceptable level for a routine screening
programme. At Children's Hospital 5,949 infants have been analysed at the time of writing this addendum and a false positive rate of 2.2% has been obtained if a cut-off point of 45 units is used, or 0.35% if a cut-off point of 70 units is used. The cut-off point has yet to be established. False positives are found in the newly born infant with jejunal atresia, biliary artresia and pancreatic achylyia.

Follow-Up Procedure

Because of the difficulties encountered in the follow-up on both the meconium albumin and fecal trypsin screening pilot projects in Vancouver, emphasis was placed on incorporating into this new screening programme the recommendations that were made as a result of the previous experience.

In order to guarantee success in the follow-up an initial request goes out to pediatricians and family physicians throughout the province for permission to test an infant (or infants) under his care. Confidence in the IRT assay is conveyed to the physician. By asking his participation in the programme, the likelihood of the physician's full co-operation following-up positive results, both chemically and clinically, is greatly increased.

A registered nurse was hired as the co-ordinator for the IRT programme. This means the programme has the manpower to follow-up all results immediately and to pursue the initial request within fairly narrow time limits. A separate telephone line was established for the CF screening programme and an answering service was incorporated in order to ensure that all enquiries regarding the programme get answered.
A request for a second dried blood specimen is made on all positive IRT levels found on the original screening card. Contact is made with the physician directly by phone requesting the collection of the second specimen at the infants "6-week check". The parents' anxiety is therefore not aroused by making a special visit to the doctor's office for the sole purpose of collecting a blood specimen. The phone call to the doctor is immediately followed with a letter and collection instructions. The physician decides whether or not the parents should be told at this stage that the blood specimen is for CF. Direct contact with the parents is not necessary because the specimen is in most cases collected at the doctor's office. At the physician's request, however, the sample collection could be made at Children's Hospital as an outpatient.

The second blood specimen is analysed immediately and the result, whether negative or positive, is phoned to the physician, followed by a confirmation letter. If the second specimen also has an increased IRT level a request for a stool chymotrypsin or sweat chloride is placed with the physician by one of the Clinicians from the CF Clinical Assessment Clinic. A pamphlet entitled "IRT Screening for Cystic Fibrosis. What is it?" is provided for the parents at this stage. In many cases this is the first time the parents make contact with the CF screening programme co-ordinator. It was felt that an R.N. would better serve the needs of the anxious parents both in person and in answering telephone queries.

The implementation of the recommendations have resulted in increasing the number of completed follow-ups and reducing the anxiety of the parents involved. It is too early to establish whether the false
positive rate is at an acceptable level and the false negative rate won't be known for some time. To date the IRT neonatal screen for CF looks very promising.
APPENDIX A

SCREENING CARD FOR COLLECTION OF STOOL SAMPLE
Fecal Trypsin CF SCREEN
C.F. SCREENING PROGRAMME
CHILDREN'S HOSPITAL

DATE OF SPECIMEN

CHILD'S NAME

BIRTH DATE

HOSPITAL

PARENT'S NAME

ADDRESS

BABY'S DOCTOR

ADDRESS

Tel.

Place pea-sized specimen of stool in each circle. Slip plastic envelope over end of card. Then press firmly to spread specimen evenly to cover circle.
APPENDIX B

LETTER TO PARENTS: REQUEST FOR COLLECTION OF INFANT'S STOOL SAMPLE FOR CF SCREENING PROGRAMME
APPENDIX C

CALCULATION OF % ERROR RESULTING FROM USE OF CROSSLEY'S FECAL PIGMENT CORRECTION PROCEDURE
1. Calculation of True Net Absorbance

It is not possible to subtract the absorbance reading at 410 nm for the fecal pigment (column b in Table III) directly from the TEST solution absorbance set 410 nm because each reading was obtained using a different sample disc with varying amounts of feces. The fecal pigment Abs 410/Abs 460 ratio should, however, remain constant despite the use of a separate sample disc.

As a result the true net absorbance value can be calculated using the fecal pigment ratio established for each sample and the fact that the p-nitroaniline absorbance at 460 nm is 4.4% of its absorbance at 410 nm in the following system of simultaneous equations.

Absorbance readings reported in Table III for the first stool sample are used in the example calculation.

\[
\begin{align*}
\text{fecal pigment} + \text{p-nitroaniline} & = 1.675 \\
\frac{1}{1.61} \text{f.p.} + 0.044 \text{ p-nitroaniline} & = 0.164 \\
\left(1.675 - 1.61(0.164)\right) \div [1-1.61(0.044)] & = \text{p-nitroaniline Abs 410} \\
& = 1.519 \\
\end{align*}
\]

Therefore, p-nitroaniline absorbance at 410 nm for the first stool sample in Table III should have been 1.519

2. Calculation of % Error

The calculation performed above results in the true net absorbance reading of p-nitroaniline at 410 nm. When Crossley's correction procedure is applied however the net absorbance represents only 91.2% of the p-nitroaniline (as discussed on page 150). Therefore 91.2% of the net absorbance calculated above was compared, with the result obtained using Crossley's correction procedure in order to calculate the % error.

\[
\frac{91.2}{100} \times 1.519 = 1.385
\]

\[
\frac{1.347 - 1.385}{1.385} \times 100 = -3\% \text{ Error}
\]
APPENDIX D

1. LETTER TO PHYSICIAN: REQUEST FOR STOOL SAMPLE FOR QUANTITATIVE CHYMOTRYPSIN ANALYSIS
2. INSTRUCTIONS FOR STOOL SAMPLE COLLECTION
STOOL COLLECTION FOR CHYMOTRYPSIN

1. Collect a few grams (walnut-sized sample) of a random stool.

2. Place stool in a closed container. Label container with:
   
   - NAME:
   - DATE OF BIRTH:
   - DATE OF SAMPLE:
   - DOCTOR'S NAME:

3. For specimens from Vancouver, send fresh or fresh frozen to:

   C.F. Screening Programme
   Children's Hospital
   250 West 59th Avenue
   Vancouver, B.C.
   V5Z 1X2
APPENDIX E

CLINICAL QUESTIONNAIRE
<table>
<thead>
<tr>
<th>CLINICAL SUMMARY</th>
<th>RECENT WEIGHT</th>
<th>DATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well - no problems</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chest infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Failure to thrive</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TESTS</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool chymotrypsin</td>
<td></td>
</tr>
<tr>
<td>Sweat electrolytes</td>
<td></td>
</tr>
</tbody>
</table>

OR

I will arrange above tests
Other suggestions

DOCTOR

N.B. Specimen for stool chymotrypsin should be walnut-sized, weighing approximately 3-5 grams, labelled and forwarded to Children's Hospital.
APPENDIX F

LETTER TO PHYSICIAN: REQUEST FOR CLINICAL FOLLOW-UP
TO PHYSICIAN WHO FELT LABORATORY FOLLOW-UP
WAS NOT WARRANTED.
APPENDIX G

CHI-SQUARE TEST FOR SIGNIFICANCE OF DIFFERENCE BETWEEN FALSE POSITIVE RATES FOR HOSPITAL AND HOME COLLECTED SPECIMENS IN FECAL TRYPsin CF SCREEN
Hospital collected samples
n = 3,210
no. of positives = 160
Home collected samples
n = 875
no. of positives = 30

<table>
<thead>
<tr>
<th></th>
<th>Hospital</th>
<th>Home</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg.</td>
<td>3050</td>
<td>845</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pos.</td>
<td>160</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3210</td>
<td>875</td>
</tr>
</tbody>
</table>

The chi-square value with one degree of freedom is
\[ \frac{4085[(3050(30) - 845(160)) - 4085/2]^2}{(3895)(190)(3210)(875)} \]
\[ = 3.41 \]

This is less than the critical value 3.84 of chi-square at the 5% level. Therefore the difference in false positives rates between hospital and home collected samples is not significant.
APPENDIX H

CHI-SQUARE TEST FOR SIGNIFICANCE OF DIFFERENCE BETWEEN FALSE POSITIVE RATES FOR LESS THAN 3-DAY OLD INFANTS AND AT LEAST 3-DAY OLD INFANTS
Hospital collected samples were checked for age of infant at time of collection:

- Number of positives checked:
  - Under 3 days: 84
  - 3 days or older: 24
  - Total: 108

- Number of negative results checked:
  - Under 3 days: 117
  - 3 days or older: 183
  - Total: 300

<table>
<thead>
<tr>
<th></th>
<th>3 days</th>
<th>3 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos.</td>
<td>84</td>
<td>24</td>
</tr>
<tr>
<td>Neg.</td>
<td>117</td>
<td>183</td>
</tr>
</tbody>
</table>

The chi-square value with one degree of freedom is:

\[ \frac{408[(184)(183) - 24(117) - 408/2]^2}{(108)(300)(210)(207)} = 46.23 \]

This is more than the critical value 10.83 of chi-square at the 0.1% level. Therefore the difference in false positive rate between the under 3 day old infant and the 3 day or older infant is highly significant.
APPENDIX I

CHI-SQUARE TEST FOR SIGNIFICANCE OF CHANGES IN RESULTS FOR HOSPITAL AND HOME COLLECTED SPECIMENS ON THE SAME INFANT
Both a hospital and a home collected specimen were collected on 692 infants. One infant gave a positive result on both specimens, 34 infants were positive on the hospital collected specimen only.

Using McNemar test for significance of changes*, corrected for continuity the chi-square value with one degree of freedom is:

\[
\frac{(21-34 - 1)^2}{21 + 34} = \frac{2.62}{21 + 34} = 2.62
\]

This is less than the critical value of 2.71 of chi-square at the 10% level. Therefore the changes in results between the two collection methods are not significant.

APPENDIX J

1. CHI-SQUARE TEST FOR SIGNIFICANCE OF DIFFERENCE BETWEEN DISTRIBUTIONS OF NET ABSORBANCE READINGS OF HOSPITAL AND HOME COLLECTED SPECIMENS

2. TEST FOR SIGNIFICANCE OF DIFFERENCE BETWEEN MEANS OF HOSPITAL AND HOME COLLECTED SPECIMENS NET ABSORBANCE VALUES
1. Distributions of Net Absorbance Values of Hospital and Home Collective Specimens

<table>
<thead>
<tr>
<th>Net Absorbance Value</th>
<th>HOSPITAL COLLECTED n = 485</th>
<th>HOME COLLECTED n = 470</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>actual</td>
<td>expected*</td>
</tr>
<tr>
<td>0 - .299</td>
<td>31</td>
<td>32.5</td>
</tr>
<tr>
<td>.300 - .599</td>
<td>46</td>
<td>57.9</td>
</tr>
<tr>
<td>.600 - .899</td>
<td>49</td>
<td>73.1</td>
</tr>
<tr>
<td>.900 - 1.199</td>
<td>104</td>
<td>116.8</td>
</tr>
<tr>
<td>1.200 - 1.799</td>
<td>255</td>
<td>204.7</td>
</tr>
</tbody>
</table>

The Chi-square value with 4 degrees of freedom is

\[ \sum \frac{(\text{Obs}_i - \text{Exp}_i)^2}{\text{Exp}_i} = 49.22 \]

This is more than the critical values of 18.46 of chi-square at the 0.1% level. Therefore the difference between distributions of net absorbance readings of hospital and home collected specimens is highly significant.

* If population distributions are the same
2. Means of Hospital and Home Collected Specimen Net Absorbance Values

Hospital collected specimens mean 1.068
Home collected specimens mean 0.941
difference 0.127

The standard error $\sigma_x$ of hospital collected specimens is 0.0175
The standard error $\sigma_x$ of home collected specimens is 0.0174
The standard error of the difference is

$$\sqrt{(0.0175)^2 + (0.0174)^2}$$

= 0.0247

Therefore the difference 0.127 between means, which due to sample size is normally distributed, is 0.127 = 5.14 standard deviations from zero and is therefore highly significant.
APPENDIX K

1. COMPARISON OF SCREENING RESULTS FROM FREEZER AND ROOM TEMPERATURE STORED SPECIMENS
2. COMPARISON OF SPECIMEN RESULTS BEFORE AND AFTER MAILING
3. COMBINED DATA FOR ROOM TEMPERATURE STORED SPECIMENS
1. COMPARISON OF RESULTS FROM FREEZER AND ROOM TEMPERATURE STORED SPECIMENS.

Using a normal approximation to the binomial distribution, with correction for continuity, the probability of getting a decrease in 12 or more specimens out of 20 due to random error alone can be calculated as follows:

\[
Z = \frac{(12-0.5) - 0.5(20)}{0.5 \sqrt{20}}
\]

\[
= 0.6708
\]

The probability of obtaining a Z value as high as 0.6708 is 25.1% which is not significant.

2. COMPARISON OF RESULTS BEFORE AND AFTER MAILING

Again using a normal approximation to the binomial distribution, with correction for continuity, the probability of getting a decrease in 14 or more specimens out of 25 due to random error alone (ignoring the single tie), can be calculated as follows:

\[
Z = \frac{(14-0.5) - 0.5(24)}{0.5 \sqrt{24}}
\]

\[
= 0.6124
\]

The probability of obtaining a Z value as high as 0.6124 is 27.0% which is not significant.

3. COMBINED DATA OF SECTIONS 1 AND 2 ABOVE

Using a normal approximation to the binomial distribution with correction for continuity, the probability of getting a decrease in 26 or more specimens out of 45 due to random error alone (again ignoring the single tie) can be calculated as follows:

\[
Z = \frac{(26-0.5) - 0.5(44)}{0.5 \sqrt{44}}
\]

\[
= 1.0553
\]

The probability of obtaining a Z value as high as 1.0553 is 14.6% which is not significant.
APPENDIX L

CHI-SQUARE TEST FOR SIGNIFICANCE OF DIFFERENCE BETWEEN DISTRIBUTIONS OF ABSORBANCE VALUES AT 460 NM OF HOSPITAL AND HOME COLLECTED SPECIMENS
**Distribution of Abs 460 Readings of Hospital and Home Collected Specimens**

<table>
<thead>
<tr>
<th>Net Absorbance Value</th>
<th>HOSPITAL COLLECTED n = 682</th>
<th>HOME COLLECTED n = 633</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>actual</td>
<td>expected</td>
</tr>
<tr>
<td>0 - .049</td>
<td>79</td>
<td>135.9</td>
</tr>
<tr>
<td>.050 - .099</td>
<td>370</td>
<td>339.7</td>
</tr>
<tr>
<td>.100 - .149</td>
<td>137</td>
<td>111.0</td>
</tr>
<tr>
<td>.150 - .199</td>
<td>40</td>
<td>38.4</td>
</tr>
<tr>
<td>.200 - .249</td>
<td>24</td>
<td>24.9</td>
</tr>
<tr>
<td>.250 - .249</td>
<td>10</td>
<td>9.9</td>
</tr>
<tr>
<td>.300 - .349</td>
<td>5</td>
<td>8.3</td>
</tr>
<tr>
<td>.350 - 1.000</td>
<td>17</td>
<td>14.0</td>
</tr>
</tbody>
</table>

The Chi-square value with 7 degrees of freedom is:

\[
\sum \left( \frac{\text{Obs}_i - \text{Exp}_i}{\text{Exp}_i} \right)^2 = 72.04
\]

This is more than the critical values of 24.32 of chi-square at the 0.1% level. Therefore the difference between distributions of Abs 460 readings of hospital and home collected specimens is highly significant.

* If population distributions are the same
APPENDIX M

COMPARISON OF THE PRECISION OF THE RESULTS
OF HOSPITAL AND HOME COLLECTED SPECIMENS
### Table: Specimens

<table>
<thead>
<tr>
<th></th>
<th>Hospital Collected</th>
<th>Home Collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>37</td>
<td>10</td>
</tr>
<tr>
<td>$\bar{x}$ S.D.</td>
<td>0.1065</td>
<td>0.1242</td>
</tr>
<tr>
<td>S.D. S.D.</td>
<td>0.0850</td>
<td>0.1469</td>
</tr>
<tr>
<td>S.D. $\bar{x}$</td>
<td>0.0140</td>
<td>0.0465</td>
</tr>
</tbody>
</table>

Difference in $x = 0.1242 - 0.1065 = 0.0177$

S.D. of difference $= \sqrt{(0.0465)^2 + (0.0140)^2} = 0.0486$

$$z = \frac{0.0177}{0.0486} = 0.364$$ S.D. which is not significant.

(i.e. no significant difference in precision between hospital and home collected repeat analysis)
APPENDIX N

CORRELATION BETWEEN POSITIVE MECONIUM SCREEN RESULTS AND PRESENCE OF NECROTIZING ENTEROCOLITIS
1. Out of 119 positive CF meconium results 23 infants were diagnosed as having necrotizing enterocolitis.

Using the Poisson distribution as an approximation to the hypergeometric distribution, with the expected NEC being 0.669 cases in 119 positives (based on 50 occurrences of NEC in the 8891 children tested at VGH), the probability of finding 23 or more NEC in 119 positives is

\[
1 - \sum_{x=0}^{22} \frac{(0.669)^x e^{-0.669}}{x!}
\]

which is insignificant.

2. Using the same procedure for the number of NEC among premature infants, where the expected number of NEC's in 82 is 1.48, (based on an incidence of 18 NEC cases per 1000 births as estimated from Stoll\(^6\)), the probability of finding 22 or more NEC in 82 premature positives is

\[
1 - \sum_{x=0}^{21} \frac{(1.48)^x e^{-1.48}}{x!}
\]

which is insignificant.
APPENDIX O

1. RANK TEST FOR SIGNIFICANCE OF THE EFFECT OF DOUBLING THE BAPNA SUBSTRATE CONCENTRATION
2. TEST FOR CORRELATION BETWEEN % INCREASE IN ABSORBANCE DUE TO DOUBLING SUBSTRATE CONCENTRATION AND THE ORIGINAL ABSORBANCE VALUE
1. The 30 samples were tested 4 times with the 0.25 mg BAPNA substrate and once with the 0.50 mg BAPNA substrate. In each case the rank of the latter among the five results was determined. In 14 out of the 30 samples, the double substrate result ranked highest of the five.

The probability of obtaining this result by chance alone, assuming equal probability for each of the 5 possible ranks, is:

\[ P(14/30 \text{ are ranked highest}) = \left( \frac{1}{5} \right)^{14} \left( \frac{4}{5} \right)^{16} \binom{30}{14} \]

\[ = 0.00067 \]

Therefore the effect of doubling the substrate concentration is significant at the 0.1% level.

2. The linear regression of the % increase in absorbance value due to doubling the substrate concentration on the mean absorbance value of the 0.25 mg BAPNA results for each sample led to the following equation:

\[ Y = 0.2047 - 0.000991X \]

with a coefficient of determination of 0.000129 and a standard error of the slope coefficient of 0.016 indicating no significant correlation.
APPENDIX P

CONDITIONS UNDER WHICH AN EQUAL PERCENT REDUCTION IN ABSORBANCE AT 410 AND 460 NANOMETERS FROM HOSPITAL TO HOME COLLECTED SPECIMENS CAN OCCUR
By the principle of additivity, the absorbance of hospital and home test solutions can be expressed as the sum of absorbances due to p-nitroaniline and fecal pigment as follows:

\[ A_{410} = A_{410}^{PNA} + A_{410}^{fp} \]
\[ A_{460} = A_{460}^{PNA} + A_{460}^{fp} \]

where e.g.

\[ A_{410}^{PNA} = a_{410}^{PNA} \times b \times c^{PNA} \]
\[ A_{460}^{fp} = a_{460}^{fp} \times b \times c^{fp} \]

and where e.g.

\[ a_{460}^{fp} = \text{molar absorptivity of fecal pigment at 460 nm} \]
\[ c^{PNA} = \text{concentration of p-nitroaniline (PNA) in the test solution (as a result of the trypsin activity in either the hospital or home collected specimens).} \]
\[ b = \text{cell depth} \]

The mean absorbance values at both 410 and 460 nanometers of test solutions prepared with home collected samples were 12% lower than the hospital collected samples.* (Sample size was 500.)

This decrease in absorbance values at both wavelengths could be as a result of one or more of the following:

1. The home collected stool smears were thinner than the hospital collected stool smears.

* For complete distributions of net absorbances and absorbances at 460 nm for home and hospital collected samples see figures II and III respectively. Note that since net absorbance

\[ A = A_{410} - 2A_{460} \]

as discussed on page 65, mean net absorbance

\[ \bar{A} = \bar{A}_{410} - 2\bar{A}_{460} , \]

and a simultaneous reduction of 12% in \( \bar{A}_{410} \) and \( \bar{A}_{460} \) results in a 12% reduction in net absorbance, \( \bar{A} \), as indicated in figure II.
2. There was a decrease in concentration of fecal pigment in home collected feces.

3. There was a decrease in trypsin concentration in home collected feces resulting in a decrease in p-nitroaniline in the test solutions.

An average 12% reduction in stool smear thickness from hospital to home (item 1 above) would have the effect of decreasing both $C_{PNA}$ and $C_{P}$ by the same amount and therefore clearly reduce $A_{410}$ and $A_{460}$ by the same amount from hospital to home.

A decrease in fecal pigment concentration in the home collected feces (item 2) would reduce $A_{410}^{P}$ and $A_{460}^{P}$ equally (proportionally to the reduction in $C_{P}$) but since $A_{410}^{P}$ is not the same fraction of the $A_{410}$ as is the $A_{460}^{P}$ of $A_{460}$, the percentage reductions in $A_{410}$ and $A_{460}$ would not be the same.

The same argument holds for item 3 above.

Of course an extraordinary coincidence of simultaneous equal changes in trypsin concentration and fecal pigment concentration could result in a 12% decrease in both.
APPENDIX Q

CALCULATION OF CONFIDENCE THAT CHILD WITH
POSITIVE FECAL TRYPsin SCREEN RESULT HAS CF
The probability that a child with a positive result from the fecal trypsin screen does in fact have CF is calculated as follows:

Let

\[ P(CF) = \text{incidence of CF in population} = 0.0005 \]
\[ P(\text{Normal}) = 1 - P(CF) = 0.9995 \]
\[ P(+/\text{Normal}) = \text{false positive rate} = 0.019 \]
\[ P(+/CF) = \text{true positive rate (unknown)} \]

We want to calculate:

\[ P(CF+/+) = \text{probability that child with positive result has CF} \]

\[ P(CF+/+) = \frac{P(+/CF) P(CF)}{P(+/CF) P(CF) + P(+/\text{Normal}) P(\text{Normal})} \]

An upper bound for the value of \( P(CF+/+) \) occurs when \( P(+/CF) = 1.0 \) i.e. the true positive rate is perfect. This would result in:

\[ P(CF+/+) = \frac{(1.0)(0.0005)}{(1.00)(0.0005)+(0.019)(0.9995)} \]

\[ = 2.6\% \]

Thus the probability that a child with a positive result does have CF is less than 2.6\%.


95. Turtle, Shirley: Unpublished data. Children's Hospital, 1975.


